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An environmental forensic approach for tropical estuaries based on metal bioaccumulation in tissues of *Callinectes danae*

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Abstract The blue crab *Callinectes danae* is distributed throughout the Atlantic coast and this study aimed to evaluate a environmental forensics approach that could be applied at tropical estuarine systems where this species is distributed, based on the metal concentrations in its tissues. For this purpose, blue crab samples were collected in 9 sites (distributed in 3 areas) along the Santos Estuarine System, state of São Paulo, Brazil. The concentrations of Al, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn were determined in gills, hepatopancreas and muscle tissues. Sediment samples were collected and analyzed in these same sites. A data distribution pattern was identified during both sampling periods (August and December 2011). In order to validate this model, a new sampling campaign was performed in March 2013 at the Santos Estuarine System and also at Ilha Grande (state of Rio de Janeiro). These data were added to the previous database (composed of the August and December 2011 samples) and a discriminant analysis was applied. The results confirmed an environmental fingerprint for the Santos Estuarine System.

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Introduction

Environmental forensics aims to identify the contamination, understand its characteristics, sources, movement and fate, allocating the responsibility among potentially responsible parties (Stout et al. 1998). It is important to clarify that environmental forensics investigations differ from traditional site assessments. Biomonitoring, for examples, approaches cosmopolite organisms to assess pollution, taking advantage that chemicals that have entered the organisms leave markers (that may be the chemical itself), reflecting this exposure (Rainbow 2002). According to Zhou et al. (2008), there is a consistency between the selected organisms and the corresponding living space. Thus, biomonitoring can directly offer information about the amounts of natural and anthropic chemicals that have entered and remained in the organisms and the corresponding effects induced, reflecting the contamination degree in the environment. Environmental forensics investigation goes beyond that. According to Mudge (2008), it aims to improve the understanding of pollutant dynamics (from the source to the environment), helping industries and other anthropogenic activities at the design stage to ensure they comply with the relevant legislation, as well as in the reconstruction of reconstructing environmental histories. This type of approach analyzes patterns of data distribution and predictions in order to characterize contamination events, enabling de development of control strategies.

In this context, some systems deserve more attention with regard to environmental forensic approaches since

they are strongly exposed to anthropogenic impacts and due to their complexity. Estuaries are environmental systems located between ocean and fresh water areas (Miranda et al. 2002). Many physical and chemical factors influence these systems, such as constant changes in pH, temperature and salinity, freshwater releases, atmospheric deposition and anthropogenic discharges (Siqueira et al. 2005; Garnier et al. 2010). Anthropogenic impacts have been increasingly intensified at tropical estuaries due to the increase of urban areas as well as industrial and agricultural activities along coastal areas in underdeveloped countries, such as Brazil (Abessa et al. 2005; Blaber 2008; Nahum et al. 2009; Duarte 2008; Barbier et al. 2010).

In the southeastern area of the state of São Paulo, Brazil, the Baixada Santista region stands out due to its distinct economic significance and its noticeable environmental importance. The most important industrial area of Brazil and the largest commercial harbor of South America are located in this region. In addition, the mangroves of the Santos Estuarine System, which surround the whole area, correspond to 43 % of the total mangrove area of the state of São Paulo (Lamparelli et al. 2001). Despite the implementation of control programs, high metal levels have still been reported in the sediment of the Santos Estuarine System (Abessa et al. 2005; Bordon et al. 2011; Hortellani et al. 2005, 2008; Luiz-Silva et al. 2008). According to Harris and Santos (2000), the local sediment may accumulate metals from industrial discharges, potentially exposing invertebrates living on or in this sediment to high concentrations through many pathways (breathing, feeding or direct contact with the sediment).

Callinectes danae Smith, 1869 (Crustacea, Decapoda, Portunidae), an omnivorous and benthic crab species, has high tolerance to salinity and can be found in brackish water environments, such as estuaries, and also in marine areas up to 75 m in depth (Melo 1996). This species is distributed throughout the Atlantic Ocean from Florida (USA) to the Southern Coast of Brazil (Costa and Negreiros-Fransozo 1998), which includes the metropolitan region of Baixada Santista. In addition, *C. danae* is an important fishery resource explored by fishermen from local communities (Severino-Rodrigues et al. 2001).

Many studies have been performed using tissues from Portunidae crab species in metal monitoring approaches (Andrade et al. 2011; Ayas and Ozugul 2011; Jop et al. 1997; Pereira et al. 2006; Reichmuth et al. 2010; Sastre et al. 1999). Among the *Callinectes* genus, the species *C. danae* has not been frequently chosen for biomonitoring purposes, and only a few studies evaluating metal concentrations in the tissues of this species (Bordon et al. 2012a, b; Virga et al. 2007; Virga and Geraldo 2008) and also in local sediment samples (Harris and Santos 2000) are available.

Since *C. danae* is a benthic species, we hypothesize that the transference of metals from the sediment to the tissues of this organism (independently from the pathways) may characterize the estuarine ecosystem where the blue crab lives. Therefore, the concentrations of metals in these tissues could reflect this distinct ecosystem, and that can, therefore, be considered an environmental fingerprint. In order to confirm if this fingerprint is local, validation procedures should be conducted.

This type of approach could be useful to confirm contamination events and/or modifications in environmental quality and alterations in this pattern could be evaluated according to the influence of the local anthropogenic activities. Effluents and/or irregular discharges would not be directly assessed and researchers would have a robust proof of the contamination event.

In this context, this study aimed to evaluate an environmental forensics approach that could be applied at tropical estuarine systems of Atlantic coast, based on metal concentration in *C. danae* tissues. The Santos Estuarine System was chosen as the study area for this purpose and the goal was to identify an environmental fingerprint for this estuary based on data distribution patterns detected by a multivariate statistic test.

Materials and methods

Sample collection

In August and December 2011, *C. danae* individuals were collected from nine sites distributed along three areas of the Santos Estuarine System, state of São Paulo, Brazil (Fig. 1; Table 1). These areas were chosen to ensure the covering of the whole estuary and crab net traps were used during these sampling campaigns. Sediment samples were collected in triplicate using a Van Veen grab sampler (sampling 10–15 cm of the superficial sediment), taken to the laboratory and stored at −20 °C until the metal determinations.

All blue crabs were collected at the intermolt stage in order to minimize the effects of lower or upper concentrations during pre-molt and molt stages (Sastre et al. 1999). The species was identified according to Melo (1996) and sex was identified according to Williams (1974). The maturation stage due to the shape and degree of adherence of the abdomen to thoracic sternites, total weight, carapace length and width were also measured (Fig. 2). Muscle tissue, hepatopancreas and gills were removed by dissection. Sediment samples were dried at

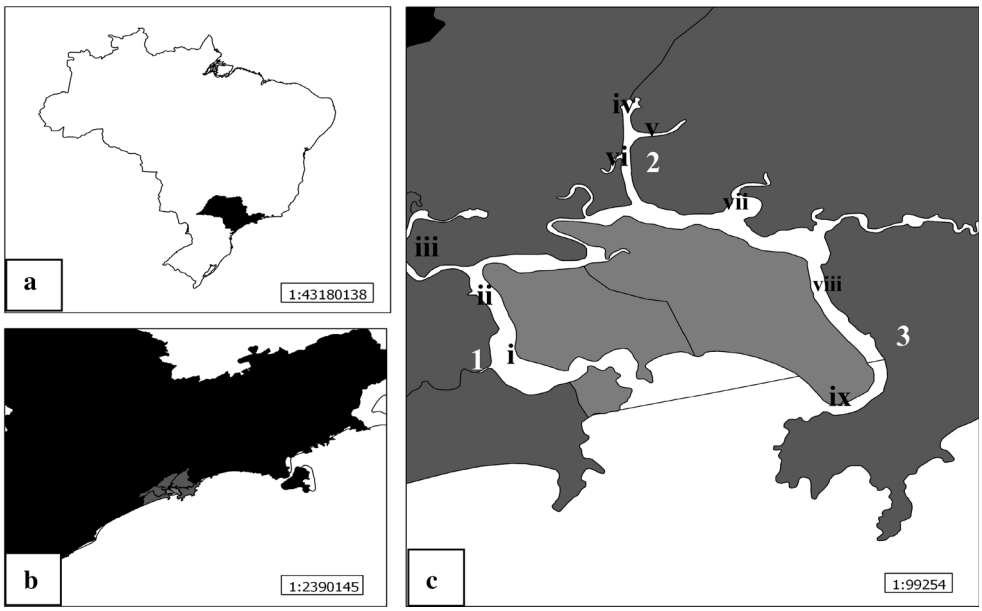

Fig. 1 Brazil (*white*) and São Paulo State (*black*) (**a**); the location of Santos Estuarine System (*gray*) in São Paulo State (**b**); and the Santos Estuarine System (**c**). The *roman numbers* indicate the sampling sites (*i–ix*); the *arabic numbers* indicate the sampling areas: São Vicente channel (*1*), the Piaçaguera channel (*2*) and the Santos channel (*3*)

Table 1 Sampling sites and their coordinates

Site	Latitude	Longitude
São Vicente channel		
i	23°58.592′S	046°25.534′W
ii	23°57.006′S	046°25.939′W
iii	23°56.093′S	046°27.832′W
Piaçaguera channel		
iv	23°52.636′S	046°22.602′W
v	23°53.059′S	046°22.056′W
vi	23°53.965′S	046°23.052′W
Santos channel		
vii	23°54.954′S	046°20.353′W
viii	23°57.157′S	046°18.303′W
ix	23°59.709′S	046°18.213′W

ambient temperature and sieved with a 2 mm net before the metal determinations.

A second sample collection was performed in March 2013 for fingerprint validation procedures. For these purposes, *C. danae* individuals were obtained from local fishermen at the Santos Estuarine System and also from a different region—Ilha Grande, Rio de Janeiro State (Fig. 3a, b). These samples were obtained from fishermen to guarantee that the fingerprint validation procedures were blind-performed and not tendentious since the exact site of collection could not be confirmed. These organisms were identified (species, sex and maturation stage), measured (carapace width and length) and dissected using the same procedures described previously.

Metal analysis

The following metals were determined in tissues and sediment samples: Al, Cd, Cr, Co, Cu, Fe, Hg, Mn, Ni, Pb and Zn. For the samples collected in August and December 2011, methodology validations regarding precision and accuracy were performed by means of certified reference materials analyses (Oyster tissue—NIST SRM 1566a; Lobster hepatopancreas—NRC TORT-2; Buffalo River sediment—NIST SRM 2704 and NIST SRM 8704—for Cd).

Samples ($\cong$1.0 g) of blue crab tissues or certified reference materials (Oyster tissue—NIST SRM 1566a; Lobster hepatopancreas—NRC TORT-2) were transferred to 100 mL glass flasks. An acid mixture consisting of sub boiling HNO_3 (1 mL), H_2SO4 (2 mL) and $HClO_4$ (1 mL) was added to the samples, which were kept for 30 min on a hot plate at 110 °C, following the method described by Lima et al. (2005).

According to U.S. EPA method 3051A recommendations (U.S. EPA 2007), an acid extraction solution consisted of sub boiling HNO_3 (9 mL) and sub boiling HCl (3 mL) was added to $\cong$1.0 g of sediment samples or certified reference materials (Buffalo River sediment—NIST SRM 2704 and NIST SRM 8704—for Cd) in microwave HP-500 vessels (PFA Teflon, fluorocarbon polymer). The digestion of the sediment samples was conducted in a high pressure microwave system (CEM Corporation, model MARS 5), according to the following method: stage-1, power: 600 W, time of temperature ramp: 9 min, temperature: 175 °C; hold time: 4.5 min.

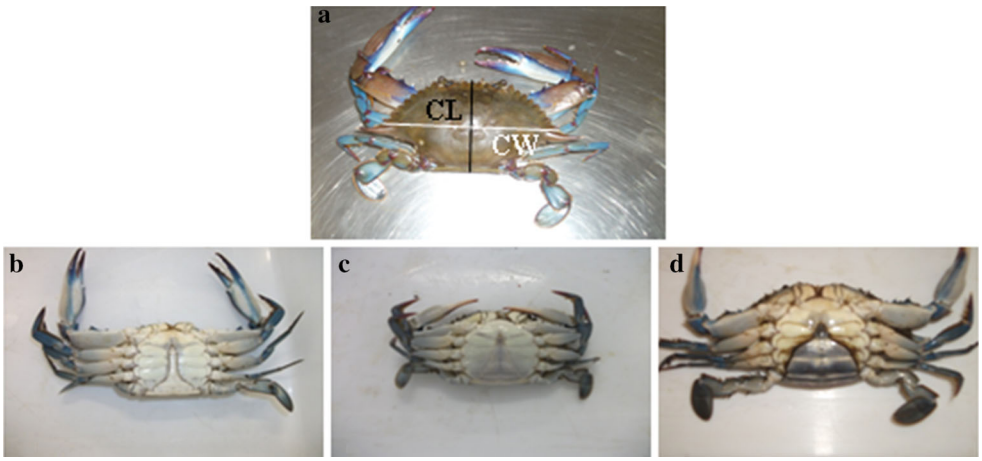

Fig. 2 *C. danae* carapace length (CL) and width (CC) (**a**); sex and maturation stage due to the shape and degree of adherence of the abdomen to thoracic sternites (**b** male, with the T form abdomen; **c** immature female, with a triangular abdomen; **d** mature female, with an oval abdomen)

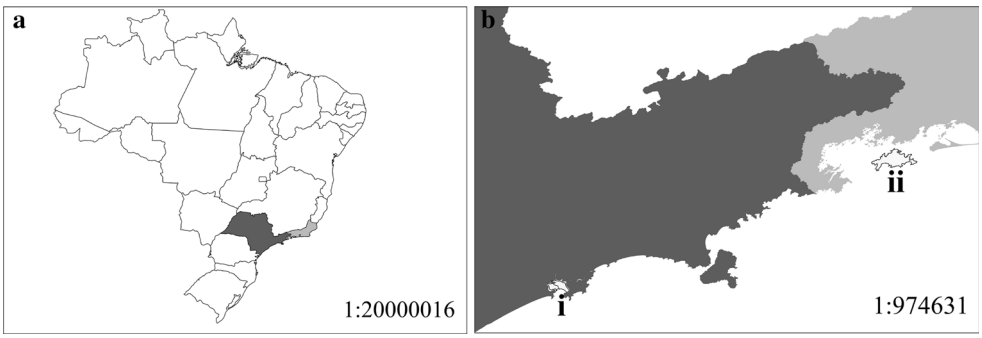

Fig. 3 São Paulo State (*black*) and Rio de Janeiro State (*gray*) (**a**); the location of the Santos Estuarine System (*i*) in São Paulo State and Ilha Grande (*ii*), in Rio de Janeiro State (**b**)

After cooling down, the extracts were transferred to 50 mL centrifuge vials and the volume was made up to 20 g (tissues) or 40 g (sediment) with Milli-Q water (resistivity 18 MΩ cm at 25 °C). The metal analyses were performed after the decanting or centrifugation of the vial contents. Before the determination of Al and Fe, the original sediment extract was diluted 100 times.

The concentrations of Al, Cd, Cr, Co, Mn, Ni and Pb in tissue samples were determined using a High-Resolution Inductively Coupled Plasma Mass Spectrometer (HR-ICP-MS) ThermoFinnigan, model Element1 and an Inductively Coupled Plasma Optical Emission Spectrometer (ICP OES) Spectro. The concentrations of Cu, Fe and Zn in tissue samples and Al, Cr, Co, Cu, Fe, Mn, Ni, Pb and Zn concentrations in sediment samples were measured using the flame mode of a Fast- Sequential Atomic Absorption Spectrometer (FS FAAS) Varian, model Spectr-AAS-220-FS. In particular, Hg concentrations in both types of samples were measured by cold vapor generation (CV AAS). The spectrophotometer was coupled to a typical FIA (Flow Analysis Injection) manifold, with a manual injection valve that injects 500 µL of digested sample at a flow of Milli-Q water (10 mL min^{-1}). The Hg^{2+} is reduced on-line by $SnCl_2$ 25 % (m/v) in HCl 25 % (v/v) at a flow rate of 1 mL min^{-1}. Argon was used as a carrier gas at a constant flow of 200 mL min^{-1}. The Cd concentration in sediment samples was determined by Graphite Furnace Atomic Absorption Spectrometer (GF AAS) Perkin Elmer, model AAnalyst 800.

The limit of detection (LOD) for each metal was calculated according to INMETRO (2011), as described below:

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

mean is mean of concentrations measured in 7 sample blanks, t is *t* Student value according to the degrees of freedom (n−1) and $\alpha = 0.05$, *SD* Standard deviation of concentrations measured in 7 sample blanks.

The obtained concentrations in sediment samples were compared to guideline levels threshold effect level (*TEL*) and probable effect level (*PEL*) of the Canadian Sediment Quality Guidelines (CCME 2001), in order to identify sediment contamination.

For samples collected in March 2013, all analyses were conducted at PUC-RIO-Pontifical Catholic University of Rio de Janeiro. The methodology validation was performed

by means of certified reference material analyses (NRC-DORM-3). The digestion mixture consisted of 2.5 mL HNO_3 (Merck), which were added to tissues or certified reference material samples (250 mg) on a hot plate at 80–100 °C. After cooling down, the extracts were transferred and the volume was made up to 25 mL with ultrapure water. Chemical analyses were conducted using an ICP–MS Perkin Elmer, model Nexion 300X (Perkin-Elmer, Norwalk, CT, USA).

Statistical analyses

Statistical tests were performed using the STATISTICA 10® software package (Statsoft). Data were log-transformed: log(x + 1). The Kruskal- Wallis and Mann–Whitney tests were applied in order to identify statistical differences between the evaluated tissues regarding metal concentrations ($p < 0.05$). A discriminant canonical analysis was performed to determine which metal coefficients distinguished gills, hepatopancreas and muscles from sediment samples and to identify any statistical similarity among data due to overlapping of statistical ellipses. This multivariate dependence technique is applied when the dependent variable is categorical (nominal or non-metric) and the independent variables are metric. After defining the groups (tissues and sediment), the individual data elements (metals) of each group were collected. Each group was plotted in the reduced multivariate space, in which the new axes (functions 1, 2, and 3) explain a proportion of the total variability in the data. Only functions that presented $p < 0.05$, variance above or close to 10 % or eigenvalues above or close to 1 were considered statistically significant. Group centroids were plotted using the canonical discriminant functions evaluated at group means, and each circle indicates the 95 % confidence ellipses. Superimposed on this plot is the association between the new axes and the determined metals. Standardized discriminant function coefficients are a measure of the association between the metals and the axes. Those groups in which ellipsoids did not overlap signify differences between groups. The correlation between the position of each group relative to the standardized discriminant function coefficients determines the metal or metals responsible for its separation.

Results and discussion

Sample description and validation of the analytical methodology

In August 2011, a total of 78 males and 4 females were analyzed (n = 82). Among them, 64 were in the immature stage and 18 in the mature stage. The mean carapace length, the mean carapace width and the mean weight were, respectively, 46.5 ± 0.6 mm, 80.2 ± 1.0 mm and 65.7 ± 2.6 g. In December 2011, a total of 83 males and 3 females were analyzed (n = 86). Of these, 9 were in the immature stage and 77 in the mature stage. The mean carapace length, the mean carapace width and the mean weight were, respectively, 48.0 ± 0.7 mm, 82 ± 1.0 mm and 72.2 ± 2.9 g. A predominance of males was observed, probably due to the migratory behavior of the females to marine areas (Barreto et al. 2006).

The certified reference materials were analyzed in triplicate. The recovery of most of the determined metals was above 70 % (Tables 2, 3). Regarding Hg, the obtained concentrations of the oyster tissue replicates were below the LOD and validation for this element was conducted according to TORT-2 results (Table 2).

Al and Cr are known as refractory elements, strongly connected with silicates that are difficult to digest, which explains low recoveries in the Buffalo River sediment replicates (Table 3). According to the 3051A method (U.S. EPA 2007), silicates may not be dissolved and in some cases may isolate target analyzed elements. However, based on the validation data presented in this method ($[Cr] = 77.1\ \mu g\ g^{-1}$), the Cr recovery in Buffalo River sediment replicates was 108 %, thus, considered an appropriated result.

Development and identification of an environmental fingerprint

For results obtained in August 2011 (Table 4), the Kruskal–Wallis test detected that Cd, Co, Cr, Ni and Zn concentrations were higher in the hepatopancreas ($p < 0.05$); Al, Cu, Fe, Mn and Pb concentrations were higher in the gills ($p < 0.05$) and only Hg concentrations were higher in muscle ($p < 0.05$). The Cd, Co, Ni and Pb concentrations in tissues of the crabs collected in December 2011 were below the limit of detection (Table 5). According to the Kruskal–Wallis test, Cr and Zn concentrations were higher in the hepatopancreas ($p < 0.05$); Al, Cu, Fe and Mn were higher in the gills ($p < 0.05$) and Hg concentrations were higher in muscles ($p < 0.05$) (Table 5). Comparing the results of this study with previous publications, Virga et al. (2007) and Virga and Geraldo (2008) observed higher concentrations of Cd, Cr, Cu, Pb and Zn in *Callinectes* sp. tissues from the Santos Estuarine System.

Cu is an integral part of the haemocyanin molecule in the haemolymph. Engel (1987) found Cu in higher concentrations during the intermolt stage due to the increase of metallothioneins during haemocyanin synthesis. Zn participates in Zn-dependent enzymatic reactions (Elumalai

Table 2 Mean metal concentrations (μg g^{-1}), certified metal concentrations (μg g^{-1}) and recoveries (%) for TORT-2 and Oyster tissue certified materials; and limits of detection (μg g^{-1})

Metal	Al	Cd	Co	Cu	Cr	Fe	Hg	Mn	Ni	Pb	Zn
Lobster hepatopancreas—TORT-2											
Mean (μg g^{-1})	a	17.6	0.43	90.8	0.58	117	0.22	10.2	1.8	0.26	179
Certif. Conc (μg g^{-1})	a	26.7	0.51	106	0.77	105	0.27	13.6	2.5	0.35	180
Recovery (%)	a	66	84	86	76	112	81	75	71	74	99
Oyster tissue											
Mean (μg g^{-1})	151.3	4.11	0.42	53.5	1.30	541	<LOD	8.7	1.81	0.262	852
Certif. Conc (μg g^{-1})	202.5	4.15	0.57	66.3	1.43	539	0.064	12.3	2.25	0.371	830
Recovery (%)	75	99	73	81	91	100	–	75	80	71	103
Limit of detection (μg g^{-1})											
	0.059	0.00002	0.00004	0.01	0.003	0.3	0.001	0.0004	0.003	0.0004	0.02

LOD limit of detection

[a] There is no certified concentration

Table 3 Mean metal concentrations (μg g^{-1}), certified metal concentrations (μg g^{-1}) and recoveries (%) for Buffalo River sediment certified materials; and limits of detection (μg g^{-1})

Metal	Al (%)	Cd	Co	Cu	Cr	Fe (%)	Hg	Mn	Ni	Pb	Zn
Buffalo River sediment											
Mean (μg g^{-1})	2.53	2.73	11.2	93.7	83.9	3.36	1.65	555.3	45.5	153.4	441.9
Certif. Conc (μg g^{-1})	6.11[a]	2.94[b]	14.0[a]	98.6[a]	135.0[a]	4.11[a]	1.47[a]	555.0[a]	44.1[a]	161.0[a]	438.0[a]
Recovery (%)	41	93	80	95	62	82	112	100	103	95	100
Limit of detection (μg g^{-1})											
	0.0001	0.001	0.014	0.029	0.025	0.00003	0.0004	0.002	0.027	0.016	0.035

LOD limit of detection

[a] NIST SRM 2704

[b] NIST SRM 8704

et al. 2007; Haya et al. 1983) that occurs in the gills and in the hepatopancreas, while Fe plays an important function in enzymatic and respiratory processes in crustaceans (Ong Che and Cheung 1998). Since haemocyanin is actively involved with the respiration process and Fe is an essential element, these are the probable reasons for the high concentrations of these metals (Cu, Fe and Zn) in the gills and the hepatopancreas.

Except for Hg, metal concentrations in gills and hepatopancreas were consistently higher than in muscle tissue. In previous studies conducted with *Callinectes sapidus* (Karouna-Reiner et al. 2007; Reichmuth et al. 2009, 2010; Sastre et al. 1999) and *Carcinus maenas* (Coelho et al. 2008), Hg concentrations were also higher in muscle tissue. For *Carcinus maenas* (Pereira et al. 2006), diet was confirmed to be the main pathway for Hg, since inorganic mercury (present mainly in the dissolved and particulate fraction) is eliminated faster than organic mercury fractions, which are incorporated mainly through the diet. According to Reichmuth et al. (2010), Hg in aquatic systems tends to be methylated, tightly bound to the cellular membrane and lipids, and, thus less likely to be depurated. Because this, Hg concentrations tend to be higher in crab muscle compared to other tissues, independent of the exposure route.

The metal concentrations in sediment samples collected in August and December 2011 are presented in Table 6. In August 2011 and December 2011, the median metal concentrations in sediment samples (except for Hg) were below the TEL value, indicating no risks to biota during the

Table 4 Median, minimum and maximum concentrations (μg g^{-1}) in *C. danae* tissues collected in August/2011 (n = 82 individuals × 3 types of tissue = 246)

	Al	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Gills											
Median	28.9[a,b]	0.021[a]	0.067[a]	0.103[b]	41.9[a]	113[a]	0.02[a]	9.8[a]	0.22[a]	0.139[a]	15.8[a]
Minimum	<LOD	0.009	0.011	<LOD	17.3	21	<LOD	1.4	0.05	0.011	7.6
Maximum	1131.7	0.083	70.496	3.888	80.2	840	0.06	84.9	14.74	4.741	29.9
Hepato											
Median	5.2[a]	0.041[a]	0.187[a]	0.110[a]	25.5[a]	47[a]	0.05[a]	5.3[a]	0.46[a]	0.058[a]	35.6[a]
Minimum	<LOD	0.004	0.014	<LOD	3.1	11	<LOD	1.9	0.10	0.005	13.8
Maximum	280	0.365	0.484	6.654	157.1	193	0.14	58.6	2.78	1.014	87.8
Muscle											
Median	3.9[b]	0.004[a]	0.019[a]	0.091[a]	9.4[a]	12[a]	0.08[a]	1.0[a]	0.14[a]	0.019[a]	27.7[a]
Minimum	<LOD	0.003	0.009	<LOD	3.1	<LOD	<LOD	0.5	<LOD	<LOD	16.4
Maximum	40.1	0.024	0.062	4.416	18.3	771	0.34	31.5	0.28	0.131	46.9

For each metal, values with the same letters indicate statistical significance (Kruskal–Wallis: $p < 0.05$)

LOD limit of detection

Table 5 Median, minimum and maximum concentrations (μg g^{-1}) in *C. danae* tissues collected in December/2011 (n = 86 individuals × 3 types of tissue = 258)

	Al	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Gills											
Median	122.0[a]	<LOD	0.400	0.490[a]	36.8[a]	268[a]	0.02[a]	15.0[a]	<LOD	0.400	13.9[a]
Minimum	14.5	<LOD	<LOD	<LOD	13.2	21	<LOD	2.7	<LOD	<LOD	5.9
Maximum	816.5	<LOD	0.437	1.309	71.7	820	0.12	193.0	<LOD	5.824	39.0
Hepato											
Median	25.5[a]	0.400	0.400	0.791[a]	29.7[b]	50[a]	0.05[a]	7.7[a]	0.40	<LOD	32.5[a]
Minimum	9.2	<LOD	<LOD	<LOD	7.7	20	<LOD	1.9	<LOD	<LOD	10.4
Maximum	107.5	0.761	0.814	4.847	186.8	242	0.28	51.6	2.37	<LOD	261.3
Muscle											
Median	12.5[a]	<LOD	<LOD	0.458[b]	9.5[a]	9[a]	0.08[a]	2.1[a]	<LOD	<LOD	23.9[a]
Minimum	7.6	<LOD	<LOD	<LOD	4.2	<LOD	<LOD	1.3	<LOD	<LOD	16.2
Maximum	73.2	<LOD	<LOD	0.947	15.3	29	0.48	12.9	<LOD	<LOD	35.9

For each metal, values with the same letters indicate statistical significance (Kruskal–Wallis: $p < 0.05$)

LOD limit of detection

sampling periods. According to Siqueira et al. (2005), although the metal concentrations in sediments may be higher than in tissues samples, this does not mean that the metals are bioavailable. Variations in the physical–chemical parameters of the water column, such as pH, temperature, salinity, dissolved oxygen, etc. (common in estuarine systems) may create this availability. According to Quináglia (2006), some variables (such as organic matter, percentage of mud, etc.) can dynamically compete to absorb metals, reducing their bioavailability.

All individuals were included in the statistical analyses in order to ensure that the information regarding environmental quality provided by the environment itself was accounted for (August 2011: n = 82 individuals × 3 types of tissue = 246; December 2011: n = 86 individuals × 3 types of tissue = 258).

Table 6 Median, minimum and maximum concentrations in sediment samples collected in August/2011 (n = 9 sites × 3 samples = 27) and December/2011 (n = 9 sites × 3 samples = 27); TEL and PEL values according Canadian Sediment Quality Guidelines (CCME 2001)

	Al %	Cd (μg g^{-1})	Co (μg g^{-1})	Cr (μg g^{-1})	Cu (μg g^{-1})	Fe %	Hg (μg g^{-1})	Mn (μg g^{-1})	Ni (μg g^{-1})	Pb (μg g^{-1})	Zn (μg g^{-1})
August/2011											
Median	1.47	0.10	5	17.8	11.1	1.13	0.40	180.6	8.72	10.1	53.3
Minimum	0.3	<LOD	<LOD	3.5	<LOD	0.18	0.06	17.5	<LOD	<LOD	6.0
Maximum	7.34	3.27	15	76.3	142.5	13.83	1.96	2858.3	63.93	253.1	1322.1
December/2011											
Median	1.57	0.10	6	20.7	5.2	1.09	0.12	163.4	8.73	5.8	35.6
Minimum	0.20	<LOD	<LOD	3.3	<LOD	0.11	0.05	13.2	<LOD	<LOD	5.1
Maximum	7.97	3.41	12	89.9	34.7	5.97	2.67	612.8	37.16	76.3	233.2
CCME (2001)											
TEL (μg g^{-1})	–	0.70	–	52.3	18.7	–	0.13	–	15.90	30.2	124.0
PEL (μg g^{-1})	–	4.21	–	160.0	108.0	–	0.70	–	42.80	112.0	271

LOD limit of detection

– There is no TEL/PEL concentration

The discriminant analysis using the concentration data of the samples collected in August 2011 detected three significant functions ($p < 0.05$) (Table 7). Scatter plots of functions are presented in Fig. 4a, b. In Fig. 4a, data of the three tissues were consistently separated from the sediment data. Pb and Zn presented the highest coefficients (0.84 and −1.05, respectively) and contributed most to the discriminatory power of function 1 (Table 7). In Fig. 4b, gill and muscle data were separated by the power of function 2 (with the contribution of Zn coefficient: −1.41). There was a correspondence between sediment and hepatopancreas data and ellipses were overlapped.

The discriminant analysis was also performed using concentration data of the samples collected in December 2011 and detected three significant functions ($p < 0.05$) (Table 8). Scatter plots of functions are presented in Fig. 5a, b. In Fig. 5a, data of the three tissues were also separated from the sediment data. The element Co presented the highest coefficient (−0.96) and contributed most to the discriminatory power of function 1 (Table 8). In Fig. 5b, gill and muscle data were separated by the power of function 2 (with the contribution of Fe and Zn coefficients: 0.86 and −1.16, respectively). As before, there was a correspondence between sediment and hepatopancreas data and ellipses were overlapped.

The results of the discriminant analyses seem to reflect a natural pathway, where metals absorbed to gills through respiration reach the hepatopancreas to be detoxified. The hepatopancreas also receives and detoxifies contaminants from feeding. It is known as a deposit organ, where low-weight proteins such as metallothioneins are found and bind to certain metals (Engel and Brouwer 1984; Harris and Santos 2000; Sastre et al. 1999). In this process, metals may be excreted as granules in order to maintain homeostasis (Hopkin and Nott 1979). Since the hepatopancreas has high concentrations of these proteins, this may explain the higher concentrations of metals in this tissue compared to others. After metabolization, the hepatopancreas distributes its content to other tissues, which include muscle tissue and gills, explaining the few overlapped data.

The median concentrations of Pb and Zn in August/2011 and the median concentration of Co in December/2011 were higher in sediment samples when compared to tissues samples. Therefore, Figs. 4a and 5a reflect the power of their coefficients in the identification of differences in magnitude between tissue and sediment concentrations.

According to Depledge (1989), Zn in the haemolymph is bound to proteins, in particular, copper-rich haemocyanin. As substantial changes in haemolymph protein concentrations occur in decapods with alterations in nutritional state,

Table 7 Results of discriminant function analysis on metal concentrations in samples of sediment and tissues of *C. danae* collected in August/2011

Wilks' λ:0.001	p < 0.0000		
Discriminant function	1	2	3
Eigenvalue	16.86	4.46	1.05
Cum. proportion	0.75	0.95	1.00
X^2 value	1401.4*	639.1*	190.1*
Degrees of freedom	33	20	9
Variables	Standardized discriminant function coefficients		
Cr	0.37	0.14	−0.23
Fe	−0.46	0.46	0.14
Zn	−1.05	−1.41	0.61
Pb	0.84	0.66	−1.46
Cu	−0.16	0.67	0.16
Mn	0.6	0.22	0.42
Al	−0.49	0.05	−0.4
Ni	0.26	−0.03	1.13
Co	0.39	−0.04	0.18
Hg	0.01	−0.21	−0.69
Cd	−0.22	0.02	0.66
	Means of canonical variables		
Gills	−1.01	3.00	−0.47
Hepatopancreas	−1.71	−0.63	1.46
Muscles	−1.31	−2.28	−1.03
Sediment	12.27	−0.25	0.16

* Significant result

changes in the relative distributions of Cu and Zn are expected. Thus, Zn may be involved in processes that lead to the distribution of metals in the organism and this explains the contribution of its coefficient in the separation of gills and muscle tissue in the Figs. 4b and 5b.

Although metal concentrations in sediment samples were much higher than in tissues samples, a correspondence between the hepatopancreas data and metal content in the sediment could be inferred. Branco and Verani (1997) recorded that sand was one of the most frequent items in the stomach contents of *C. danae* and suggested that ingestion occurred with preys such as other crustaceans and polychaete worms. Mantelatto and Christofoletti (2001) reported that 48.7 % of the natural diet of *Callinectes ornatus* was composed by sediment. Reichmuth et al. (2009) reported that 60 % of the stomach contents of *Callinectes sapidus* corresponded to ingested sediment. Based on this information and on the graphical correspondence between data from sediment in the hepatopancreas in the canonical discriminant analysis, it could be inferred that these organisms ingest the surrounding sediment. However, the metal content in the sediment is not completely absorbed, as losses may occur during digestive and other metabolic processes.

These results allowed the identification of a data distribution pattern that was repeated in August and December 2011, suggesting the existence of an environmental fingerprint for the Santos Estuarine System that could be related to the sediment. Most studies have focused on the assessment of metal concentrations absorbed by organisms, either in natural or simulated conditions in the laboratory (Jop et al. 1997; Sastre et al. 1999; Reichmuth et al. 2009,

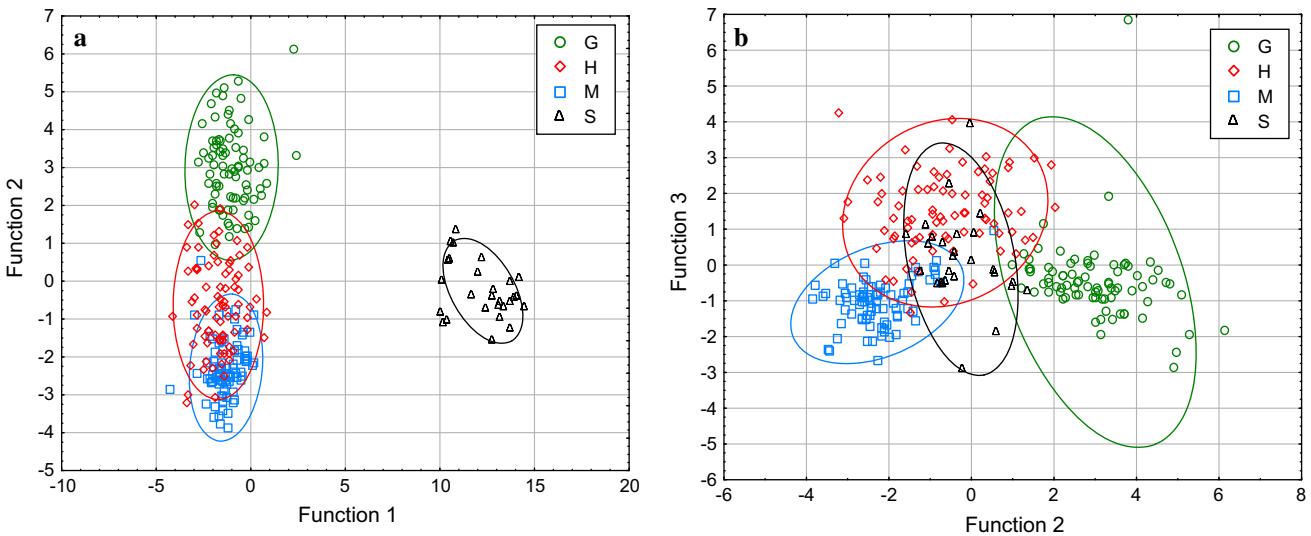

Fig. 4 Scatter plots of discriminant function 1 versus discriminant function 2 (**a**) and discriminant function 2 versus discriminant function 3 (**b**) using samples collected in August/2011 (Legend: *G* gills; *H* hepatopancreas; *M* muscles; *S* sediment)

Table 8 Results of discriminant function analysis on metal concentrations in samples of sediment and tissues of *C. danae* collected in December/2011

Wilks' λ:0.001	$p < 0.0000$		
Discriminant function	1	2	3
Eigenvalue	37.61	9.17	1.05
Cum. proportion	0.79	0.98	1.00
X^2 value	1850.9*	840.7*	199.5*
Degrees of freedom	33	20	9
Variables	Standardized discriminant function coefficients		
Co	−0.96	0.33	0.55
Fe	0.11	0.86	−0.15
Zn	0.54	−1.16	−0.53
Hg	0.44	−0.57	0.51
Cu	0.20	0.41	−0.48
Cr	−0.44	−0.02	−1.04
Al	0.44	0.01	0.45
Pb	−0.32	0.01	0.54
Mn	−0.01	0.31	−0.20
Cd	0.25	0.21	−0.16
Ni	−0.10	0.39	0.01
	Means of canonical variables		
Gills	2.69	3.96	0.62
Hepatopancreas	2.15	−0.58	−1.50
Muscles	1.02	−3.68	0.90
Sediment	−18.71	0.96	−0.11

* Significant result

2010). Recently, Bastami et al. (2012) (assessing Cd, Co, Cu, Ni and Pb), Hosseini et al. (2012) (assessing Fe, Hg, Ni and P) and Khoei and Bastami (2013) (assessing Hg) observed that the metal concentrations in the sediment were also higher than those observed in the carapaces, muscle and hepatopancreas of the *Portunus pelagicus* crab species from the Persian Gulf.

The present study not only assessed the metal concentrations, but also investigated the relationship between these tissues, the local sediment and the study area, and identified a pattern of data distribution. This approach adds new information about using these three tissues together to evaluate a bioindicator species. The assessment based only on the metal concentrations or only on one tissue could lead to a misinterpretation of what is actually happening in the estuarine ecosystem. Thus, the risk of false positives of a contamination event is reduced, since the concentration variations in each tissue are included. These variations are related to the complexity of the Santos Estuarine System, according to the different processes that rapidly alter metal availability.

In general, the use of the three tissues is more robust to identify real changes in levels of individual exposure. This approach considers that the complexity of the system can lead to rapid changes in metal levels without, however, being related to an insertion resulting from anthropogenic means.

Validation procedures of this model were performed using samples collected in March 2013 from the other study area, Ilha Grande Bay, in order to test the robustness of the model throughout the time (based on the sampling campaigns conducted in August and December 2011) with regard to the local specificity of the model.

Nine males were collected at the Santos Estuarine System. All individuals were considered in the mature stage. The mean carapace length and width and the mean weight were, respectively, 50 ± 1 mm, 87 ± 2 mm and 83 ± 8 g. Seven males were collected at Ilha Grande and all the individuals were also considered in the mature stage. The mean carapace length and width and the mean weight were, respectively, 49.3 ± 0.7 mm, 113 ± 2 mm and 80 ± 4 g.

The certified reference material DORM-3 was analyzed and recoveries were above 70 % (Table 9). For the samples collected at the Santos Estuarine System, Cd, Co, and Cu concentrations were higher in the hepatopancreas; Al, Fe and Pb concentrations were higher in gills; Hg and Zn concentrations were higher in the muscle tissue (Table 10). For the samples collected at Ilha Grande, all concentrations in the muscle tissue were below the LOD (Table 11). Therefore, the Mann–Whitney test was performed to compare metal concentrations in gills and hepatopancreas. Cd and Ni concentrations were higher in the hepatopancreas; Al, Fe and Pb concentrations were higher in gills. In general, the concentrations of samples collected at the Santos Estuarine System were higher than those obtained from samples collected at Ilha Grande.

In order to test the robustness throughout time and the local specificity of the environmental fingerprint model, the concentration data of samples collected from Santos Estuarine System (Fig. 6a, b) and data obtained from Ilha Grande samples (Fig. 7a, b) in March 2013 were separately added to the previous discriminant analysis database (August and December 2011).

In Figs. 6a and 7a, function 1 contributed to the separation of tissues and sediment samples. In Fig. 6b, the overlapping of the new data with the previous database was clearly observed, which was not observed in Fig. 7b. Also, the statistical ellipses were inverted in Fig. 7b. The results confirmed the robustness throughout

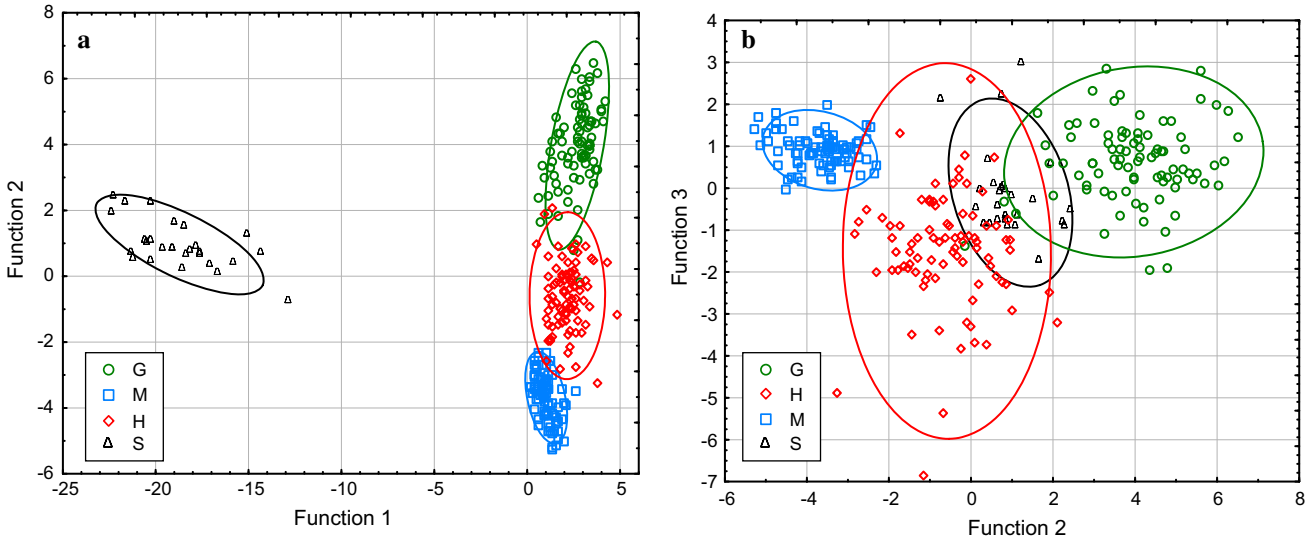

Fig. 5 Scatter plots of discriminant function 1 versus discriminant function 2 (**a**) and discriminant function 2 versus discriminant function 3 (**b**) using samples collected in December/2011 (Legend: *G* gills; *H* hepatopancreas; *M* muscles; *S* sediment)

Table 9 Mean metal concentrations (μg g^{-1}), certified metal concentrations (μg g^{-1}) and recoveries (%) for DORM-3 certified materials; and limits of detection (μg g^{-1})

DORM-3										
Metal	Al	Cd	Cu	Cr	Fe	Hg	Mn	Ni	Pb	Zn
Mean (μg g^{-1})	1981	0.30	2.36	14.5	363	0.38	2.8	1.13	0.282	50.6
Certif. Conc. (μg g^{-1})	1700	0.29	1.89	15.5	347	0.382	4.6	1.28	0.395	51.3
Rec (%)	117	104	125	93	105	100	61	88	71	99

Limit of detection (μg g^{-1})											
Metal	Al	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
	0.2	0.001	0.001	0.024	0.01	0.5	0.001	0.003	0.003	0.025	0.05

Table 10 Median, minimum and maximum concentrations (μg g^{-1}) in *C. danae* tissues collected from the Santos Estuarine System in March/2013 (n = 9 individuals × 3 types of tissue = 27)

	Al	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Gills											
Median	$85.4^{a,b}$	0.019^{a}	0.049	0.972	28.7^{a}	152^{a}	0.01^{a}	5.5	0.16	0.387^{a}	$11.5^{a,b}$
Minimum	30.7	0.013	0.033	0.796	19.5	50	<LOD	2.8	0.15	0.193	8.9
Maximum	105.6	0.043	0.073	1.639	66.9	280	0.03	20.8	1.72	0.949	21.1
Hepato											
Median	4.7^{a}	$0.063^{a,b}$	0.111^{a}	1.146	41.4^{b}	75	0.03	4.4	0.31	0.199^{b}	20.5^{a}
Minimum	2.4	0.020	0.047	0.500	22.3	31	0.02	1.1	0.15	0.112	16.0
Maximum	11.8	0.183	0.180	1.422	64.8	151	0.05	23.2	0.89	0.218	33.2
Muscle											
Median	1.1^{b}	0.014^{b}	0.027^{a}	1.055	$10.9^{a,b}$	18^{a}	0.04^{a}	2.6	0.14	$0.093^{a,b}$	27.6^{b}
Minimum	<LOD	<LOD	0.007	0.796	8.7	5	0.02	0.2	0.04	<LOD	15.4
Maximum	6.5	0.036	0.072	1.819	23.9	139	0.06	56.7	1.06	0.136	52.5

For each metal, values with the same letters indicate statistical significance (Kruskal–Wallis: $p < 0.05$)

LOD limit of detection

Table 11 Median, minimum and maximum concentrations (μg g^{-1}) in *C. danae* tissues collected from the Ilha Grande in March/2013 (n = 7 individuals × 3 types of tissue = 21)

	Al	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Gills											
Median	18.1[a]	0.007[a]	0.015	0.083	8.4	64[a]	0.002	2.2	0.07[a]	0.223[a]	5.0
Minimum	4.6	0.004	0.006	<LOD	2.9	5	<LOD	1.6	0.03	<LOD	2.4
Maximum	34.4	0.018	0.021	0.178	26.4	110	0.004	8.6	0.08	0.613	6.5
Hepato											
Median	0.6[a]	0.041[a]	0.025	0.092	10.5	6[a]	0.002	1.9	0.18[a]	<LOD[a]	9.6
Minimum	<LOD	0.013	0.004	0.080	2.1	2	<LOD	0.2	0.02	<LOD	2.2
Maximum	0.8	0.117	0.051	0.154	17.3	12	0.007	11.2	0.23	<LOD	11.6
Muscle											
Median	<LOD	<LOD	<LOD	<LOD	0.04	<LOD	<LOD	<LOD	<LOD	<LOD	0.2
Minimum	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Maximum	<LOD	<LOD	<LOD	<LOD	0.06	<LOD	<LOD	<LOD	<LOD	<LOD	0.2

For each metal, values with the same letters indicate statistical significance (Mann–Whitney: $p < 0.05$)

LOD limit of detection

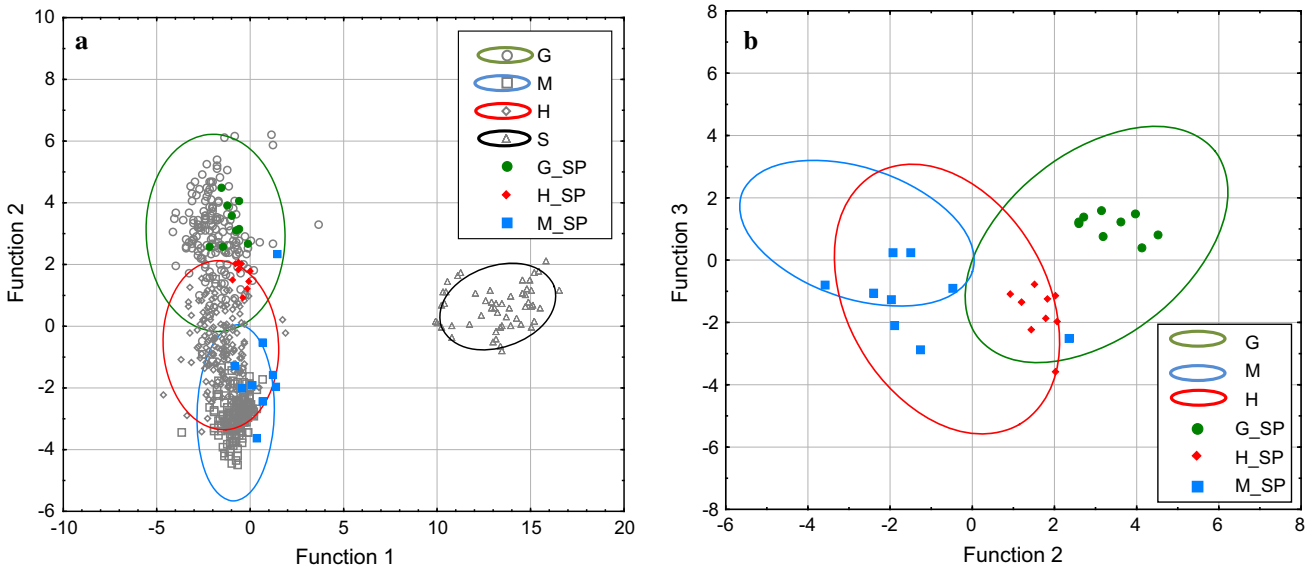

Fig. 6 Scatter plots of discriminant function 1 versus discriminant function 2 (**a**) and discriminant function 2 versus discriminant function 3 (**b**) using previous database (August and December/2011: *G* gills; *H* hepatopancreas; *M* muscles; *S* sediment and their respective *ellipses*) and samples from the Santos Estuarine System collected in March/2013 (M_SP = muscle; H_SP = hepatopancreas; G_SP = gills)

time and the local specificity of the environmental fingerprint model.

Thus, it was concluded that the presented model could identify an environmental fingerprint, representing the Santos Estuarine System conditions. According to the conducted validation procedures and the fact that the blue crab *Callinectes danae* is distributed throughout the Atlantic coast, this model could be applied at other estuarine systems in order to detect contamination events or irregular discharges due to alterations in the data distribution patterns.

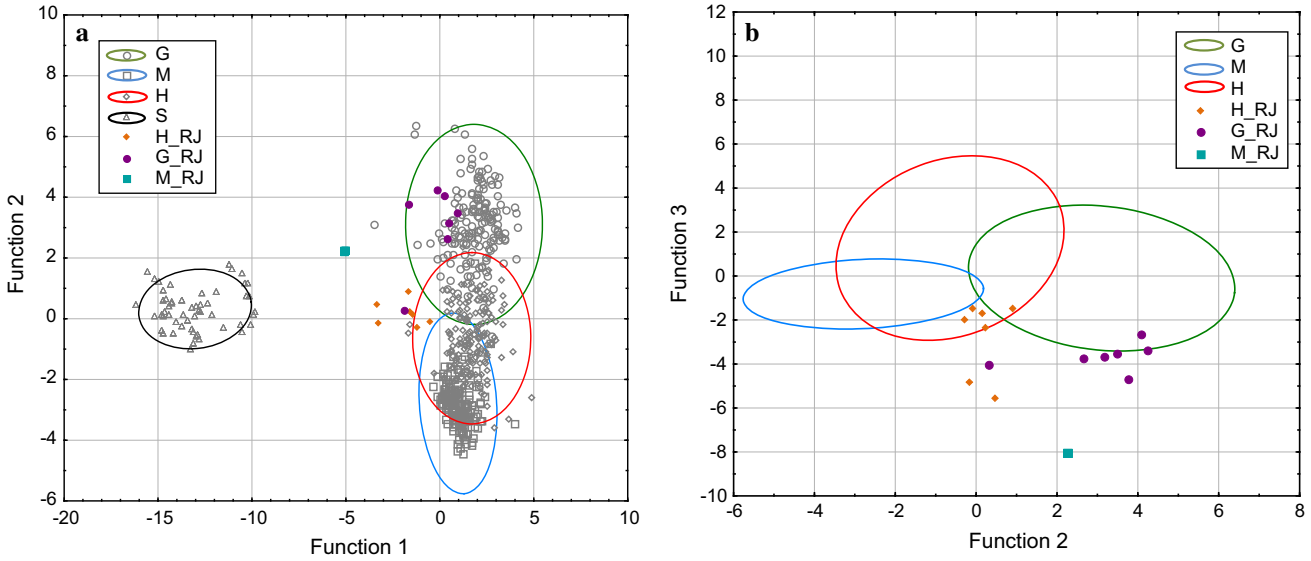

Fig. 7 Scatter plots of discriminant function 1 versus discriminant function 2 (**a**) and discriminant function 2 versus discriminant function 3 (**b**) using previous database (August and December/2011: *G* gills; *H* hepatopancreas; *M* muscles; *S* sediment and their respective *ellipses*) and samples from the Ilha Grande collected in March/2013 (M_RJ = muscle; H_RJ = hepatopancreas; G_RJ = gills)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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