PANAMJAS
Pan-American Journal of Aquatic Sciences

Lead and cadmium contamination in sediments and blue crabs *Callinectes danae* from a Ramsar wetland in southeastern Brazil

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Abstract. The Cananéia-Iguape-Peruíbe Environmental Protection Area (APA-CIP), on the southeastern coast of Brazil has been designated as a Ramsar wetland site. This estuary is impacted by inputs of contaminated water and sediments from the Ribeira de Iguape River (RIR), in São Paulo state. Inputs of metals are mostly from historical mining and metallurgy activities in the RIR watershed. This study aimed to evaluate the concentrations of Pb and Cd in the soft tissues of blue crabs (*Callinectes danae*) from the APA-CIP, and to determine if the levels of these metals in crab tissues are correlated with the concentrations of these metals in sediments from the estuary. Sediments and crabs were collected during the rainy (March) and dry (September) seasons. Pb concentrations in sediments at two stations in the estuary exceeded sediment quality guidelines, at concentrations of 13.08 and 40.24 $\mu g.g^{-1}$ dry weight, respectively. Despite these high Pb concentrations in sediments, the levels of Pb in gill and hepatopancreas tissues of the crabs were relatively low. Cd concentrations in sediments from the estuary were relatively low, but the concentrations in crab tissues were elevated. For instance, the Cd concentrations in the hepatopancreas of crabs collected in the March survey at two stations were 21.73 and 4.24 $\mu g.g^{-1}$, respectively. These findings indicate that *C. danae* may rapidly depurate Pb, while these crabs may rapidly accumulate and poorly depurate Cd. There was some indication that the concentrations of Cd in the hepatopancreas of *C. danae* were correlated with sediment concentrations, but factors such as varying sand and mud contents of the sediments, and the potential for migration of the crabs within the estuary may constitute confounding factors. These data indicate that governmental action is required to control contamination of this World Heritage Site.

Key words: bioaccumulation, Marine Protected Area, Ramsar site, metal contamination, crustaceans.

Resumo: Contaminação de chumbo e cadmio em sedimentos e siris *Callinectes danae* provenientes de uma Zona Úmida de Importância Internacional Ramsar no sudeste do Brasil. A Área de Proteção Ambiental Cananéia-Iguape-Peruíbe (APA-CIP), na costa sudeste do Brasil, foi designada como Zona Úmida pela conferência de Ramsar. Esse estuário é impactado por aportes de água contaminada e sedimentos do Rio Ribeira de Iguape (RIR), no estado de São Paulo. O aporte de metais provém principalmente de atividades históricas de mineração e metalurgia na bacia hidrográfica do RIR. Este estudo teve como objetivo avaliar as concentrações de Pb e Cd nos tecidos moles de siri-azul (*Callinectes danae*) da APA-CIP, e determinar se os níveis desses metais nos tecidos dos siris estão correlacionados com as concentrações de metais nos sedimentos do estuário. Sedimentos e siris foram coletados durante as estações chuvosa (Março) e seca (Setembro). As concentrações de Pb nos sedimentos em duas estações no estuário excederam as diretrizes de qualidade dos sedimentos, nas concentrações de 13.08 e 40.24 $\mu g.g^{-1}$ de peso seco, respectivamente. Apesar dessas altas concentrações de Pb nos sedimentos, os níveis de Pb nas brânquias e nos tecidos (hepatopâncreas) dos siris eram relativamente baixos. As concentrações de Cd nos sedimentos do estuário foram relativamente baixas, mas as concentrações nos tecidos dos siris foram elevadas. Por exemplo, as concentrações de Cd no hepatopâncreas de siris coletados em Março em duas estações de coleta foram de 21.73 e 4.24 $\mu g.g^{-1}$, respectivamente. Esses resultados indicam que *C. danae* pode depurar Pb rapidamente, enquanto são capazes de acumular e mal depurar Cd. Há indicações de que concentrações de Cd no hepatopâncreas de *C. danae* estão correlacionadas com as concentrações nos sedimentos, mas fatores como a variação do conteúdo de areia e lama dos sedimentos e o potencial de migração dos siris dentro do estuário podem apresentar fatores de variabilidade. Esses dados indicam que uma ação governamental é necessária para controlar a contaminação desta Zona Úmida como Patrimônio Mundial.

Palavras-chave: bioacumulação, Área Marinha Protegida, área Ramsar, contaminação por metais, crustáceos.

Introduction

The Cananéia-Iguape-Peruíbe Environmental Protection Area (APA-CIP) is recognized as a World Heritage site by the United Nations Educational, Scientific and Cultural Organization (UNESCO), as part of UNESCO's Biosphere Atlantic Rainforest Reserve. This area was recently included in the Ramsar List of Wetlands of International Importance (https://rsis.ramsar.org/ris/2310) and also it is part of the Mata Atlântica Biosphere Reserve (RBMA). Mata Atlântica is a protected biome by specific federal law (Law nº 11.428/2006).

However, recent studies have demonstrated that APA-CIP is contaminated with a range of metals (Cruz *et al.* 2014; 2019), with lead identified as the contaminant of main concern (Gusso-Choueri *et al.* 2018, 2015; Tramonte *et al.* 2018), as well as substances originating from sewage (Aidar *et al.* 1997). Historical lead mining activities in the basin of the Ribeira de Iguape River (RIR) represent the main source of metals contaminating the APA-CIP (Guimarães and Sígolo 2008; Rodrigues *et al.* 2012; Abessa *et al.* 2014). Mine tailings deposited directly or close to the rivers of the basin continue to be carried downstream, causing contamination along the estuary, especially in sediments (Mahiques *et al.* 2009, 2013).

Seasonality can be an important factor influencing the distribution of metals in the environment, as well as their bioavailability and toxicity. In particular, the rainfall regime can modify freshwater flows, and consequently, the mobility of metals. We recently documented the influences of season on the deposition of metals in sediments in the APA-CIP (Araujo *et al.* 2020a). The RIR is the main contributor of freshwater, sediments, nutrients and contaminants to the APA-CIP estuary. The RIR is 470 km long and its regime is strongly controlled by rainfall seasonality (Mahiques *et al.* 2013). The rainy season lasts from October to March while the dry season extends from April to September (Abessa *et al.* 2014). The Valo Grande canal connecting the estuary to the RIR was constructed to facilitate agricultural trade, but the construction of this system caused the main water flux from the RIR to be directed into the canal, causing drastic changes to the hydrology of the estuary and an increase in suspended particulate loads discharged into the APA-CIP (Mahiques *et al.* 2013). As a secondary effect caused by opening of the Valo Grande canal, increased levels of metals have been observed in sediments and organisms from the APA-CIP (Rodrigues *et al.* 2012).

Metals can be actively or passively taken up by aquatic organisms and accumulate in their tissues (Arnot & Gobas 2006). Crustaceans, in particular, can be useful biomonitoring organisms for evaluating the distribution of metals in aquatic environments (Vogt *et al.* 2018). Swimming crabs from the family, Portunidae, including those belonging to the *Callinectes* genus, have been used as biomonitors in several studies worldwide (Freire *et al.* 2011; Masui *et al.* 2005). The blue crab, *Callinectes danae* is widely distributed along the western Atlantic coast, from Florida (USA) to Argentina and is an important species for the flow of energy and biomass within coastal trophic systems (Harris & Santos 2000; Andrade *et al.* 2015). This crab species is also an important fishery resource in Brazil (Bordon *et al.* 2012a), including in the APA-CIP (Mendonça *et al.* 2010). *C. danae* has been recommended as a biomonitor species for environmental assessments and ecotoxicological studies (Sastre *et al.* 1999; Bordon *et al.* 2016, 2018). Such organisms can potentially contribute to the trophic transfer of contaminants (Ferrer *et al.* 2006), including to humans through the ingestion of contaminated seafood (Lavradas *et al.* 2014).

The research objectives of this study were to evaluate the levels of metals in tissues of *C. danae* collected from the APA-CIP and to determine if metal bioaccumulation in these crabs is correlated with the concentrations of metals present in sediments. For this study, we focused our analysis on the levels of lead (Pb) and cadmium (Cd) in sediments and crab tissues; lead is the main contaminant of interest in APA-CIP (Tramonte *et al.* 2018, Mahiques *et al.* 2009), while Cd was previously found in sediments of the region, probably associated with local sources (Cruz *et al.* 2014). Additionally, we aimed to determine if seasonality was a factor influencing on metal accumulation in crabs. Finally, this study aimed to contribute on the use of C. *danae* as a biomonitor for metal contamination in estuarine environments, providing information if crabs responds to short term (i.e. seasonal) changes in metal contamination. Concomitantly, this study assessed the level of Pb and Cd contamination in a Ramsar wetland within a World Heritage site.

Materials and Methods

Study area and sampling sites: The study area is presented in the Figure 1, including the APA-CIP estuary and the lower reaches of the RIR watershed. Anthropogenic influences are greater in the northeastern portion of the estuary because of urbanization in the cities of Iguape and Comprida Island. Sediments and *C. danae* were collected at six stations throughout the APA-CIP (Figure 1). Sampling stations included the region close to the entrance to the Valo Grande canal (VG) where higher levels of metal contamination were expected, and the southern region close to Cardoso Island (IC) where lower metal contamination was expected. Sampling surveys were conducted in March (summer, rainy season) and September (winter, dry season) of 2013.

A stainless steel Van Veen (0.036 m^2) grab sampler was used for sediment collection, and 3-6 samples were taken at each site. Subsamples of sediments were stored in plastic containers at 4ºC for analysis of sedimentological properties and at -20ºC for analysis of geochemical parameters and metals. Adults of *C. danae* were collected by otter-trawling, lasting for about 10-15 min at each station. The collection was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), through the System for Authorization and Information of Biodiversity (SISBIO), by the Licenses 28550-2 and 28550-3. When trawling was not possible, crab traps were used. In each sampling survey, eight crabs from each station were collected and taken to the field laboratory, where organisms were immobilized and anesthetized by being put on ice to reduce their metabolism, then, anterior and posterior gill and hepatopancreas tissues were removed for analysis of metals. In field, the samples were stored frozen in liquid nitrogen until being transported to the laboratory, where they were stored at -20°C.

Analysis of sediments: Sediment grain size was determined based on the protocol developed by McCave and Syvitski (2007). Briefly, 30 g of dry sediment were wet sieved (0.060 µm) to isolate fine particles (i.e. mud and silt), and then dried again in a stove at 60 ºC. Mud content was calculated by the difference between initial and final weights. Subsequently, the sandy fraction, containing the particles retained on the sieve, were dried (60ºC), weighed and sieved through different meshes (ϕ scale), according to the Wentworth scale, in order to determine sand fractions. Organic matter (OM) was determined by loss from ignition, in which 5 g of dry sediment were incinerated in a muffle furnace at 500 ºC for 4 hours (Luczak *et al.* 1997). To determine calcium carbonate ($CaCO_3$) content, 10 mL of hydrochloric acid (5 M) were added to 5 g of dry sediment and left to digest for 24 h. Subsequently, samples were washed with distilled water and dried (60 ºC), and the $CaCO_3$ content was calculated from the difference between the initial and final weights, as described by Hirota and Szyper (1975).

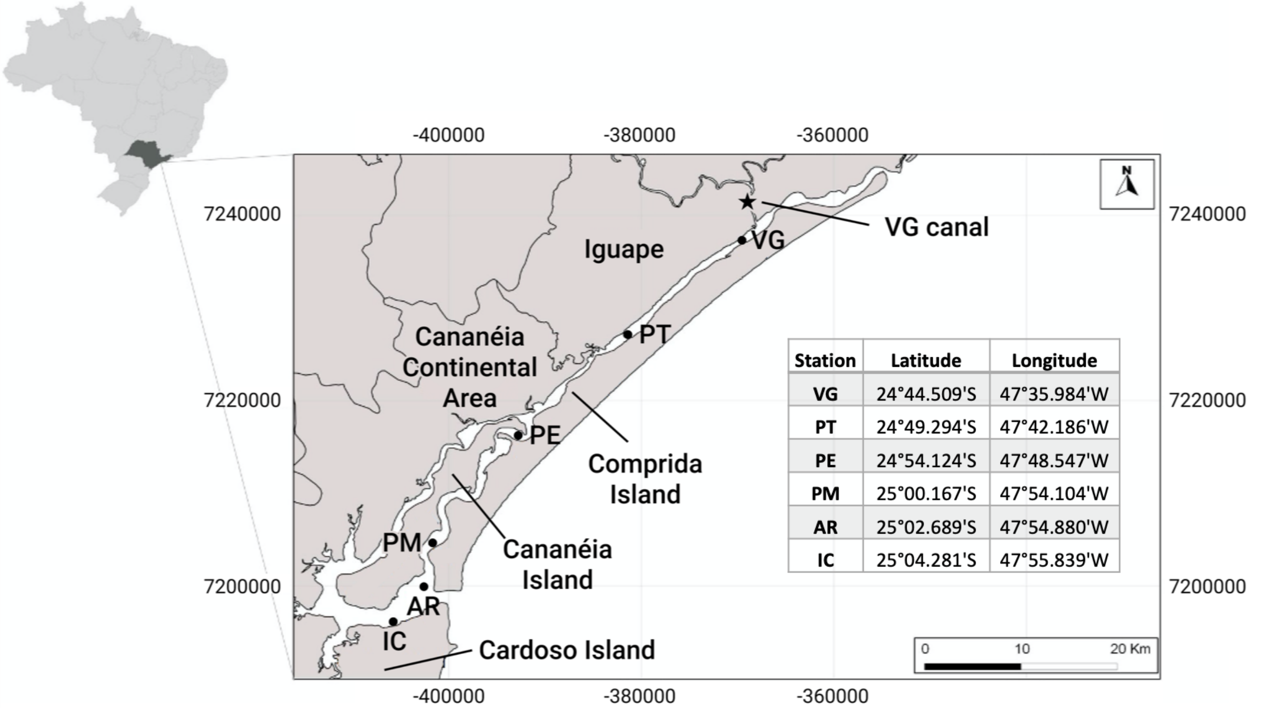


Figure 1. Map and geographic coordinates of the sampling stations for sediments and in *Callinectes danae* within the APA-CIP. The inset shows the location of the study area along the southeastern coast of Brazil.

Sediments from the March survey (2 g wet sediment) were digested using a weak acid extraction (i.e. simultaneously extracted metals; SEM) using 6 M hydrochloric acid (HCl), and the released H_2S was trapped in 0.5 mol L^{-1} NaOH, using N_2 as a carrier gas, as described by Allen *et al.* (1993). The choice of using this weak digestion was to obtain results comparable to a previous study (Cruz *et al.* 2014). These digests were analyzed for lead (Pb) and cadmium (Cd) by inductively coupled plasma-optical emission spectroscopy on an Ultima 2 Horiba ICP-OES (São Paulo, Brazil). QA/QC procedures included the use of blanks, replicates, and standards. For both Pb and Cd, curves were constructed using fortified solutions (Specsol, Quimlab, Brazil) with known concentrations (7-point curves for each element). The Limits of Detection (LOD) were calculated as 3sd/S, where sd was the standard deviation for the blanks and S was the sensitivity obtained in the calibration curve; such values were 0.01 mg Kg^{-1} for Cd and 0.02 mg Kg^{-1} for Pb.

Sediments from the September survey (1 g, dry weight) were digested with sub-distilled nitric acid (HNO_3) (67 % v/v, Sigma-Aldrich, São Paulo) at 100 ºC for approximately 4 h. To monitor plasma stability, ^{103}Rh (PerkinElmer, USA) at 20 µg L^{-1} was monitored online during the analyses as the internal standard. The sediment sample digests from the September survey were analyzed for Pb and Cd by inductively coupled plasma-mass spectrometry using a PerkinElmer ELAN DRC II ICP-MS (Norwalk, CT, USA) with no reaction cell. QA/QC procedures included the analysis of replicates and laboratory blanks, as well as the analysis of a PACS-2 marine sediment certified reference material (CRM), supplied by the National Research Council of Canada (Ottawa, ON, Canada). An external standard method was used for quantification of Pb and Cd in samples using an 8-point calibration curve using multi-elemental external calibration, by appropriate dilutions of a mixed standard solution (Merck IV). The instrument LOD and limit of quantification (LOQ) were estimated as 3 sd/S and 10 sd/S, respectively, where sd is the standard deviation for the blank measures and S is the method sensitivity. The LOD values were 0.006 mg Kg^{-1} for Cd and 0.007 mg Kg^{-1} for Pb for the NIST 2976 standard material and 0.01 mg Kg^{-1} for Cd and 0.003 mg Kg^{-1} for Pb for the PACS-2 certified reference material. See Table I for more details.

Quality control/quality assurance: Table I summarizes the results from the analyses of mussel tissue and sediment CRMs. These data indicate that Cd and Pb were recovered efficiently from the tissue samples. Mean Pb recoveries from sediment samples were 74% of the certified value, while Cd recoveries were 124% (Table I). These data indicate confidence in the accuracy of the both sediments and tissue analyses, although the concentrations of Pb in sediment may have been slightly underestimated.

Analysis of tissues: C. *danae* tissues were freeze-dried, and 0.5 g subsamples were placed in Nalgene digestion vessels. Volumes of 4 mL of nitric acid (HNO3) P.A. 65% (Synth) and 1 mL of hydrogen peroxide (H_2O_2) P.A. 35% (Synth) were added to each vessel for digestion. Following pre-digestion overnight at room temperature, the vessels were placed for 3 h in a digestion block at 90° C. The extracts were then transferred to Falcon tubes and the volumes were made up to 25 mL with Milli-Q water (resistivity 18 MΩ.cm at 25 °C). Metal concentrations were determined by Graphite Furnace Atomic Absorption spectrometry using a PerkinElmer Analyst 800 GF-AAS. For each metal of interest, a calibration curve of at least five different concentrations was performed, diluting aliquots of 1000 µg/mL - AAS single element calibration standard (PerkinElmer) in ultrapure water. The limit of detection (LOD) for each metal was calculated according to INMETRO (2016) using the following equation:

$$LOD = mean + t\,(n\text{-}1;\,1\text{-}\alpha) \times SD$$

Where:

mean = concentration means determined from 7 sample blanks; t = t'-Student value according to the degrees of freedom (n-1) and α=0.05; SD= Standard deviation of the concentrations determined in 7 sample blanks.

To validate the analytical method for tissues, a NIST 2976 standard reference material of freeze-dried mussel tissue supplied by the National Institute for Standards and Testing (Gaithersburg, MD, USA) was analyzed for Cd and Pb.

Statistical analyses: Bioaccumulation data were analyzed using permutational analysis of variance (PERMANOVA) (Anderson 2001), considering each metal (Pb and Cd) in two fixed factors: (1) Site and (2) Season for each tissue analyzed (AG, PG and Hep). Differences between the metal contents (Pb and Cd) in tissues were assessed considering all situations. Pairwise a posteriori analysis was performed for all data to detect significant differences ($p < 0.05$). PERMANOVA tests were conducted on Euclidean-distance similarity matrices. The residuals were permutated using unrestricted permutation of raw data. Monte Carlo p-values were used when the number of permutations were lower than 50 (Anderson *et al.* 2008).

The Pb and Cd results (µg.g^{-1} dry weight) in all sediment and *C. danae* soft tissue samples were also compared using Pearson correlation analysis. The Statistica® version 6.0 software was used for all statistical analysis.

Results

Sediments: The percent contents of $CaCO_3$, OM, sand and mud in sediments collected from the APA-CIP are presented in Table II. Data from the March survey indicate that the sediments were predominantly sandy (>80%) at the VG, PE, AR and IC sites. The sediments at the other two stations (PT and PM) had a higher percentage of fine material (i.e. mud) at >59%. Sediments from the September survey presented similar properties, with the exception of station PM, where the sand content of the sediments increased to >77% in September relative to >40% in the March survey (Table II).

Metal concentrations (Pb and Cd) in sediments are also presented in the Table II. In the summer survey, Pb concentrations exceeded Canadian interim marine sediment quality guidelines (ISQGs = 30.20 µg.g^{-1}; CCME, 2001) in PT station, achieving 32.84 µg.g-1. Cd concentrations were low and remained below the toxic thresholds.

Table I. Mean concentrations (µg.g^{-1} dry weight) of Pb and Cd in the PACS-2 sediment CRM and the NIST 2976 mussel tissue SRM relative to the certified values.

	Mussel tissue standard reference material (NIST 2976)	
	Pb	Cd
Mean ± SD (n = 3)	1.22 (0.08)	0.85 (0.04)
Certified concentration	1.19	0.82
Recovery (%)	102	104
	Sediment certified reference material (PACS-2)	
Mean ± SD (n = 3)	135.13 (0.6)	2.61 (0.06)
Certified concentration	183	2.11
Recovery (%)	74	124

Table II. Sedimentological analysis (percent contents of $CaCO_3$, Organic Matter (OM), sand and mud), metal concentrations (Pb and Cd) in sediments and Callinectes danae soft tissues (posterior and anterior gills and hepatopancreas) collected from 6 sites in the APA-CIP in the March and September sampling campaigns. Bold values indicates metal content exceeding sediment guidelines for metals (CCME 2001; Choueri et al. 2009).

	March				September			
Stations	$CaCO_3$	OM	Sand	Mud	$CaCO_3$	OM	Sand	Mud
VG	0	0.2	95.4	4.6	0	0.3	98.9	1.1
PT	8.2	13.8	1.8	98.2	6.4	14.4	3.8	96.2
PE	1.6	0.6	91.5	8.5	0	0.8	96.7	3.3
PM	8.8	0.6	40.7	59.3	1.3	6.1	77.2	22.8
AR	5.0	16.8	83.6	16.4	2.2	1.0	92.5	7.5
IC	1.8	2.0	89.8	10.2	2.5	2.1	89.6	10.4

Stations	Pb	Cd	Pb	Cd
	Sediment			
VG	8.27 ± 045	<0.02	0.83± 0.05	<0.01
PT	**32.84 ± 4.95**	0.17 ± 0.05	**13.08 ± 0.51**	0.07 ± 0.01
PE	2.23 ± 0.09	<LOD	2.93 ± 0.09	<0.01
PM	8.68 ± 0.22	0.04 ± 0.00	**40.24 ± 0.30**	0.14 ± 0.00
AR	1.52 ± 0.16	<0.01	3.08 ± 0.26	<0.02
IC	1.83 ± 0.06	<0.01	2.04 ± 0.02	<0.01
LOD	0.02	0.01	0.01	0.003
	Posterior Gill			
VG	0.03 ± 0.00	0.11 ± 0.00	<LOD	0.10 ± 0.00
PT	0.02 ± 0.01	0.28 ± 0.00	0.36 ± 0.00	0.26 ± 0.00
PE	0.08 ± 0.01	0.09 ± 0.00	0.65± 0.01	0.18 ± 0.00
PM	0.93 ± 0.00	0.13 ± 0.00	1.26 ± 0.04	0.43 ± 0.00
AR	1.37 ± 0.01	0.17 ± 0.00	0.50 ± 0.02	0.17 ± 0.00
IC	1.90 ± 0.03	0.13 ± 0.00	2.23 ± 0.07	0.17 ± 0.00
	Anterior Gill			
VG	0.06 ± 0.00	0.13 ± 0.00	0.30 ± 0.00	0.31 ± 0.00
PT	0.47 ± 0.00	0.70 ± 0.02	0.69 ± 0.05	0.44 ± 0.02
PE	0.19 ± 0.02	0.20 ± 0.00	0.52 ± 0.07	0.21 ± 0.00
PM	1.99 ± 0.07	0.16 ± 0.00	1.31 ± 0.02	0.40 ± 0.00
AR	1.41 ± 0.02	0.16 ± 0.00	0.45 ± 0.02	0.11 ± 0.00
IC	3.22 ± 0.22	0.16 ± 0.00	1.77 ± 0.03	0.26 ± 0.00
	Hepatopancreas			
VG	0.47 ± 0.00	3.85 ± 0.05	0.30 ± 0.06	0.45 ± 0.00
PT	2.05 ± 0.04	21.73 ± 0.56	0.39 ± 0.02	4.88 ± 0.03
PE	0.20 ± 0.03	4.24 ± 0.02	0.24 ± 0.03	0.76 ± 0.00
PM	0.32 ± 0.02	1.47 ± 0.00	0.18 ± 0.00	3.36 ± 0.01
AR	0.83 ± 0.09	0.71 ± 0.03	0.17 ± 0.01	0.82 ± 0.00
IC	0.15 ± 0.05	0.83 ± 0.00	0.54 ± 0.03	2.51 ± 0.00

In winter, sediments from PT and PM presented the higher Pb concentrations (13.08 and 40.24 µg.g^{-1}, respectively).

Crab tissues: For Pb, no difference between summer and winter was shown for all tissues tested. Statistical difference between stations was shown for both gill tissues: for anterior gills, the only difference was between crabs from VG and PT, while for posterior gills, differences were detected between IC and VG, PT and PE, and between PT and PM. No statistical difference was shown for Hepatopancreas (Table III, S1).

For Cd, no differences between summer and winter were shown for all tissues tested (Table III). Statistical differences between stations were shown for posterior gill, between VG and PT and AR (Table S2), while no significant difference occurred for both anterior gill and hepatopancreas (Table S2). The maximum mean Cd concentration of 21.73 µg.g^{-1} dry weight was observed in the hepatopancreas of crabs collected from the PT station in March, 2013 (Table III).

Considering Pb content in tissues, no statistical difference was shown among tissues. However, Cd tissue content differed for comparisons between PG vs. Hep (p = 0.046) and AG vs. Hep (p = 0.043). No statistical difference was presented among gills.

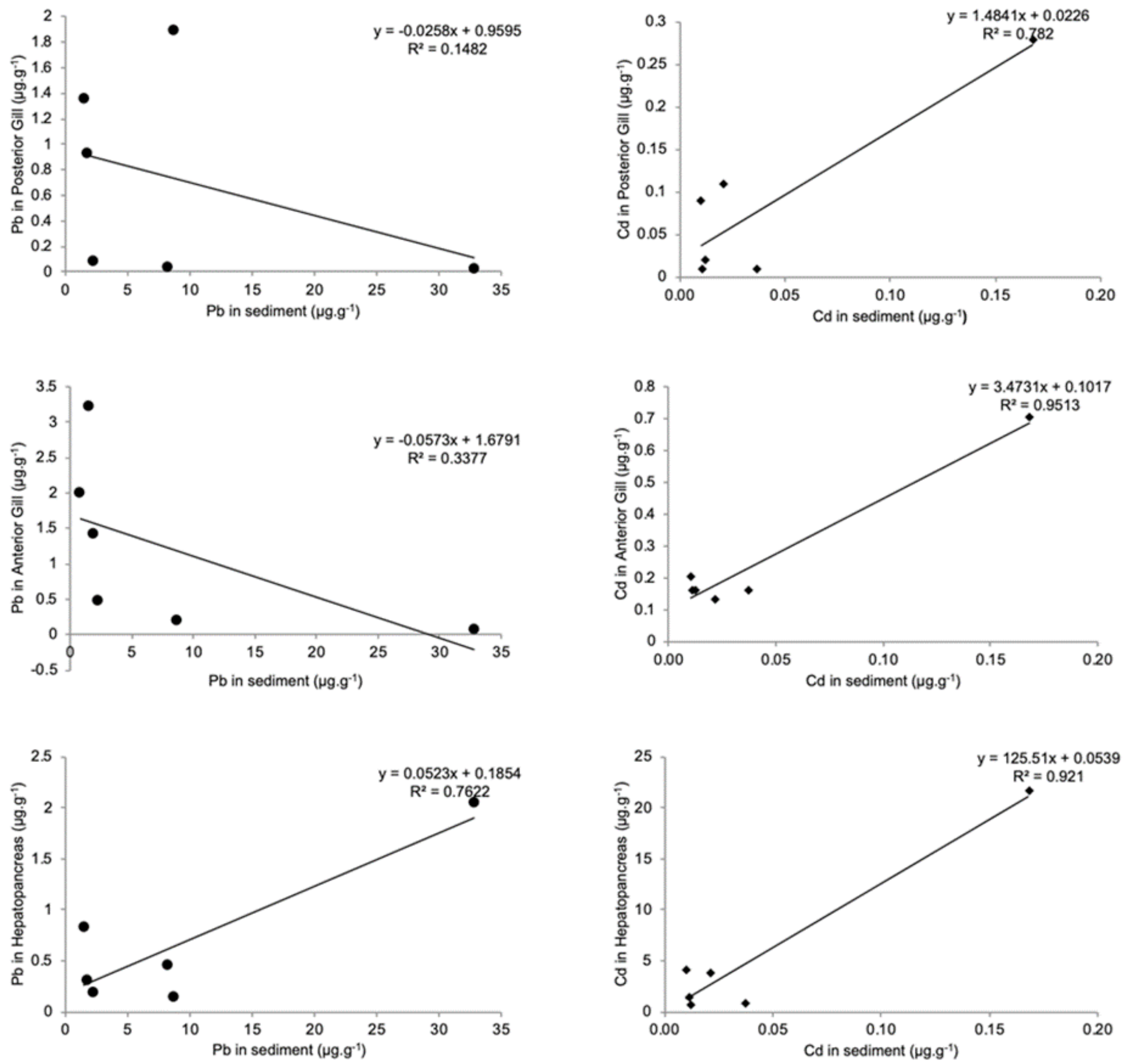
Comparisons between sediment and tissue metal concentrations: Pearson correlation coefficient analysis was used to determine if there were statistically significant relationships between the concentrations of Pb and Cd in sediments and *C. danae* soft tissues. The correlations and linear regressions are presented in Figures 2 and 3 for the March and September sampling campaigns, respectively. In March samples, correlations between Pb in sediment and *C. danae* tissues were significant only for the hepatopancreas (0.87; p=0.02), as shown in Figure 2. There were no significant correlations for Pb for samples collected in September. For Cd measured in samples collected in March (Fig. 2), significant correlations were observed between concentrations in sediment and concentrations in all crab tissues (Posterior gill: 0.91, p= 0.02; Anterior gill: 0.98, p= 0.01 and Hepatopancreas: 0.96, p= 0.0008). For Cd measured in samples collected in September (Fig. 3), the correlations were only significant for concentrations in the sediment and the samples of posterior gill (0.966; p=0.0017). However, the positive correlations are driven to a large extent by the high concentrations of Cd in sediments and tissues from crabs collected at the PT station. The Cd concentrations were much lower in sediments and crabs collected at all other stations in both the March and September sampling campaigns.

Table III. Permanova results obtained after comparisons between metals (Pb and Cd), tissues (anterior gill (AG), posterior gill (PG) and hepatopancreas (Hep)), sampling sites (VG, PT, PE, PM, AR, IC) and seasons (summer and winter) in *Callinectes danae.*

	Df	MS	Pseudo-F	P (perm)
PG ·Pb · Sites	5	1.146	8.578	0.021
PG · Pb · Season	1	3.97E-02	0.297	0.619
Residuals	5	0.1336		
AG · Pb · Sites	5	1.569	5.587	0.032
AG · Pb · Season	1	0.437	1.557	0.263
Residuals	5	0.28		
HEP · Pb · Sites	5	0.28	1.076	0.478
HEP · Pb · Season	1	0.4	1.54	0.271
Residuals	5	0.26		
PG · Cd · Sites	5	1.06E-02	1.447	0.267
PG · Cd · Season	1	1.33E-02	1.8132	0.282
Residuals	5	7.35E-03		
AG · Cd · Sites	5	4.75E-02	2.9272	0.121
AG · Cd · Season	1	4.03E-03	0.24877	0.629
Residuals	5	1.62E-02		
HEP · Cd · Sites	5	44.17	1.7882	0.156
HEP · Cd · Season	1	33.5	1.3563	0.345
Residuals	5	24.7		
Pb tissues	2	0.89281	1.5332	0.21
Residuals	33	0.58232		
Cd tissues	2	51.097	4.4576	0.001
Residuals	33	11.463		

Discussion

Relatively high Pb concentrations were detected in sediments collected from the APA-CIP estuary, with the highest concentrations observed at the PM and PT sites, which were also the sites with the highest percentage of fine particulates (i.e. mud) and OM. This might indicate that these sites are deposition zones for fine particulates with a high organic content originating from the RIR watershed that are carrying Pb from upstream abandoned



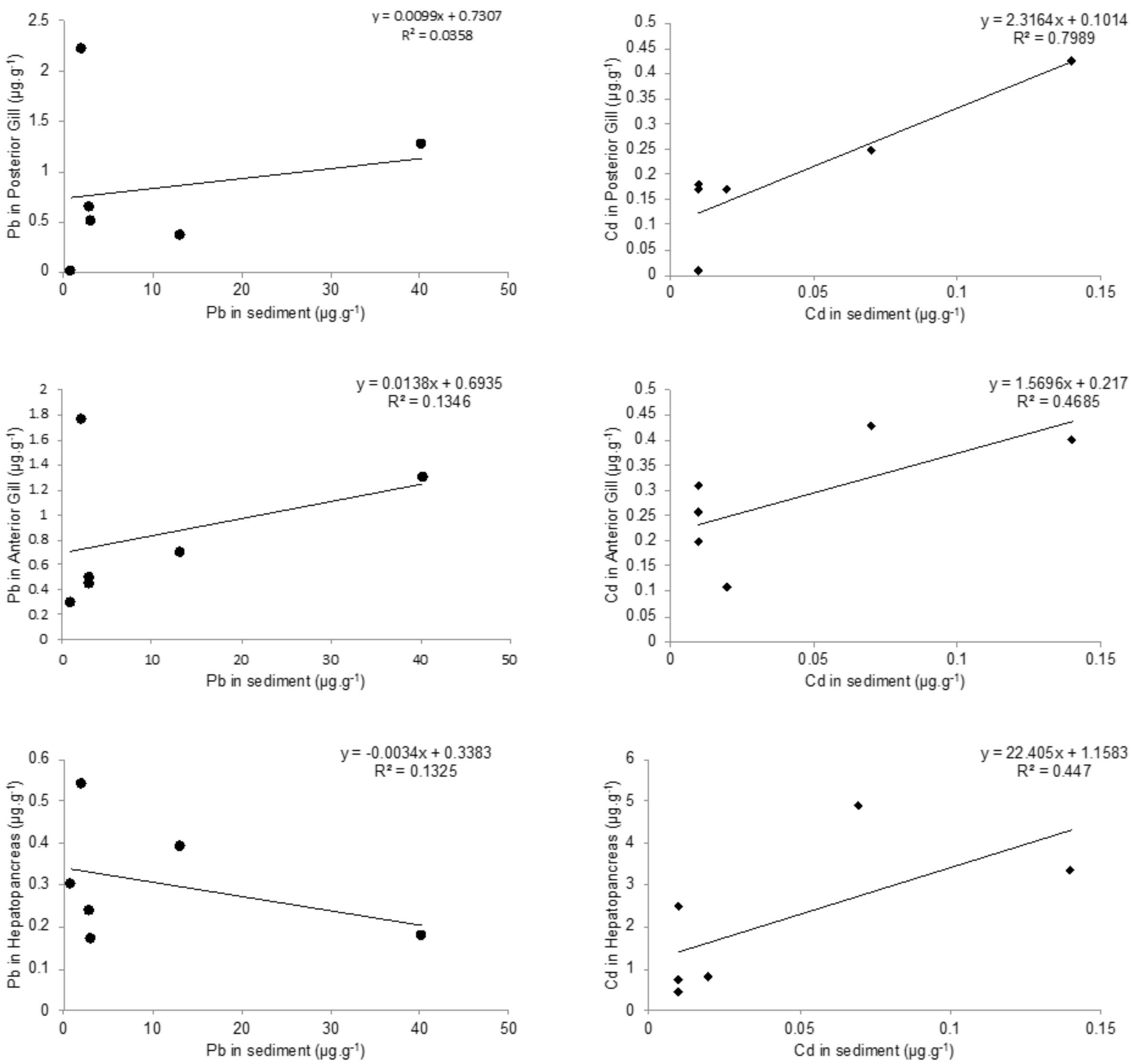
Correlation	Pb	Cd
PG x Sed	-0.38	**0.91***

Correlation	Pb	Cd
AG x Sed	-0.24	**0.98***

Correlation	Pb	Cd
Hep x Sed	**0.87***	**0.96***

Figure 2. Correlations between Pb and Cd concentrations in (µg.g^{-1}dry weight) in sediments (SED) and *Callinectes danae* posterior gill (PG), anterior gill (AG) and hepatopancreas (HEP) collected from the APA-CIP during March, 2013. Linear regression equations and R^2 are presented in each graph and correlations are presented in the box below the graphs. An asterisk (*) within the respective boxes located below the graphics indicates significant correlation values.

mining sites. There were differences in the distribution of Pb in sediments collected in March (i.e. rainy season) and September (i.e. dry season), but it is difficult to speculate on whether these differences were due to seasonal changes in rainfall. In addition, these differences between the two sampling campaigns may have been due to the two different digestion and analysis methods used for sediments collected in March and September. In contrast, the concentrations of Pb in the tissues of crabs were low. In fact, the highest Pb concentrations (not significant) in crab gills were observed in *C. danae* collected at stations PM, AR and IC, which are the sites furthest away from the



	Pb	Cd
PG x Sed	0.041	**0.966***

	Pb	Cd
AG x Sed	0.371	0.68

	Pb	Cd
H x Sed	-0.367	0.669

Figure 3. Correlations between Pb and Cd concentrations in ($\mu g.g^{-1}$dry weight) in sediments (SED) and *Callinectes danae* posterior gill (PG), anterior gill (AG) and hepatopancreas (HEP) collected from the APA-CIP during September, 2013. Linear regression equations and R^2 are presented in each graph and correlations are presented in the box below the graphs. An asterisk (*) within the respective boxes located below the graphics indicates significant correlation value.

influence of the RIR. The high sand content at these stations may have contributed to high mobility of Pb associated with sediments and uptake into crabs. In this case, the Pb uptake could be from porewater or sediment-water interface, as Tramonte *et al.* (2018) showed that this element is bioavailable in the APA-CIP. In samples of hepatopancreas, the Pb levels were slightly elevated in crabs collected in March from site PT (not statistically), but the high concentrations in hepatopancreas were not observed in crabs collected at this site in September. Overall, it appears that Pb in sediments in the APA-CIP estuary have a low bioaccumulation potential in the tissues of crabs.

On the other hand, Cd concentrations were relatively low in sediments, but were elevated to concentrations >1 µg.g^{-1} dry weight in the hepatopancreas of crabs collected at some of the monitoring stations. The concentrations of Cd in sediments were comparable to those observed in the APA-CIP by Cruz *et al.* (2019), for SEM, but the maximum concentrations were slightly lower than those reported some years before (Cruz *et al.* 2014). Such differences corroborate that the distribution of metals in sediments of APA-ICP is highly variable, as further discussed in this MS. Higher Cd values were observed in *C. danae* hepatopancreas at stations PM and PE in the March sampling campaign and at PM and PT in September. Although no statistical difference was detected, the concentrations of Cd in hepatopancreas of animals from PM were more than 25 times higher than the content in crabs from IC, for example. The concentrations of Cd were lower in the gill tissues of the crabs. There was some indication that the concentrations of Cd in sediments are correlated with the concentrations of Cd observed in the hepatopancreas and gill tissues of crabs (all tissues in summer and PG in winter). Since low concentrations of Cd were observed in sediments, feeding is likely the exposure route for this element. These data indicate that there is a threat of bioaccumulation of Cd in *C. danae*, despite the relatively low concentrations in sediments. Our data also indicate that there are different mechanisms for the bioaccumulation of Cd and Pb in these crabs.

The APA-CIP estuarine system was once considered a “reference” site with relatively low levels of chemical contamination (Azevedo *et al.* 2012; Albergaria-Barbosa *et al.* 2016), or low to moderate levels of contamination (Gusso-Choueri *et al.* 2016; Rodrigues *et al.* 2012). However, a recent monitoring study of the sea catfish, *Cathorops spixii* from APA-CIP showed that Pb and Cd contents exceeded the recommended levels for human consumption of 0.3 µg.g^{-1} for both metals (Gusso-Choueri *et al.* 2018). *C. danae* from APA-CIP presented higher Pb concentrations (up to 3.22 µg.g^{-1}) than individuals from the Santos Estuarine System (SES), a known contaminated area from São Paulo State, where the average concentrations in crabs were 0.54 µg.g^{-1} (Bordon *et al.* 2012b). Lima *et al.* (2011) also detected lower levels of Pb (0.31 µg.g^{-1}) in *C. danae* from SES than this study. The concentrations of Cd in gills and hepatopancreas from crabs in APA-CIP were comparable to those observed in crabs from SES (Bordon *et al.* 2012b).

In sediments, the concentrations of Pb in some sites of APA-CIP were two-fold higher than Pb in sediments from the SES (Mahiques *et al.* 2009). When compared to regional sediment quality guidelines (Choueri *et al.* 2009), Pb concentrations in sediments from PT exceeded the threshold for moderately polluted sites (i.e. >10.3 µg.g^{-1}) while sediment from PM was classified as highly polluted (i.e. >22.2 µg.g^{-1}). Previous studies indicate that considerable amounts of suspended sediments and contaminants are discharged into the estuary through the VG canal (Martinez *et al.* 2013; Tramonte *et al.* 2018). However, the concentrations of Pb and Cd in sediment and *C. danae* collected at the VG site closest to the canal were relatively low. This probably reflects the high sand content of the sediments, which have low capacity for adsorbing metals. On the other hand, the highest concentrations of metals were detected in the sediments and crabs collected at the PT and PM sites (Table III), which also had the highest mud content. This investigation indicates that there is contamination of the APA-CIP by Cd and Pb. This estuary is an important Marine Protected Area and actions to control pollution sources and mitigate chemical impacts should be taken.

Non-essential metals may bioaccumulate in different ways in different tissues (Martín-Díaz *et al.* 2005). In decapod crustaceans, a thin epithelium specialized in respiration and gas transfer is observed on the anterior gills, while the posterior gills have a thicker epithelium specialized in osmoregulation and ion transport (Péqueux 1995). Thus, posterior gills can participate in metal depuration (Davanso *et al.* 2013). Once metals are absorbed by the gills, they tend to be translocated to the hepatopancreas through the hemolymph in decapods (Bordon *et al.* 2016). The hepatopancreas is involved in the absorption, storage and excretion of metals, displaying hepatic, intestinal and pancreatic functions (Al-Mohanna & Nott 1989; Bhavan & Geraldine 2000).

This study indicates that gills and hepatopancreas tissues of *C. danae* exhibit relatively similar levels of Pb, while hepatopancreas tended to present higher concentrations of Cd, in both the March and September surveys. Consistent with these findings, Bordon *et al.* (2016) detected higher Cd content in *C. danae* hepatopancreas and higher Pb in gills. Cd can be taken up via Ca^{2+} channels, and high Cd uptake was seen in *Carcinus maenas* crab specimens in their post-molting period (Bondgaard & Bjerregaard 2005). Laboratory studies of blue crabs exposed to water-borne Cd have demonstrated that this metal rapidly crosses the gill epithelium and is transported via the hemolymph to the hepatopancreas (Brouwer & Lee 2007). The hepatopancreas is the major detoxifying organ in invertebrates and is used to sequester and detoxify both dietary and water-borne metals (Ahearn *et al.* 2004; Bordon *et al.* 2018). This organ can produce high amounts of chelating agents, such as metallothionein (MT) or reduced glutathione (GSH) which may complex with Cd, and this could explain the higher Cd concentrations observed in the hepatopancreas (Sastre *et al.* 1999). MT is thought to be an important ligand for Cd (Bebianno *et al.* 1993), including in *C. danae* (Bordon *et al.* 2018). However, in a previous study, we detected reduced GSH in crabs from the APA-CIP (Araujo *et al.* 2020b), which could indicate metal scavenging by this tripeptide. Duarte *et al.* (2019) observed detoxified forms of Cd in the gills and hepatopancreas of mangrove crabs. *Callinectes* individuals exposed to waterborne metals mainly take up contaminants through gills (Harris and Santos 2000). Thus, gill accumulation may be expected (Lima *et al.* 2011; Bordon *et al.* 2012b, 2016; Lavradas *et al.* 2014; Çoğun *et al.* 2017).

However, in the environment, *C. danae* may be exposed through the dietary route (Bordon *et al.* 2018), which could also explain the Cd accumulation in the hepatopancreas. Overall, it appears that crabs can take up Cd readily from water or the diet and have low capacity for detoxifying and excreting Cd. This may explain the relatively high concentrations of this metal in crabs from the estuary. However, organisms belonging to the *Callinectes* genus are highly migratory and therefore, metal concentrations in their tissues might reflect contamination from other stations (Sastre *et al.* 1999). This could be a reason for the general lack of correlations between sediment Cd contamination and the concentrations of Cd in the soft tissues of *C. danae* during winter. Another explanation could be avoidance behaviour (Burnett & Stickle 2001), in which the organisms move from the most contaminated estuarine sites to the cleaner ones in an attempt to avoid contaminated sediments. It is important to notice that two distinct methodologies were used for the quantification of metals in sediments and that the winter analysis (strong acid digestion) may have accounted not only for bioavailable elements but also for elements linked to sulfites and organic matter for example.

Bordon *et al.* (2018) demonstrated that Pb accumulation in blue crab tissues is mainly due to waterborne exposure, with a secondary contribution of exposure due to contaminated food ingestion. However, when Pb is in excess in gills, it may be re-located to other tissues via the hemolymph (Bordon *et al.* 2018; Henry *et al.* 2012). Recently, another study on Pb in mangrove crabs showed that this species has a high ability to transform Pb into detoxified forms, such as in granules or MT-bound forms in both gills and hepatopancreas (Duarte *et al.* 2020). Invertebrates possess multiple routes for detoxification of metals, which include MT binding and biomineralization (Amiard *et al.* 2006). The crab *Uca pugnax,* for example, is capable of shifting Pb from soft tissues to the carapace prior to molting, in order to depurate Pb (Bergey & Weis 2007). Moreover, induction of MT synthesis has been reported in *C. danae* gills after Pb exposure, suggesting that this metalloprotein plays a detoxification role and is involved in the Pb excretion (Bordon *et al.* 2018).

Reichmuth *et al.* (2010) observed that blue crabs transported from a contaminated area to a clean estuary displayed rapidly declining Pb concentrations in the hepatopancreas, indicating efficient Pb depuration, which these authors suggested was occurring through the molting process. The estuarine crab *Uca rapax* collected from metal-contaminated areas has been observed to exhibit compensatory biochemical and physiological adjustments, such as stronger hyper and hypo-osmotic regulatory abilities and greater gill Na+/K+-ATPase activities (Capparelli *et al.* 2016). Overall, previous studies indicate that Pb can be detoxified and excreted in crabs relatively quickly, which explains the low concentrations observed in crabs from the estuary.

A variation in *C. danae* tissue metal concentrations was noticed, mainly for the hepatopancreas. The literature reports seasonal variations in crab soft tissue metal concentrations, with higher levels during the summer: Firat *et al.* (2008) detected higher concentrations of five metals (Zn, Cu, Cr, Fe and Cd) in the crab, *Charybdis longicollis*, and Çoğun *et al.* (2017) observed higher concentrations of four metals (Cu, Cd, Zn and Pb) in *C. sapidus* soft tissues. However, the influence of seasonality may differ among different elements. For example, Pb concentrations were higher in fish and crustaceans during the dry season, while Cd was higher in fish during the rainy season (Ruelas-Inzunza *et al.* 2011).

Although this study did not assess metal concentrations by sex, a proposed pattern for *C. danae* is that couples mate inside the estuary, and females then migrate to areas under higher marine influence, while males remain in estuarine regions (Araújo *et al.* 2011).

This study demonstrates that the APA-CIP estuary is contaminated with Pb and moderately contaminated by Cd originating from discharges from the RIR of water and sediments. The content of organic matter, sand and mud in the sediments in the estuary appears to be a factor influencing the distribution of these metals. The profiles of accumulation of these metals in the soft tissues of *C. danae* indicates that these crabs show high potential for bioaccumulation of Cd and lower potential for bioaccumulation of Pb. Moreover, the results show that APA-CIP is not being effective in protect this biome from external sources of contamination and measures should be taken to mitigate contamination. Since the sources of Pb are the former mining sites located at the upper Ribeira de Iguape river Basin, the removal of the residues deposited close to the river could reduce the amount of Pb discharged in the APA-CIP. For Cd, the control of local sources, such as sewage and urban drainage, could be done by improving the local sanitation systems.

Acknowledgements

The authors would like to thank the São Paulo Research Foundation (FAPESP, grant #09/52762-6) for financial support and the National Council for Scientific and Technological Development (CNPq) for Giuliana S. Araujo's master's fellowship (grant #479899/2013-4). Paloma K. Gusso-Choueri thanks the Coordination for the Qualification of Higher-Level Staff in Brazil (CAPES). The authors are also grateful to the USP Oceanographic Institute for providing field work support at Cananéia. DMSA would like to thank CNPq for fellowships and financial support (grants #303620/2008-0, #308649/2011- and #311609/2014-7). The authors declare no conflict of interest.

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Received: Jannuary 2021
Accepted: May 2021
Published: July 2021

PANAMJAS
Pan-American Journal of Aquatic Sciences

Lead and cadmium contamination in sediments and blue crabs *Callinectes danae* from a Ramsar wetland in southeastern Brazil

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Supplementary material

Table S1: PERMANOVA´s post-hoc statistical analysis of Pb content in tissues of *Callinectes danae* among stations of APA-CIP.

	PG	AG	HEP
Groups	P(MC)	P(MC)	P(MC)
VG vs. PT	0.525	**0.02**	0.484
VG vs. PE	0.463	0.157	0.36
VG vs. PM	0.094	0.196	0.08
VG vs. AR	0.274	0.445	0.712
VG vs. IC	**0.055**	0.199	0.897
PT vs. PE	0.374	0.181	0.433
PT vs. PM	**0.008**	0.243	0.414
PT vs. AR	0.417	0.657	0.399
PT vs. IC	**0.001**	0.26	0.576
PE vs. PM	0.102	0.228	0.776
PR vs. AR	0.583	0.535	0.59
PE vs. IC	**0.051**	0.252	0.578
PM vs. AR	0.830	0.118	0.527
PM vs. IC	0.800	0.288	0.781
AR vs. IC	0.322	0.098	0.827

Table S2: PERMANOVA´s post-hoc statistical analysis of Cd content in tissues of *Callinectes danae* among stations of APA-CIP.

	PG	AG	HEP
Groups	P(MC)	P(MC)	P(MC)
VG vs. PT	**0.024**	0.358	0.332
VG vs. PE	0.673	0.881	0.078
VG vs. PM	0.45	0.305	0.938
VG vs. AR	**0.043**	0.591	0.568
VG vs. IC	0.325	0.863	0.889
PT vs. PE	0.254	0.211	0.339
PT vs. PM	0.958	0.412	0.445
PT vs. AR	0.062	0.154	0.377
PT vs. IC	0.131	0.309	0.413
PE vs. PM	0.383	0.61	0.984
PE vs. AR	0.577	0.235	0.506
PE vs. IC	0.668	0.932	0.805
PM vs. AR	0.604	0.497	0.321
PM vs. IC	0.487	0.498	0.107
AR vs. IC	0.494	0.517	0.478