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Matching metal pollution with bioavailability, bioaccumulation and biomarkers response in fish (*Centropomus parallelus*) resident in neotropical estuaries

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ABSTRACT

Two neotropical estuaries affected by different anthropogenic factors were studied. We report levels of metals and metalloids in water and sediment as well as their influence on genetic, biochemical and morphological biomarkers in the native fish *Centropomus parallelus*. Biomarkers reflected the fish health status. Multivariate statistics indicated both spatial and temporal changes in both water and sediment, which are linked to the elemental composition and health status of inhabitant fish, showing the biggest influence of surface water, followed by sediments and interstitial water. Bioaccumulation in fish muscle was useful to identify elements that were below detection limits in water, pointing out the risk of consuming fish exceeding allowance limits for some elements (As and Hg in this case). Multivariate statistics, including physical, chemical and biological issues, presents a suitable tool, integrating data from different origin allocated in the same estuary, which could be useful for future studies on estuarine systems.

1. Introduction

Metal pollution in aquatic ecosystems can be related to sanitary effluents, coal plants burning energy, non-ferrous mining foundries, mining, steel and dumping of sewage sludge (Lesage et al., 2007; MacDonald et al., 2011). In such ecosystems, metals are freely dissolved or complexed in the water and may be present in colloidal or solid systems in the sediment. Metal speciation depends on biological processes, redox potential, ionic strength, pH, activities of organic and inorganic chelators and scavenging processes. Thus, bioavailable metals may be hazardous for aquatic biota (Larocque and Rasmussen, 1998).

Estuaries, defined as a transition zone between river and ocean environments, represent highly variable environment and it may be aggravated by human activities. Dredging, harbor implantation, coastal and basin industries, leisure and tourism are among the human activities that promote modifications on the physical and chemical characteristics of estuaries, reducing the water quality due to continuous discharge of industrial and sanitary effluents (Borja et al., 2012). In the last decades, an intense urban occupation and industry, mining, harbor and marina installations have occurred in the southeast coast of Brazil; in the Espirito Santo State, estuaries have been occupied by metallurgical and mining complexes, including harbors for iron exportation resulting in an increasing pollution of water and sediment by metals and other compounds. Nowadays, the Vitoria harbor is the main place in Brazil for exporting iron to the world (Souza et al., 2011).

Although different approaches have been used to evaluate the presence of pollutants in aquatic environments, few studies correlate their presence, bioavailability and effects on the aquatic biota in neotropical estuaries with mangroves (Sundaray et al., 2011; Bonanno, 2012; Costa et al., 2012; Sakuragui et al., 2013). The

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integrate use of chemical analysis, with the concurrent evaluation of biochemical and morphological biomarkers, provides with a more precise data pool, evidencing the presence of pollutants in the aquatic ecosystem as well as their negative effects on the aquatic biota, enabling actions to preserve/restore the environment and its resident organisms (Ahmad et al., 2006; Lacerda et al., 2009). Fish have been considered suitable organisms for biomonitoring as they are sensible to changes in the aquatic environment, while their biological responses change, even at low levels of pollution. Thus, some genetic, biochemical, morphological and behavioral responses, measured in exposed fish are useful biomarkers for environmental biomonitoring (Pesce et al., 2008; Ballesteros et al., 2009).

Genetic biomarkers such as micronucleus (MN) and nuclear abnormalities (NA) in fish erythrocytes are considered effect biomarkers (Matsumoto et al., 2006), indicating genotoxic damage (Carrasco et al., 1990; Ayylon and Garcia-Vazquez, 2000; Bombail et al., 2001).So, both MN and NA are strongly recommended for biomonitoring (Duarte et al., 2012; Souza et al., 2011). Among biochemical biomarkers, the activity of enzymes related to the biotransformation and antioxidant processes has been frequently used for biomonitoring. Some antioxidant enzymes, like superoxide dismutase (SOD) and catalase (CAT) as well as the biotransformation enzyme glutathione-S-transferase (GST) help controlling the oxidative stress induced by pollutants (Atli et al., 2006; Sakuragui et al., 2013). SOD catalyzes the reduction of superoxide radical to hydrogen peroxide (H_2O_2), CAT is the main metabolic route for reducing levels of H_2O_2 degrading it to O_2 and H_2O. Additionally, GST catalyzes the conjugation of glutathione to pollutants, contributing to eliminate them from the cellular system. Morphological biomarkers include specific organ histopathological markers, which may express acute or chronic exposure, evidencing tissue damage that may impair malfunction of affected organs (Fernandes and Mazon, 2003; Triebskorn et al., 2008).

In fish, gills and liver are frequently studied to assess the impact of pollutants during environmental monitoring. The gills are the main organs for respiration, ionic and acid-base balance, being firstly affected by pollutants due to its large surface, which is in direct contact with the surrounding water, in addition to the narrow water-blood distance (Mazon et al., 2002a,b; Fernandes and Mazon, 2003). Physical and chemical changes in the aquatic environment may alter the gill epithelium, leading to changes in the number of chloride cell (CC) and in the activity of Na^+/K^+-ATPase, an enzyme related to the ion transport, which maintains the osmotic and ionic homeostasis and fish osmoregulation (Paulino et al., 2012a; Fernandes et al., 2013). The liver is a key organ for the biotransformation of xenobiotics, also involved in the excretion of metals. Metals and other xenobiotics accumulate in the liver, exposing its cells to a high level of chemical agents, which may damage this organ (Johnsen et al., 1998; Pacheco and Santos, 2002). Biochemical and morphological markers evidence the contact and effects of pollutants on these organs, being important tools to evaluate the fish health status (Schmalz et al., 2002; Oliveira Ribeiro et al., 2005).

The fat snook, *Centropomus parallelus* Poey 1860 (Centropomidae), a protandric predator fish (Taylor et al., 2000), is widely distributed throughout the Occidental Atlantic, from tropical and subtropical coasts in Florida (USA) to Brazilian southern coasts (Rivas, 1986). So far, *C. Parallelus* could be a useful bioindicator for evaluating the effect of complex pollutants mixtures present in estuaries. *C. parallelus* have a relative fast development and does not present major migratory cycles (Volpe, 1959). This species has a carnivore diet, being in the top of the aquatic food chain (Gilmore et al., 1983). During the juvenile stage (up to 45 cm in length) this fish has benthic habits, feeding with crustaceans (Volpe, 1959; Cháves, 1963; Gilmore et al., 1983), while adult fish is benthic-pelagic, feeding mainly with small fish (Carvajal, 1975).

In this context, the main goal of this study was evaluating two neotropical estuaries with different levels of metals-metalloids pollution as well as the effect of such pollution on the health of a fish inhabiting these estuaries, using several biological endpoints, looking for links between environmental issues and biomarker responses.

2. Materials and methods

Ultra pure water (<5 μg L^{-1} TOC) was obtained from a purification system Arium 61316-RO plus Arium 611 UV (Sartorius, Germany). Multi-element standard solution Merck VI CertiPUR® was obtained from Merck Química Argentina (Buenos Aires, Argentina). Nitric acid (63.7%) sub-boiling grade was prepared from analytical grade acid using a distiller (Figmay Sub-boiling distiller, Córdoba, Argentina). Purity of nitric acid was verified by ICP-MS. Filters (0.45 μm, HAWG04756) were obtained from Millipore (São Paulo, Brazil). All glassware and plastic bottles/containers were left with sulfuric/nitric acids solution overnight and washed with ultra-pure water. ICP probes and pipes were of PTFE (Teflon) previously washed with nitric acid (2% v v^{-1}).

2.1. Study areas

The study was conducted in two neotropical estuaries located in the State of Espirito Santo, Brazil: Vitoria bay and Santa Cruz that were selected because they represent areas affected by different pollution sources and ocean influence (Fig. 1). Vitoria bay (20° 19′S e 40° 20′W) is an estuarine complex formed by five rivers, which receives the influence of the Espirito Santo bay. This estuary has strong environmental degradation caused by harbors, which activities were not accompanied by an increase of the urban infrastructure (run-off, sewage, etc.). Santa Cruz (19° 58′S e 40° 07′W) is an estuary formed by two rivers and has an extensive mangrove area (Souza et al., 2011), which add complexity to this ecosystem. Both studied areas were georeferenced during field sampling, using a portable GPS 368 (Garmin Vista, USA), considering at least five satellites with an accuracy between10 and 50 m.

2.2. Water, sediment and fish sampling

In field, dissolved oxygen (DO) and conductivity of surface and bottom water were determined using a multiparameter probe (YSI model 85, USA) operated either 20 cm below the surface and ca. 20 cm above the sediment.

Water and sediment samples were taken throughout the experiment simultaneously with fish sampling. Sample collection, containers, stabilization, and transportation to the laboratory as well as sample storage were done in accordance with previously described methods (Monferrán et al., 2011). Water samples for metals analysis were collected into acid washed plastic bottles, from approximately 20 cm below the surface of the estuary. In the laboratory, interstitial water was extracted from sediment samples by centrifugation (3000 rpm; 40 min) and the supernatant was filtrated using 0.45 μm nitrocellulose filters. Both kind of water samples were acidified with ultra pure HNO3 (sub-boiling) and stored at 4 °C until analysis. Prior to measurement, the samples were filtered using 0.45 μm nitrocellulose.

Sediment samples (approximately 20 cm depth interval) were collected from the location, using a plastic spoon. Sediment samples were quickly transferred into clean 1-L plastic containers (without head space) for metal analyses. Subsequently they were dried at room temperature and sieved through nylon meshes (63 microns) with an acrylic frame to avoid the transfer of metals from metallic meshes/frames during sieving.

Ten juvenile male *C. parallelus* (Body mass = 150 ± 30 g; Total length = 15 ± 5 cm) were collected using hook and line. Blood samples were immediately taken via the caudal vein. Fish were then sacrificed by medullar section. Gills and liver were removed and divided (sub-sampled) for enzymatic and morphological analyses. So, a sub sample was stored at −80 °C until enzymatic analysis, while the other sub sample was immediately fixed in Bouin solution. White muscle was also sampled, dried at 40 °C until constant weight and stored at −20 °C until analysis.

For metal analyses, biological samples were ground and homogenized with a mortar. Sediment and fish samples (0.5 g each) were digested with nitric and hydrochloric acids (ultra pure, sub boiling grade) in pre-cleaned quartz close-vessel using a microwave oven (Anton PaarMultiwave3000, Austria). Controls were prepared using the same protocol without sample (only reagents). The assay of organic matter in the sediment was performed in according to Walkley and Black (1934) method.

2.3. Multielement analyses

The analysis of metals and metalloids (Al, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Ag, Cd, Hg and Pb) in both abiotic and biotic digested samples, was performed with a Mass Spectrometer Inductively Coupled Plasma (ICP-MS), Agilent 7500cx, USA, equipped with an ASX-100 autosampler (CETAC Technologies, Omaha, NE).

All samples were digested in triplicate. Concentrations of elements were determined in triplicate; the repeatability of ICP-MS measurements was generally ≥

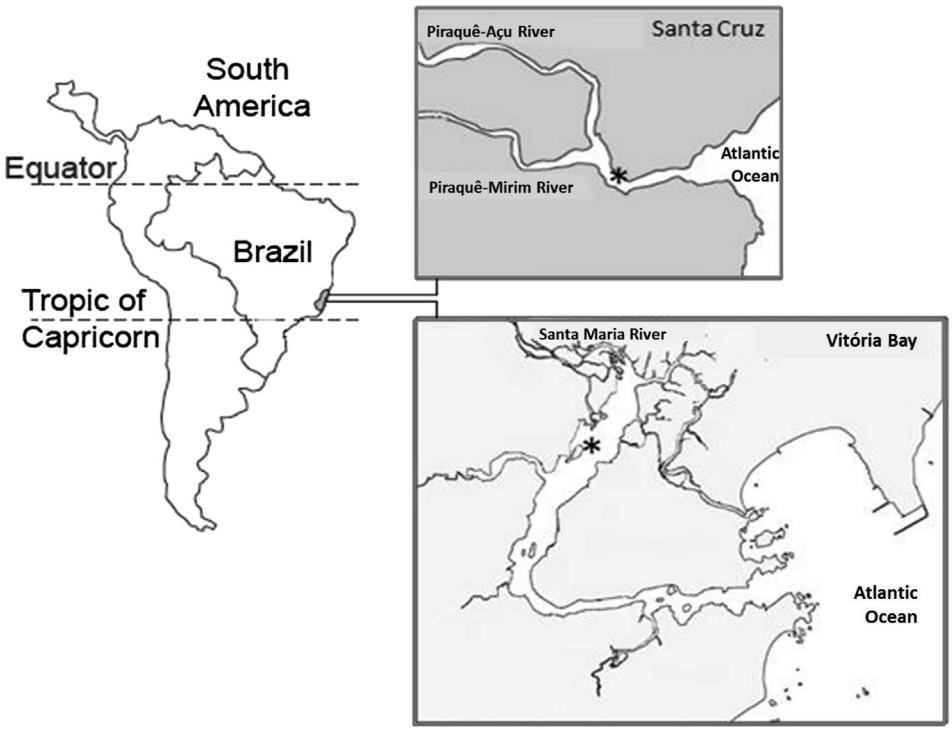

Fig. 1. Localization of the State of Espírito Santo (South America, Brazil), showing Santa Cruz and Vitoria bays.

97%. Quality assurance (QA) and quality control (QC) were done using certified reference materials (CRMs):

NIST 1646a (Bovine Muscle) and NIST 1573a (sediment sludge). Recoveries from CRMs were 102 ± 18% and 98 ± 11%, respectively. CRMs were selected according to the elements measured in the samples. Spiked samples were also prepared. Variable amounts of mix standard solutions, containing all the elements analyzed in the samples, were added to 0.3–0.5 g of dried fish muscle or sediment samples, doubling the starting concentration for each element. The rest of the procedure was the same as used for non-spiked samples. The average recovery of these assays was 98 ± 17%

2.4. Biochemical analyses

Enzyme extracts were prepared from gill and liver samples. The total content of protein in each sample was determined in according to the Bradford method (Bradford, 1976), at 595 nm using bovine serum albumin as standard in a Dynex MR-HD microplate reader (Dynex Technologies Ltd., USA). SOD, CAT and GST activities were measured as described by Paulino et al. (2012b) and Na^+/K^+-ATPase (NKA) activity were determined in according to Paulino et al. (2012a). All measurements were performed in triplicate.

2.5. Morphological analyses

For histopathological analysis, the fixed gill and liver samples were dehydrated in ethanol and embedded in historesin (Leica, Germany). Semi-serial (1:10) sections (3 μm thick) were stained with toluidine blue (gill) and toluidine blue and basic fuchsin (liver). The chloride cells (CC) in the gills were identified and quantified by immunohistochemistry against NKA, in according to the avidin–biotin–peroxidase complex (ABC) technique (Dang et al., 2000). All sections were analyzed using images obtained in an Olympus BX51 microscope (Olympus, Denmark) with a digital video-camera connected to a computer with the Motic Image Plus 2.0 software (Hong Kong, China). Histopathological changes were analyzed in both filaments and lamella using 5 random microscope fields/section ($n = 25$)/animal and the gills and liver histopathological change indexes (HCI) were determined in according to Poleksic and Mitrovic-Tutundzic (1994), modified by Cerqueira and Fernandes (2002) and Camargo and Martinez (2007). Chloride cells were quantified in the filament in 25 random microscope fields/section/animal.

2.6. Genotoxicity analyses

Blood smears were fixed in methanol and analyzed in according to Feulgen and Rossenbeck (1924) and Mello and Vidal (1978). A total of 4000 erythrocytes from each fish were scored. Mutagenic (MN) and genotoxic (NA) damages were classified following the Al-Sabti and Metcalfe (1995) and Carrasco et al. (1990) methodologies, respectively.

2.7. Statistical analysis

Data are reported as mean ± standard deviation. The statistical packages, STATISTICA 7.1 from StatSoft Inc. (2005), and Infostat (Di Rienzo et al., 2010) were used for the statistical analysis. All data were tested for normal distribution. One-way analysis of variance (ANOVA) was applied to compare data followed by Tukey's post-test with significance $P < 0.05$.

Multivariate statistical methods were applied to datasets: lineal discriminant analysis (LDA), generalized procrustes analysis (GPA) and canonical correlation analysis (CCA). Multivariate statistical methods evidenced the contribution of each variable to the model, and its capacity to discriminate one category from another. LDA is a supervised procedure that maximizes the variances between categories and minimizes the variances within categories. LDA was performed in the stepwise mode to verify statistical differences in global parameters measurement during each season (dry and wet), sites, and interaction considering both seasonal and spatial responses. LDA was performed on experimental data with or without standardization obtaining the same discrimination in agreement with our previous experience (Wunderlin et al., 2001). In addition to LDA, GPA was used to evidence both spatial and temporal segregation. Specifically, GPA constructs the consensus configuration of a group of datasets by applying transforms in an attempt to superimpose them. Therefore, GPA theory and algorithms can be applied to match water elemental data (both surface and interstitial) to the corresponding sediment and fish data (both elemental and biomarkers). CCA was also applied for assessing the relationship between diverse data matrix (surface and interstitial waters, sediment and fish) using a more formal mathematical approach (Di Paola-Naranjo et al., 2011).

3. Results and discussion

3.1. Physical and chemical characterization of studied estuaries

The organic matter percentage in the sediment of Vitoria Bay was significantly higher than in Santa Cruz without significant temporal variation (winter vs. summer) (Table 1). Water DO and conductivity show stratification in both studied areas; DO is lower and the conductivity is higher in the water layer close to the bottom (Table 1). The surface water is, in general, associated with continental water arising from river basins, while bottom water is

Table 1
Physical and chemical characterization of Santa Cruz and Vitoria Bays. Metal concentrations in surface and interstitial water, sediment and in fish muscle ($n = 9$ in each site). Values are expressed as mean ± SD.

Physical and chemical parameters	Santa Cruz									Vitoria Bay								
	Winter								Summer	Winter								Summer
OM (mg g^{-1} sediment)	20 ± 1^{a}								19 ± 2^{a}	24 ± 2^{b}								26 ± 2^{b}
Cond (mS) surface water	40 ± 3^{a}								28 ± 2^{b}	43 ± 3^{a}								28 ± 2^{b}
Cond (mS) interstitial water	72 ± 4^{a}								72 ± 7^{a}	40 ± 4^{b}								54 ± 5^{c}
DO (mg L^{-1}) Interstitial	4.8 ± 0.3^{a}								1.3 ± 0.1^{b}	4.7 ± 0.4^{a}								1.2 ± 0.1^{b}
DO (mg L^{-1}) surface	9.5 ± 0.8^{a}								9.3 ± 0.7^{a}	9.2 ± 0.8^{a}								9.4 ± 0.8^{a}
Metals	Surface water (μg L^{-1})	Interstitial water (μg L^{-1})	Sediment (μgg^{-1} dry mass)	*C. parallelus* (μg g^{-1} muscle dry mass)	Surface water (μg L^{-1})	Interstitial water (μg L^{-1})	Sediment (μg g^{-1} dry mass)	*C. parallelus* (μg g^{-1} muscle dry mass)	Surface water (μg L^{-1})	Interstitial water (μg L^{-1})	Sediment (μg g^{-1} dry mass)	*C. parallelus* (μg g^{-1} muscle dry mass)	Surface water (μg L^{-1})	Interstitial water (μg L^{-1})	Sediment (μg g^{-1} dry mass)	*C. parallelus* (μg g^{-1} muscle dry mass)		
Al	38.3 ± 3.2^{a}	$3422 \pm 615^{a,b}$	29986 ± 1854^{b}	3.8 ± 0.7^{c}	109.2 ± 25.3^{b}	2576 ± 471^{a}	29970 ± 1146^{b}	2.7 ± 0.3^{b}	138.9 ± 2.8^{c}	6999 ± 3157^{c}	22567 ± 1009^{a}	4.0 ± 0.6^{c}	92.6 ± 6.9^{b}	5363 ± 692^{b}	22297 ± 1622^{a}	1.4 ± 0.4^{a}		
Cr	<LOD	<LOQ	28.1 ± 1.5^{a}	$0.09 \pm 0.01^{a,b}$	<LOD	<LOQ	28.4 ± 1.4^{a}	0.07 ± 0.00^{a}	<LOQ	9.7 ± 7.3^{b}	45.0 ± 29.0^{a}	0.07 ± 0.01^{a}	50.3 ± 0.9^{a}	3.7 ± 4.3^{a}	24.8 ± 3.4^{a}	0.11 ± 0.04^{b}		
Mn	10.2 ± 0.8^{a}	1168 ± 269^{b}	90.3 ± 11.4^{a}	0.83 ± 0.02^{c}	20.4 ± 0.2^{b}	2159 ± 804^{c}	78.4 ± 11.0^{a}	$0.95 \pm 0{,}10^{d}$	57.8 ± 2.8^{c}	125 ± 122^{a}	58.8 ± 6.8^{b}	0.55 ± 0.02^{b}	69.0 ± 0.5^{d}	192 ± 100^{a}	53.1 ± 8.4^{b}	0.39 ± 0.03^{a}		
Fe	150 ± 1^{a}	13751 ± 4021^{a}	20697 ± 2185^{a}	8.4 ± 0.6^{c}	145 ± 116^{a}	13189 ± 2137^{a}	20759 ± 1510^{a}	7.8 ± 0.5^{c}	355 ± 6^{b}	13520 ± 3691^{a}	24064 ± 4537^{a}	6.8 ± 0.9^{b}	320 ± 2^{b}	10847 ± 1085^{a}	27413 ± 3115^{b}	5.0 ± 0.3^{a}		
Ni	<LOD	8.6 ± 17.5^{a}	8.7 ± 1.2^{a}	0.10 ± 0.05^{c}	<LOD	<LOD	7.5 ± 0.4^{a}	<LOQ	34.9 ± 1.1^{b}	<LOD	11.6 ± 6.9^{a}	0.04 ± 0.04^{ab}	66.7 ± 1.6^{c}	6.0 ± 7.1^{a}	6.9 ± 0.8^{a}	0.05 ± 0.02^{b}		
Cu	<LOD	5.3 ± 10.7^{a}	3.7 ± 0.2^{a}	0.77 ± 0.09^{a}	<LOD	<LOD	7.8 ± 13.4^{a}	$0.66 \pm 0.04^{a,b}$	16.6 ± 0.5^{b}	4.0 ± 3.4^{a}	4.4 ± 0.8^{a}	0.67 ± 0.07^{b}	22.7 ± 1.1^{c}	7.3 ± 6.7^{a}	4.3 ± 0.4^{a}	0.57 ± 0.08^{a}		
Zn	<LOD	<LOD	<LOD	12.4 ± 0.8^{a}	<LOD	<LOD	<LOD	14.2 ± 0.7^{b}	27.9 ± 21.6^{b}	<LOD	<LOD	14.4 ± 0.4^{b}	358.4 ± 6.0^{c}	<LOD	<LOD	12.0 ± 0.8^{a}		
As	<LOD	<LOD	8.8 ± 1.8^{b}	0.69 ± 0.48^{a}	<LOD	<LOD	8.3 ± 1.0^{b}	0.82 ± 0.48^{a}	<LOD	<LOD	6.0 ± 1.5^{a}	0.77 ± 0.01^{a}	<LOD	<LOD	6.2 ± 0.6^{a}	0.51 ± 0.02^{a}		
Se	<LOD	7.0 ± 17.0^{a}	<LOQ	0.21 ± 0.03^{a}	<LOD	6.4 ± 15.6^{a}	<LOQ	0.29 ± 0.05^{a}	<LOD	8.7 ± 21.2^{a}	<LOQ	0.74 ± 0.05^{b}	<LOQ	$8{,}8 \pm 21{,}5^{a}$	<LOQ	0.69 ± 0.16^{b}		
Ag	48.0 ± 3.3^{c}	<LOD	<LOD	<LOQ	56.7 ± 13.5^{c}	<LOD	<LOD	<LOQ	13.1 ± 2.5^{b}	6.1 ± 9.4^{a}	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOQ		
Cd	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD		
Hg	<LOD	<LOD	<LOD	0.18 ± 0.02^{a}	<LOD	<LOD	<LOD	0.24 ± 0.07^{b}	<LOD	2.2 ± 3.5^{a}	<LOD	0.35 ± 0.01^{c}	<LOD	<LOD	<LOD	0.22 ± 0.01^{b}		
Pb	19.8 ± 0.6^{d}	<LOD	6.4 ± 0.2^{a}	$0.08 \pm 0.06^{a,b}$	12.6 ± 1.6^{c}	<LOD	5.9 ± 0.2^{a}	$0.05 \pm 0.03^{a,b}$	11.1 ± 0.6^{b}	<LOD	12.1 ± 4.5^{b}	0.03 ± 0.01^{a}	7.5 ± 0.8^{a}	<LOD	13.9 ± 2.5^{b}	0.10 ± 0.10^{b}		

Sediments correspond to the fraction <63 μm <LOD (below detection limit); <LOQ (below quantification limit). LODs: Fe, Zn, As, Se and Hg (0.15 μg L^{-1}); Al, Cr and Ni (0.03 μg L^{-1}); Mn, Cu, Ag, Cd and Pb (0.015 μg L^{-1}). Equal letter in the same line data do not differ significantly (Tukey test; $P < 0.05$).

related to the sea water and sediment (Pereira et al., 2007). Low DO levels close to the sediment indicate hypoxic environment at the bottom in the summer. The conductivity of the surface water was higher in the winter, showing seasonal differences in both studied area; however, such seasonal differences were not observed in the bottom water. The higher conductivity of the bottom water in Santa Cruz evidences stronger marine influence in this area, which is favored by its localization (Fig. 1).

In the environment, the degradation of chemical substances occurs by chemical and biochemical processes; the stability of a given compound is related to its structure and numerous environmental factors such as temperature, solar radiation, pH and concentration of organic matter. In sediment and soil, metals and other inorganic substances can be adsorbed to the surface of oxides or organic matter reducing their mobility and availability; however, in the water, the oxygen content determines the nature and the velocity of the chemical and biochemical transformations of substances which may increase or decrease the availability of metal species (Fernicola et al., 2003). The lower DO level combined with the higher organic matter concentration in Vitoria Bay (Table 1), probably decreases the bioavailability of metals in this estuary. Conversely, Santa Cruz probably presents higher metal bioavailability (Table 1).

3.2. Metals-metalloids

Metals-metalloids concentrations in the interstitial water were significant higher ($P < 0.05$) than in the surface water in both Santa Cruz and Vitoria bays, excepting Ag and Pb in Santa Cruz and Ni, Cu, Zn, Ag and Pb in Vitoria bay. In general, the concentration of measured elements in the interstitial water in Vitoria bay was higher than in Santa Cruz (Table 1). Some metals such as Cr, Ni, Cu, Zn and Hg were detected in the surface water in Vitoria bay, but not in Santa Cruz (Table 1). These differences evidence that Vitoria bay presents a higher metal pollution level compared to Santa Cruz, which is in agreement with more severe anthropic pollution at the first estuarine area.

Concentrations of measured elements in the muscle of *C. parallelus* did not show significant differences in Cr, Ni, Cu, Zn, As, Ag, Cd, Pb and Hg between winter and summer at each site. The concentration of Al was significantly higher in the winter in both estuaries; Mn levels in Santa Cruz were higher than in Vitoria bay in both seasons; conversely, Se concentration was higher in Vitória bay (Table 1). Manganese is used to estimate the continental influence on the marine environment as this element is a component of rocks and soil and, in general, it is transported by watercourses to the sea adsorbed on suspended particulate matters (Pereira et al., 2007). So far, high levels of Mn found in interstitial water, sediments and fish muscle from Santa Cruz bay could be the result of the influence of continental water.

A notable point is the levels of some elements bioaccumulated in fish muscle, although they were not detected in surface or interstitial waters. For instance, arsenic (As) and mercury (Hg) were below analytical limits in waters and sediment but above these limits in fish muscle (Table 1). Considering that juveniles *C. parallelus* has benthic habits, being at the top of the food chain during this stage of life, biomagnification of some elements that cannot be detected in the sediment or the water is expected. This is the case observed with As and Hg during the current study. Accumulation of Hg was also found in the same species in other estuarine complex, less affected by human influence (Curcho et al., 2009). Both elements (As and Hg) were above the suggested daily intake (DIA) for humans recommended by USEPA (2009). Namely, DIA for As is 21 $\mu g\ day^{-1}$, while DIA for Hg is 11.2 $\mu g\ day^{-1}$ (considering 70 kg body mass). Levels of As in the fish muscle were between 4 and 6 fold higher than DIA, while Hg exceeded by 3–5 fold the corresponding DIA, considering a daily ingestion of 150 g fish muscle, posing a serious risk to the health of people eating fish from these estuaries.

3.3. Biochemical, morphological and genotoxic biomarkers

Micronucleus frequency (mutagenicity) did not differ ($P > 0.05$) in fish collected from both estuaries (Table 2), evidencing the absence of potential mutagenic pollutants as pointed out by Matsumoto et al. (2006). Nuclear abnormalities (genotoxic damage) did not differ in fish captured in Santa Cruz in summer and winter (Table 2); conversely, NA were higher in those captured during summer in Vitoria bay. Despite the genotoxic effect observed in fish from Vitoria bay in summer, it is important to emphasize that NA are reversible damages that may be repaired (Oliveira et al., 2007; Lemos et al., 2005).

The HCI calculated for gills and liver damages did not show significant differences (spatial or temporal) in both estuaries, but the liver HCI was higher than those calculated for the gills (Table 2). The gill HCI (lower than 10) indicates that lesions detected in the gills did not affect the function of this organ, in agreement with results reported by Poleksic and Mitrovic-Tutundzic (1994). This is reinforced by the CC density observed in the gill filament of *C. Parallelus*, which did not show significant changes (spatial or temporal) in both estuaries (Table 2). These results suggest that the metal-metalloid pollution of Santa Cruz and Vitoria bays did not cause CC malfunction or death. The NKA is the main enzyme creating a driving force for NaCl excretion in marine fish. NKA levels

Table 2
Biomarkers measured in *Centropomus parallelus* ($n = 9$ in each site) captured in the estuaries Santa Cruz and Vitória Bay in the summer and winter. Values are expressed as mean ± SD. Equal letter in the same line data do not differ significantly (Tukey test; $P < 0.05$).

Biomarkers	Sites			
	Santa Cruz		Vitória Bay	
	Winter	Summer	Winter	Summer
Genotoxicity (Frequency %)	4.0 ± 2.3a	3.0 ± 1.6 a,b	1.7 ± 0.9 a,b	6.1 ± 2.8a,c
Mutagenicity (Frequency %)	0.1 ± 0.1a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
Histopathological change index (HCI) – Gills	3.5 ± 1.9a	3.7 ± 0.5a	4.6 ± 0.9a	4.8 ± 1.1a
Histopathological change index (HCI) – Liver	67 ± 13a	40 ± 9a	41 ± 12a	50 ± 16a
Chloride cells (number mm^{-2} filament)	1165 ± 477a	951.3 ± 368a	1018 ± 280a	1469 ± 195a
Na^+/K^+-ATPase activity ($\mu M\ Pi\ mg\ pt^{-1}\ h^{-1}$)	0.4 ± 0.2a	0.6 ± 0.2 ab	0.6 ± 0.4 ab	1.0 ± 0.5b
Superoxide Dismutase – Gills ($U\ mg\ pt^{-1}$)	22 ± 11a	60 ± 30b	17 ± 7a	34 ± 18a
Gluthatione-S-Transferase – Gills ($\mu M\ mg\ Pt^{-1}\ min^{-1}$)	13 ± 4a	26 ± 8b	10 ± 5ac	5 ± 3c
Superoxide Dismutase – Liver ($U\ mg\ Pt^1$)	33 ± 19a	49 ± 16a	63 ± 13b	62 ± 21b
Gluthatione-S-Transferase – Liver ($\mu M\ mg\ Pt^{-1}\ min^{-1}$)	127 ± 46a	64 ± 34b	49 ± 13b,c	34 ± 10c,d
Catalase – Liver ($\mu M\ g\ Pt^{-1}\ min^{-1}$)	223.81 ± 246.87a	78,5 ± 23.69b	203,87 ± 87.67 a,b	189.02 ± 46.63 a,b

in gills were lower during the winter in Santa Cruz and higher in fish collected in the Vitória bay in the summer (Table 2). Such differences in the NKA activity may be related to ion diffusion into the fish, or due to an increased amount of water drunk by the fish, resulting in an ionic overload, and thus requiring a higher activity of this enzyme to deal with this imbalance. Additionally, differences in concentrations from several elements between waters of Santa Cruz and Vitória bay may be related to NKA responses. In general, *in vitro* and *in vivo* laboratory studies, but also field studies, have shown that metals such as Cu, Zn and Pb inhibit the activity of NKA (Stagg et al., 1992; Fernandes et al., 2013). However, in Vitoria bay these metals were present at higher concentrations in the summer, when the NKA activity was higher. The absence of NKA inhibition in Vitória bay may be due to compensatory processes, involving the homeostatic mechanisms to recover the enzyme loss (Stagg et al., 1992).

Liver HCI (40–67) indicates moderate to heavy damages. Although the gill is the first organ facing dissolved pollutants, this pollutants are usually transferred to the blood, reaching other organs such as the liver, which is the main detoxification organ of fish (Paulino et al., 2012b; Santos and Martinez, 2012). Thus, metals accumulated in the liver may cause more severe cell damage, affecting its function.

Metals may also generate reactive oxygen species (ROS), which lead to a higher activity of biotransformation and antioxidant enzymes, such as GST, CAT and SOD, thereby keeping ROS levels within physiological limits (Formigari et al., 2007). In general, the activity of SOD and GST in gills of *C. parallelus* were lower than in the liver (Table 2). This results is expected considering that gills can transfer absorbed metals to the blood, reaching the liver, which is the main detoxification organ. Bioaccumulation of metals arising from other sources (food, water drunk by fish, suspended materials, etc.) in the liver cannot be discarded. Comparing the activity of SOD and CAT in the liver of fish from both estuaries, higher values were observed in Vitória bay during both winter and summer, indicating the activation of these antioxidant defenses. SOD and CAT are the primary defense against ROS. SOD activation is related to the increased production of the superoxide radical, which is transformed to H_2O_2 catalyzed by this enzyme. Additionally, CAT is activated at high levels of H_2O_2 (Sanchez et al., 2005). CAT activation has been reported in the liver of fish from polluted environments, showing presence of metals and other pollutants (Carvalho et al., 2012; Sakuragui et al., 2013). The GST activity in the liver was lower in fish from Vitória bay than those from Santa Cruz, suggesting a possible inhibition of this biotransformation enzyme. These results evidence a higher impact of stressors in the Vitoria Bay, which may be related to the presence of higher levels of metals and other toxic elements at this site (Table 1).

3.4. Overall discussion using multivariate statistics

Interpreting data from environmental biomonitoring is complex, particularly when multiple pollution sources are present. The multivariate analysis can provide an integrated view of the overall situation, pointing out differences between seasons, monitoring areas as well as associations between spatial and temporal variations and their effects on the biota (Wunderlin et al., 2001; Monferrán et al., 2011).

In order to point out which parameters may explain spatial and temporal differences between studied areas, the LDA was applied, including chemical parameters analyzed in superficial water, interstitial water, sediment and fish in addition to biomarkers as independent variables. Results from LDA are shown in Table 3.

Table 3 presents results from stepwise LDA, showing classification functions for both monitoring stations, separated by season.

Table 3
Classification functions corresponding to LDA of studied parameters, considering both spatial and temporal variations.

Classification functions LDA	Sites			
	Santa Cruz		Vitória Bay	
	Winter	Summer	Winter	Summer
Al-SW	−5.24	24.40	28.9	22.1
Mn-SW	−40.47	5.72	−30.7	−4.0
Fe-SW	3.57	−6.41	−0.6	−1.0
Pb-SW	117.77	−129.07	−215.7	−176.1
Mn-Fish	−2054.56	−467.70	−31,010.3	−23,049.0
Fe-Fish	59.40	−358.31	94.3	113.0
Cu-Fish	−1018.12	5551.91	5676.3	3549.8
Zn-Fish	197.00	218.44	1445.5	1053.1
As-Fish	−86.37	−1126.16	−4092.7	−2935.1
Se-Fish	717.58	2291.73	10,084.2	7154.5
Hg-Fish	1731.93	9724.84	59,790.8	43,471.1
Pb-Fish	−646.01	−8942.98	−17709.2	−11,932.8
Constant	−1517.32	−2686.35	−16249.3	−9126.0

SW = Surface water; Fish = fish muscle.

Twelve of 59 parameters were necessary to distinguish between areas and seasons with 100% correct classification (classification matrix, data not shown). Noteworthy is that parameters pointed out by LDA include four metals measured in surface water and eight metals measured in fish, without including biomarkers. Thus, the complex data matrix obtained by performing chemical and biological monitoring at both estuaries, could be reduced to only 12 parameters, which should allow spatial and temporal differentiation. Considering that 8 out of 12 parameters belong to fish (metals), it is clear that fish condition can evidence differences between studied areas considering different seasons. However, LDA operates on the data matrix to evidence differences between areas-seasons without considering the importance of biological issues to the health of inhabiting biota.

Fig. 2 shows box plots with patterns representing four out of 59 measured parameters. The concentration of Mn in interstitial water (Fig. 2A), fish muscle (Fig. 2B) and surface water (Fig. 2C) evidence that the Mn content in fish muscle (Fig. 2B) match the Mn levels in the interstitial water (Fig. 2A) but not the surface water (Fig. 2C). This fact could lead to conclusion that the composition of interstitial water is affecting the bioaccumulation of metals in fish, while surface water did not. However, the high levels of Pb found in the winter in both surface water and fish muscle at both monitored sites, without the concomitant presence of Pb in the interstitial water (Table 1), together with the CAT activity pattern in fish liver (Fig. 2D), which is quite close to that of Mn in the surface water (Fig. 2C) suggest different pollution sources for surface and interstitial water, with both contributing to the global bioaccumulation of metals in fish. These results are in agreement with the concentration of metals and metalloids in surface and interstitial waters in both Vitória and Santa Cruz bays, highlighting a higher pollution degree at Vitoria Bay. So far, LDA seems to be useful to point out parameters that allow spatial and temporal differentiation, without considering the effect of such parameters on the biota.

Thereafter, a generalized procrustes analysis (GPA) was performed, looking to demonstrate matching between water (surface and interstitial), sediment and fish, considering spatial and temporal differences. Fig. 3 shows a graphical representation of the correspondence between surface water, interstitial water, sediment and fish (metals and biomarkers). Significant differences between studied areas were described primarily by the first axis, which explained 59.2% of the total variance, while the second function, described by the second axis (CP2), accounts for additional 25.3%. Also from Fig. 3 it can be observed that the first function (CP1) contributes to the spatial differentiation between both studied

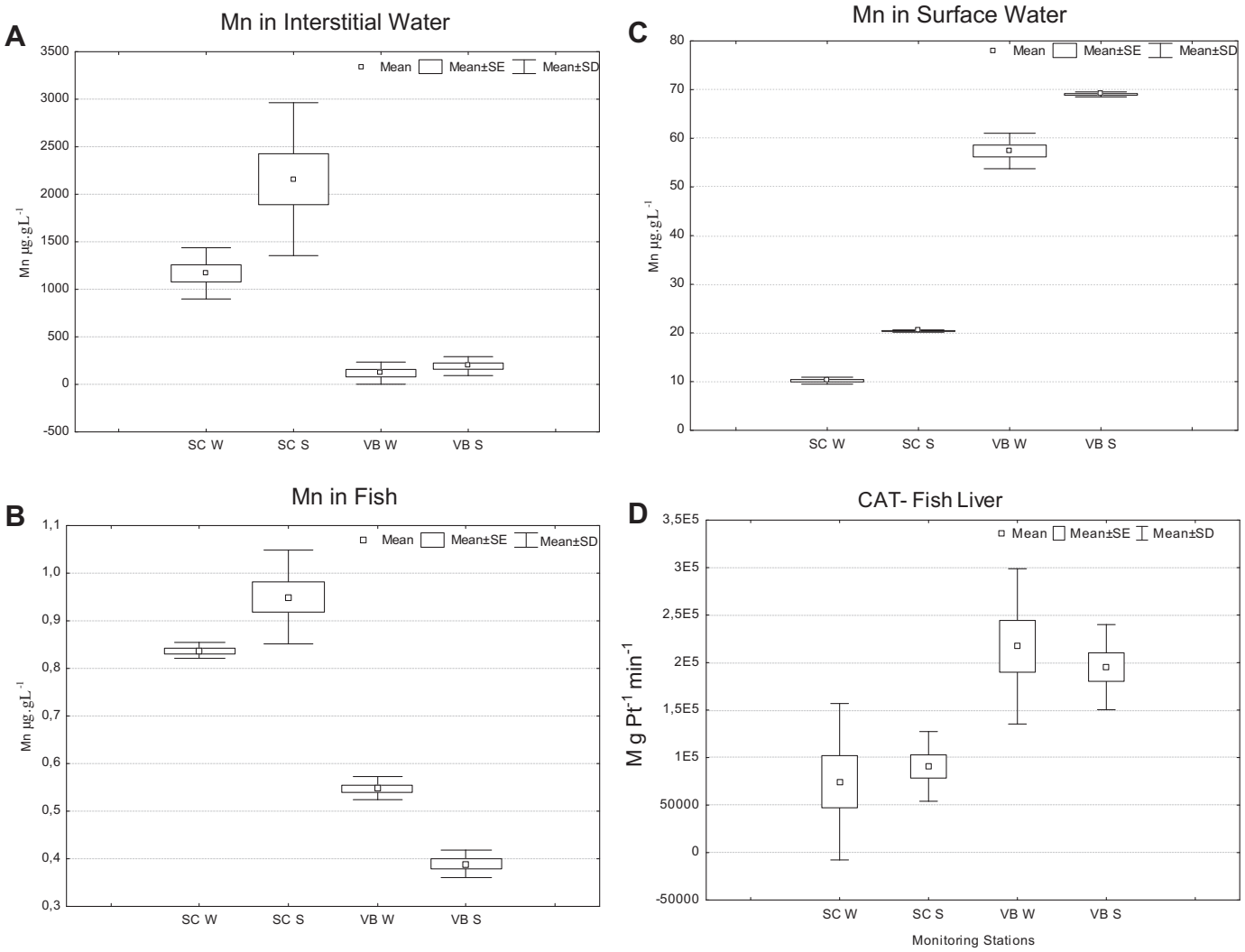

Fig. 2. Box & whisker plots from some selected parameters measured in surface water, interstitial water and fish. Values are reported as mean ± SD and SE.

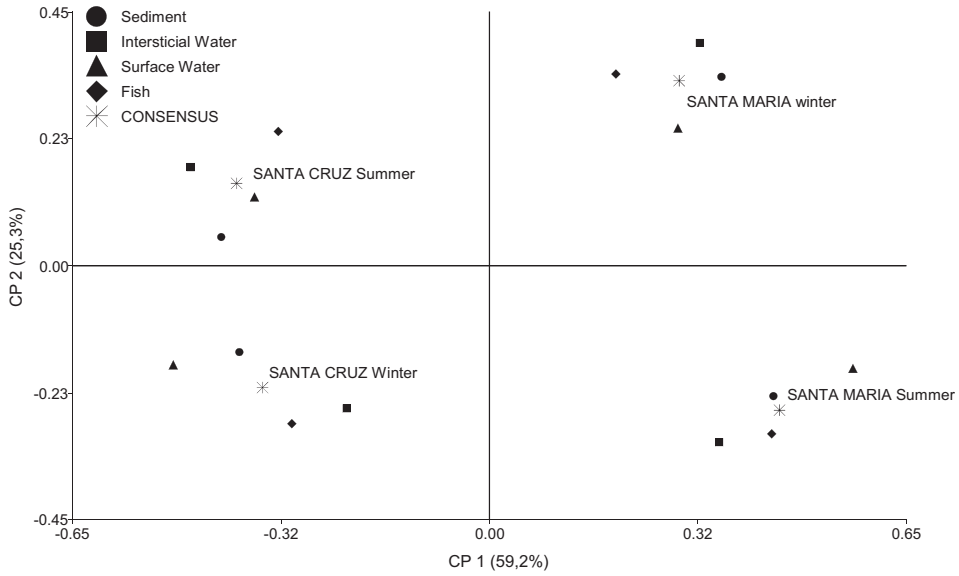

Fig. 3. Generalized procrustes analysis (GPA) of studied parameters from each sampling site.

areas (Santa Cruz and Vitória bay), while CP2 further contributes to seasonal differentiation (winter vs. summer). So far, a second independent multivariate method confirms spatial and temporal differences between studied areas but showing that metals measured in waters (surface and interstitial) and sediments match with levels of metals in fish muscle inhabiting both areas, including temporal differences. Furthermore, biomarkers are also pointed out by GPA close to the characteristics of water, sediments and metals-metalloids in the fish muscle.

However, neither LDA nor GPA indicates which environmental compartment is affecting fish to a bigger extent. To elucidate it, we carried out CCA considering paired data matrix, namely levels of metals in: surface water − fish; interstitial water − fish and sediment − fish. Results from CCA show that the surface water presented the better correlation with levels of metals in fish muscle ($R^2 = 0.99$; $p = 0.0$), followed by sediments ($R^2 = 0.94$; $p = 1.4\ 10^{-9}$) and interstitial water ($R^2 = 0.87$; $p = 4\ 10^{-9}$). So far, CCA points out to the surface water as the main responsible for levels of metals found in fish muscle (Table 1) and, probably, to the associated biotransformation (GST) and antioxidant enzymes (SOD e CAT) (Fig. 2D; Table 2). Sediments seem to play also an important role, followed by interstitial water.

Multivariate statistical analyses indicated that fish from each station exhibited a distinctly response during both studied seasons, pointing out that metal bioaccumulation significantly contribute to discriminate among studied areas, reinforcing the need of integrated monitoring, using both physical–chemical parameters and biomarkers to improve results during water quality assessments. This approach is especially important in neotropical estuaries, considering variations in salinity, DO, radiation, that affect environmental parameters and their influence on inhabiting biota (Canário et al., 2007; Ram et al., 2009).

4. Conclusion

The present study confirms the utility of *Centropomus parallelus* as bioindicator for neotropical estuaries, mainly considering its ability to adapt to diverse environments, reflecting environmental changes. The use of several biomarkers responded quite well to different pollution scenarios in Santa Cruz and Vitoria Bay estuaries, matching their response to the physical and chemical parameters, measured at different partitions within the estuary. Metal bioaccumulation in fish muscle was useful to identify some metals that were below analytical detection limits, showing the importance of fish or other biota components to fully evaluate the pollution degree in estuaries and pointing out the risk for people consuming fish exceeding allowance limits for some elements (As and Hg in this case).

The use of multivariate statistics greatly contributes to extrapolate results from field and laboratory measurements, pointing out parameters that help to differentiate sites with different water quality and, consequently, different risks for the aquatic biota. Furthermore, statistical methods like LDA, GPA and CCA contribute to integrating the knowledge coming from different scientific disciplines, like biology and chemistry, producing more complete results complementing both field and laboratory efforts. Further integrated studies using different species from several trophic levels should be necessary to fully evaluate changes in studied estuaries, looking for additional bioindicators and biomarkers that reflect the pollution degree and its toxicity to the estuarine biota.

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