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Differential biochemical responses to metal/metalloid accumulation in organs of an edible fish (*Centropomus parallelus*) from Neotropical estuaries

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ABSTRACT

Metal/metalloid accumulation in fish organs elicits biochemical responses indicating the overall fish and environmental health status. This study evaluated the bioaccumulation of metals and metalloid in relation to a suite of biochemical biomarkers (superoxide dismutase, catalase, glutathione-S-transferase, Na+/K+-ATPase, H+-ATPase, acetylcholinesterase activities and the levels of glutathione, metallothionein, lipid peroxidation and oxidized protein) in different organs of fish, *Centropomus parallelus*, in Vitória Bay and Santa Cruz estuaries (State of Espírito Santo, Brazil) with distinct contamination levels. Metal and metalloid concentrations differ in each organ and were significantly higher in winter than in summer. Chemometric evaluation performed between metal/metalloid accumulation and the biomarkers revealed a complex scenario in which the biomarker responses depend on both metal accumulation and organ/tissue sensitivity. The metal levels in gills indicate fish contamination mainly via water and the low sensitivity of this organ to most metals. Biomarker responses suggested that the metal elimination pathway is through the gills and kidney. The hepatopancreas and kidneys were the most important detoxification organs while muscle was the less reactive tissue. In general, the finding suggested that, *C. parallelus* is partly able to tolerate such metal contamination. However, it is emphasized that the biomarker responses imply an energetic cost and may affect the growth rate and reproduction. Given the ecological and economic importance of *C. parallelus*, the level of toxic metals/metalloids in juvenile fish is an important early-warning for the maintenance, conservation and commercial use of this species.

1. Introduction

Estuaries are characterized by high physical and chemical variability as they constitute an interface between the continent and the sea (Elliott and Whitfield, 2011) and many are influenced by anthropogenic activities, including main contamination sources (Borja et al., 2012; Wolanski and Elliott, 2015). Metal/metalloid inputs in estuarine areas, and their transfer through the trophic web, may disrupt biological processes resulting in toxicity, which may affect the structure of population and community, even in those organisms well-adapted to tolerate such stressors (Elliott and Quintino, 2007). Essential metals play important roles in biological systems, but they become toxic at high levels and non-essential metals can be toxic, disturbing biological processes, even at trace amounts (Mazon et al., 2002; Hartl, 2013; Rosabal et al., 2015).

Metal/metalloid bioaccumulation may differ among organs/tissues depending on the mode of exposure (dietary and/or water), uptake, regulation and excretion mechanisms as well as their roles in these processes (Jarić et al., 2011). Metals may interfere in cellular enzymatic pathways by generating reactive oxygen species (ROS), which promote oxidative stress and degenerative processes in the cells (Oliveira et al., 2010; Carvalho et al., 2012; Sakuragui et al., 2013; Barbee et al., 2014; Brandão et al., 2015; Cappello et al., 2016a, 2016b). ROS can be detoxified by enzymatic and non-enzymatic cell defence systems, including the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and

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levels of glutathione (GSH) and metallothioneins (MT), among others enzymes and compounds (Storey, 1996). The activation and/or inhibition of these systems reflect the exposure to metals and/or their toxicity (Oliveira et al., 2010; Souza et al., 2013). Studies on wild fish have long emphasized the metal accumulation in the muscles, the edible part of fish consumed by humans (Bosch et al., 2016; Cerveny et al., 2014). However, it is important to analyse other organs/tissues to evaluate metal distribution, clarifying the links between the contamination and adverse responses in fishes (Elliott et al., 1988).

Among fish organs, the gills have important functions such as gas exchange, ion transport and nitrogen excretion, being the first organs exposed to waterborne chemicals; contaminant uptake (including metals) is facilitated by the large surface area and thin water-blood diffusion distance in gills (Fernandes and Mazon, 2003). As well as representing the main route of entry of contaminants in fishes, the gills can also be directly affected by metal and metalloids, triggering antioxidant defence systems and disturbing its metabolic pathways (De Domenico et al., 2013; Cappello et al., 2016b). Furthermore, the high osmoregulatory activity in estuarine fish in the gills and, to a lesser extent, the kidney, increases the tissue susceptibility to water contaminants (Elliott et al., 1988; Monserrat et al., 2007). The liver, the main detoxification organ in all vertebrates, effectively takes up metals/metalloids from the bloodstream, and its dysfunction is an early indicator of the presence of toxicants in the environment (Fonseca et al., 2011; Paul et al., 2014).

In Brazil, the coast of Espírito Santo state (ES) has been impacted by metal and metalloids, such as As, Pb, Cr, Cu, Fe, Al, Zn and Mn, arising from metallurgical industries and harbours for iron export (Santos et al., 2017). The estuaries of Santa Cruz and Vitória Bay have different levels and types of metal contamination. Contamination with K, Ag and Mn has been reported in Santa Cruz, while high levels of Fe, Al and Pb were found in Vitória Bay (Arrivabene et al., 2015; Souza et al., 2013, 2014a, 2014b). Despite the lower levels of As, Pb and Cu reported in Santa Cruz, the bioavailability of such elements was higher in Santa Cruz than in Vitória Bay (Souza et al., 2014b). Numerous biological changes in the biota from these areas were associated with metal contamination (Souza et al., 2014a, 2014b, 2015; Arrivabene et al., 2014, 2015).

In a previous study, we reported that the fat snook fish (*Centropomus parallelus*) inhabiting these areas showed biological anomalies, such as changes in the activity of antioxidant enzymes (SOD and CAT) and the biotransformation enzyme (GST) in the hepatopancreas and gills, in addition to erythrocyte anomalies, moderate damage in liver and metal bioaccumulation in muscles (Souza et al., 2013). These findings led to the following questions: 1) Does metal distribution and accumulation differ among fish organs? 2) How does each organ respond to metal accumulation? 3) Are there seasonal differences in the bioaccumulation and physiological responses in fish?

In this context, metals and metalloid accumulation in the gills, hepatopancreas, kidneys and muscle have been determined together with biochemical enzymatic and non-enzymatic biomarkers in juvenile fat snook, *Centropomus parallelus* Poey 1860 (Centropomidae) from Vitória Bay and Santa Cruz estuaries, ES, Brazil. This was allowed all aspect to be integrated to identify metal and metalloid accumulation and organ biochemical and physiological responses. *C. parallelus* is a protandric top-predator fish, which does not undergo migratory cycles during its juvenile stage (Volpe, 1959; Taylor et al., 2000). As an estuarine resident, *C. parallelus* has been proposed as a possible bioindicator in Brazilian coastal regions (Rocha et al., 2007).

2. Materials and methods

2.1. Fish sampling

Samples from gills, kidney, hepatopancreas and muscles used in this study were from the same juvenile male *C. parallelus* of the previous study (Souza et al., 2013). *C. parallelus* (n = 40, ten fishes for each site and season; body mass = 150 ± 30 g; total length = 15 ± 5 cm) were collected in two seasons (winter 2009 and summer 2010) in Vitória Bay (20° 19′S and 40° 20′W) and Santa Cruz estuary (19° 58′S and 40° 07′W), ES, Brazil (Fig. 1) in September of 2009 and March of 2010. Fishes were killed by medullary section and field-dissected. The gills, hepatopancreas, kidneys (posterior region) and muscle were removed using plastic instruments to avoid contaminating the samples, stored in plastic tubes (Eppendorf or Falcon tubes depending on the size), immersed in liquid nitrogen immediately after collection to stop the metabolic activity and so transported to the laboratory, and stored at − 80 °C until analysis.

Vitória Bay is an estuarine complex formed by five rivers showing environmental degradation caused by harbour and industrial activities, including air pollution by smoke metallic particles; the Santa Cruz estuary is formed from two rivers with a large mangrove area (natural reserve). Water samples were taken approx. 1 m below the surface in pre-cleaned recipients. Physical and chemical data corresponding to water samples were reported in Souza et al. (2013).

2.2. Multi-elemental analyses

Ultra-pure water (resistivity > 18.2 MΩ cm^{-1}; ≤ 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). The purity of nitric acid was verified by Mass Spectrometry Inductively Coupled Plasma (ICP-MS), Agilent 7500cx, USA, equipped with an ASX-100 autosampler (CETAC Technologies, Omaha, NE). Filters (0.45 µm, HAWG04756) were obtained from Millipore (São Paulo, Brazil). All glassware and plastic bottles and containers were cleaned overnight in dextran 10%, scrubbed, washed with tap water, then left overnight in nitric acid 10%, washed first with deionized water and finally with ultrapure water. ICP probes and pipes were of PTFE, previously washed as described above, changing the concentration of nitric acid to 35% and then in 20% sulphuric acid, as the method EPA 200.8 for metal analysis (EPA, 1994).

For multi-elemental analysis tissue samples (n = 5 animals per site and season) were dried at 37 °C until constant weight and stored at room temperature. Samples in triplicate were ground and homogenised with a mortar and digested (0.1 g from each organ) according to Chappaz et al. (2012), using 4 mL nitric acid (ultra-pure, sub boiling grade) and 1 mL hydrogen peroxide (30%, Merck), in pre-cleaned PTFE tubes (Savillex) at constant temperature (90 °C) during 24 h. Controls were prepared using the same protocol without sample (only reagents) (Monferrán et al., 2016). Digested samples were stored at 4 °C until analysis for B, Al, V, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Ag, Cd, Hg and Pb.

The concentrations of elements were determined in triplicate from each sample and the repeatability of ICP-MS measurements was generally ≥ 97%. Analytical quality assurance (QA) and quality control (QC) were done using a certified reference material (CRM): typical diet NIST1548a) and bovine muscle (NIST 8414) and the recoveries of each material were 94 ± 17% and 91 ± 13%, respectively. Three spiked samples were prepared for gills, kidney, hepatopancreas and muscle samples by adding standard solutions (10 mg/L and 10 µg/L) containing all the elements analysed to 0.1 g of dried tissue. Spiked samples are important to verify whether the digested matrix can influence in the metal analysis (Monferrán et al., 2016). The average recovery of these assays was 87 ± 16%.

2.3. Biochemical analyses

Individual samples of gills, hepatopancreas, kidney and muscle from each animal were homogenised (n = 5 animals per site and season). The total protein in each sample was determined according to Bradford

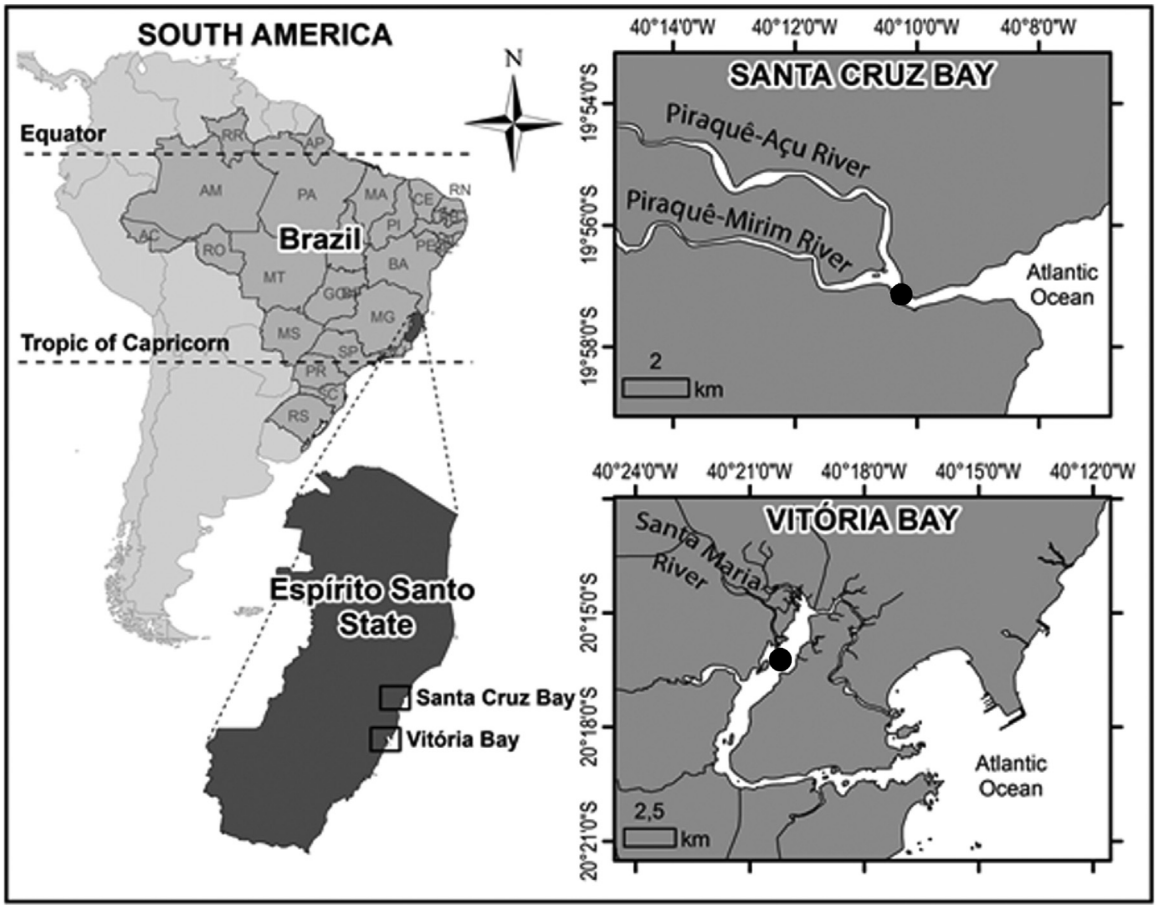

Fig. 1. Map of Brazil (South America) showing the State of Espírito Santo and the location of estuaries Santa Cruz (S 19°56`26.2``; W 40° 12`87``) and Vitória Bay (S 20°14`31.5``; W 40°19`84.7``) sampling sites (●).

(1976), in a microplate reader (SpectraMax M5, Molecular Devices, USA) using bovine serum albumin as standard. Four biomarkers were measured in all sampled organ/tissues: the phase II biotransformation enzyme GST was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and following the change in absorbance at 340 nm (Habig and Jakoby, 1981); the activity of antioxidant enzymes SOD was measured following the inhibition of the cytochrome c reduction by the superoxide radicals at 550 nm (McCord and Fridovich, 1969) and CAT was measured by monitoring H_2O_2 decomposition using the decrease in absorbance at 240 nm (Beutler, 1982); the level of GSH was determined using Ellman's reagent (5,50-dithiobis-2-nitrobenzoic acid, DTNB) and measuring the thiolate anion formation at 412 nm (White et al., 2003). The MT concentration was quantified following Viarengo et al. (1997) in hepatopancreas and muscle. The oxidative stress biomarkers were measured in hepatopancreas and muscle; lipid peroxidation (LPO) was determined following Jiang et al. (1991, 1992) and oxidized protein (OP) following Levine et al. (1994). Acetylcholinesterase activity (AChE) was assessed only in muscle following Ellman et al. (1961). Na^+/K^+-ATPase (NKA) and H^+-ATPase activities (HAT) (Gibbs and Somero, 1989) were measured in the gills and kidneys, considering the osmoregulatory and excretory functions of these organs in fish. All measurements were performed in triplicate.

2.4. Statistical analysis

Data are reported as the mean ± standard deviation and analysed using the statistical packages, STATISTICA 7.1 from StatSoft Inc. (2005), and Infostat. All data were tested for a normal distribution using Shapiro-Wilk test and for homogeneity of variance using Levene's test and Brown & Forsythe's test. The one-way analysis of variance (ANOVA) was applied followed by Tukey's post-hoc test to compare data from each organ between sites and seasons (significance $p < 0.05$). The outliers were removed. Principal Component Analysis (PCA) was performed crossing all metal concentrations and biological effects data to identify potential relationships between these variables in each organ and studied areas; Pearson correlation coefficients were employed to determine the significant correlations ($p < 0.05$) and presented as a correlation matrix.

In addition, Generalized Procrustes Analysis (GPA) (Di Paola et al., 2011) was performed to evaluate the correspondence between biomarkers and metal and metalloid concentrations in organs/tissues of fish with metal and metalloid data in sediment and water samples from the studied estuaries reported by Souza et al. (2013) as the fish samples were the same used in the previous study. GPA is based on PCA results by transforming data in a consensus configuration of data set groups. The Grower algorithm was used to minimize within-sample variance by applying translation, scaling and rotation and generate a dimensional average configuration Yc. A q-dimensional group average space ($q \leq p$) was constructed from Yc by PCA (Wunderlin et al., 2001).

3. Results

3.1. Metal accumulation and biochemical biomarker responses

There were significant differences between metal and metalloid concentrations measured in the gills, hepatopancreas, kidneys and muscle of *C. parallelus* from Vitória Bay and Santa Cruz estuaries (Table 1). In fish from Vitória Bay, the concentration of B and Cr was higher in gills, Fe in hepatopancreas and Cu, As and Se in both, hepatopancreas and kidneys. In contrast, fish from Santa Cruz had higher

Table 1
Metal concentrations (μg g^{-1} dry mass) in different organs/tissues of *Centropomus parallelus* (n = 5 in each site per season) from Vitória Bay and Santa Cruz estuaries. Values are mean ± SD. VBW: Vitória bay winter; VBS: Vitória bay summer; SCW: Santa Cruz winter; SCS: Santa Cruz summer.

		Metals						
		B	Al	V	Cr	Mn	Fe	Ni
Gill								
	VBW	4.66 ± 3.77^{b}	55.6 ± 6.4^{a}	0.20 ± 0.03^{a}	9.42 ± 6.59^{a}	11.5 ± 2.3^{a}	128 ± 28^{a}	< LOQ^{a}
	VBS	1.26 ± 0.52^{a,b}	74 0.3 ± 10.9^{a}	0.20 ± 0.03^{a}	7.02 ± 2.02^{a}	11.1 ± 1.6^{a}	121 ± 13^{a}	3.03 ± 0.99^{b}
	SCW	< LOD^{a}	279.5 ± 72.5^{c}	0.25 ± 0.06^{a}	10.23 ± 3.83^{a}	28.9 ± 10.1^{b}	117 ± 32^{a}	4.53 ± 1.74^{b}
	SCS	1.17 ± 0.15^{a}	187.8 ± 69.3^{b}	0.21 ± 0.03^{a}	9.38 ± 0.27^{a}	40.6 ± 9.8^{b}	151 ± 4^{a}	4.05 ± 0.14^{b}
Hepatopancreas								
	VBW	< LOD	7.7 ± 2.2^{b}	0.25 ± 0.14^{b}	< LOQ^{a}	7.4 ± 2.2^{b}	1571 ± 414^{c}	< LOD
	VBS	< LOD	3.7 ± 0.8^{a,b}	0.11 ± 0.05^{a,b}	< LOQ^{a}	5.0 ± 0.6^{a,b}	777 ± 383^{b}	< LOD
	SCW	< LOD	2.8 ± 2.8^{a}	0.17 ± 0.01^{a,b}	< LOQ^{a}	4.3 ± 0.9^{a}	98 ± 68^{a}	< LOD
	SCS	< LOD	4.0 ± 2.9^{a,b}	0.10 ± 0.04^{a}	0.33 ± 0.27^{a}	3.5 ± 2.3^{a}	43 ± 11^{a}	< LOD
Kidney								
	VBW	< LOD^{a}	5.9 ± 0.8^{a}	0.29 ± 0.05^{a}	4.44 ± 3.74^{b}	< LOQ^{a}	378 ± 75^{a}	< LOD
	VBS	< LOD^{a}	13.2 ± 8.6^{a}	0.19 ± 0.04^{a}	< LOD^{a}	3.3 ± 0.6^{a,b}	405 ± 29^{a}	< LOD
	SCW	< LOD^{a}	31.6 ± 6.8^{b}	1.39 ± 0.45^{b}	< LOD^{a}	4.5 ± 0.9^{b}	1095 ± 309^{b}	< LOD
	SCS	2.05 ± 0.27^{a}	43.4 ± 11.8^{b}	1.06 ± 0.12^{b}	< LOD^{a}	4.3 ± 0.7^{b}	670 ± 279^{a}	< LOD
Muscle								
	VBW	< LOD	37.4 ± 15.0^{a}	0.07 ± 0.02^{a,b}	4.58 ± 3.31^{a,b}	0.8 ± 0.1^{a}	34 ± 16^{a}	1.87 ± 1.74^{a}
	VBS	< LOD	15.8 ± 6.2^{a}	0.04 ± 0.01^{a}	2.26 ± 0.89^{a}	0.7 ± 0.1^{a}	20 ± 6^{a}	0.78 ± 0.51^{a}
	SCW	< LOD	53.2 ± 39.5^{a}	0.12 ± 0.06^{b}	13.21 ± 9.21^{b}	2.7 ± 1.4^{b}	84 ± 38^{b}	6.52 ± 5.11^{a}
	SCS	< LOD	22.7 ± 10.9^{a}	0.04 ± 0.01^{a}	2.60 ± 0.49^{a}	1.3 ± 0.3^{a}	30 ± 7^{a}	0.84 ± 0.18^{a}

	Metals							
	Cu	Zn	As	Se	Ag	Cd	Hg	Pb
Gill								
	0.52 ± 0.22^{a,b}	92 ± 12^{a}	0.50 ± 0.22^{b,c}	1.46 ± 0.16^{b}	< LOD	< LOD	< LOQ^{a}	0.31 ± 0.12^{a}
	0.84 ± 0.14^{b}	82 ± 3^{a}	0.64 ± 0.17^{c}	1.29 ± 0.08^{b}	< LOD	< LOD	< LOD^{a}	< LOQ^{a}
	0.43 ± 0.04^{a}	96 ± 47^{a}	0.26 ± 0.15^{a,b}	0.60 ± 0.19^{a}	< LOD	< LOD	0.81 ± 0.26^{a}	0.38 ± 0.12^{a}
	0.70 ± 0.31^{a,b}	83 ± 16^{a}	< LOQ^{a}	0.76 ± 0.01^{a}	< LOD	< LOD	0.83 ± 0.46^{a}	0.41 ± 0.04^{a}
Hepatopancreas								
	10.28 ± 3.02^{a}	88 ± 21^{b}	4.34 ± 1.99^{b}	5.90 ± 1.07^{c}	< LOD	< LOD	< LOD	0.45 ± 0.35^{a}
	8.47 ± 1.85^{a}	55 ± 10^{a,b}	2.84 ± 1.27^{b}	4.10 ± 0.35^{b}	< LOD	< LOD	< LOQ	< LOD^{a}
	11.63 ± 6.10^{a}	46 ± 27^{a}	0.33 ± 0.14^{a}	1.50 ± 0.31^{a}	< LOD	< LOD	< LOD	< LOD^{a}
	9.78 ± 6.97^{a}	28 ± 14^{a}	< LOD^{a}	0.98 ± 0.21^{a}	< LOD	< LOD	< LOD	< LOD^{a}
Kidney								
	3.74 ± 1.20^{a}	569 ± 98^{a}	2.49 ± 0.86^{b,c}	5.74 ± 1.18^{b}	< LOD	< LOD	< LOD	< LOD^{a}
	5.57 ± 0.50^{a}	787 ± 155^{a}	3.11 ± 0.51^{c}	5.29 ± 0.67^{b}	< LOD	< LOD	< LOD	< LOD^{a}
	5.72 ± 1.38^{a}	1398 ± 559^{b}	1.95 ± 0.53^{b}	5.65 ± 0.60^{b}	< LOD	< LOD	< LOD	< LOQ^{b}
	9.94 ± 3.29^{b}	381 ± 111^{a}	< LOQ^{a}	3.59 ± 0.13^{a}	< LOD	< LOD	< LOD	< LOQ^{b}
Muscle								
	0.71 ± 0.41^{a}	37 ± 5^{a}	1.22 ± 0.52^{a,b}	1.16 ± 0.18^{b}	< LOD	< LOD	0.68 ± 0.34^{a}	1.28 ± 1.20^{a}
	< LOD^{a}	30 ± 2^{a}	0.67 ± 0.11^{a}	1.18 ± 0.13^{b}	< LOD	< LOD	< LOQ^{a}	< LOQ^{a}
	< LOD^{a}	235 ± 196^{b}	1.75 ± 0.48^{b}	0.61 ± 0.12^{a}	< LOD	< LOD	< LOQ^{a}	< LOQ^{a}
	< LOD^{a}	29 ± 20^{a}	0.66 ± 0.43^{a}	0.52 ± 0.03^{a}	< LOD	< LOD	< LOQ^{a}	1.93 ± 1.83^{a}

< LOD (below detection limit); < LOQ (below quantification limit). LOQs: B (2.84 μg g^{-1}); Al, Cr, Ni, Cu, Zn, As, Ag, Cd, Pb (0.46 μg g^{-1}); V (0.03 μg g^{-1}); Mn (0.86 μg g^{-1}); Fe (4.42 μg g^{-1}); Se (1.60 μg g^{-1}) and Hg (0.81 μg g^{-1}). Different letters indicate significant difference between site/seasons for each organ ($p < 0.05$).

Table 2
Activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and level of glutathione (GSH) in gills, liver, kidney and muscle; activity of H^+-ATPase, Na^+/K^+-ATPase in gills and kidneys; level of metallothionein (MT), lipid peroxidation (LPO) and oxidized protein (OP) in liver and muscle and, activity of acetylcholinesterase (AChE) in muscle of *Centropomus parallelus* (n = 5 for each site and season) from Vitória Bay and Santa Cruz estuaries. Values are mean ± SD. VBW: Vitória bay winter; VBS: Vitória Bay summer; SCW: Santa Cruz winter; SCS: Santa Cruz summer; ND: not detected; -: not analysed. Different letters indicate significant difference between site/seasons for each organ ($p < 0.05$).

		SOD (U mg Pt^{-1})	**CAT** (μmol g Pt^{-1} min^{-1})	**GST** (nmol mg Pt^{-1} min^{-1})	**GSH** (nmol mg Pt^{-1})	**H^+-ATPase** (μmol Pi mg Pt^{-1} h^{-1})	**Na^+/K^+-ATPase** (μmol Pi mg Pt^{-1} h^{-1})
Gills							
	VBW	16.05 ± 1.64^{a}	11.48 ± 2.28^{a,b}	8,76 ± 2,40^{b}	92.76 ± 28.37^{c}	0.12 ± 0.08^{a}	0.05 ± 0.03^{a,b}
	VBS	26.95 ± 7.73^{a}	14.65 ± 2.53^{b}	4,58 ± 0,90^{a}	68.24 ± 22.64^{b,c}	0.12 ± 0.10^{a}	0.05 ± 0.01^{a,b}
	SCW	27.46 ± 7.49^{a}	10.57 ± 1.47^{a}	11,69 ± 1,54^{b}	44.03 ± 10.61^{a,b}	0.14 ± 0.06^{a}	0.08 ± 0.03^{b}
	SCS	71.61 ± 18.01^{b}	11.48 ± 2.72^{a,b}	30,00 ± 3,32^{c}	33.29 ± 6.74^{a}	0.24 ± 0.08^{a}	0.03 ± 0.01^{a}
Hepatopancreas							
	VBW	70.63 ± 5.66^{b}	225.72 ± 21.17^{d}	49.63 ± 4.09^{a}	57.27 ± 25.29^{a}	–	–
	VBS	47.41 ± 6.23^{b}	186.78 ± 28.97^{c}	35.18 ± 6.67^{a}	71.70 ± 17.87^{a}	–	–
	SCW	22.08 ± 9.44^{a}	31.08 ± 14.52^{a}	141.30 ± 29.92^{b}	100.74 ± 12.20^{a,b}	–	–
	SCS	49.37 ± 5.78^{b}	80.48 ± 14.56^{b}	48.27 ± 19.08^{a}	132.67 ± 51.40^{b}	–	–
Kidney							
	VBW	1.12 ± 0.59^{a}	12.36 ± 2.99^{b}	33.21 ± 4.47^{c}	30.11 ± 2.20^{a}	1.00 ± 0.07^{c}	1.27 ± 0.04^{c}
	VBS	1.07 ± 1.14^{a}	9.79 ± 1.05^{a,b}	18.34 ± 5.57^{b}	31.93 ± 3.56^{a}	0.56 ± 0.36^{a,b}	0.69 ± 0.39^{a,b}
	SCW	2.77 ± 1.80^{a}	8.78 ± 1.80^{a}	13.69 ± 4.18^{a,b}	56.60 ± 39.50^{a}	0.84 ± 0.10^{b,c}	0.94 ± 0.09^{b,c}
	SCS	1.16 ± 0.74^{a}	10.44 ± 1.90^{a,b}	10.56 ± 1.56^{a}	44.15 ± 7.89^{a}	0.41 ± 0.21^{a}	0.54 ± 0.14^{a}
Muscle							
	VBW	5.81 ± 2.22^{a}	0.79 ± 0.07^{a,b}	9.47 ± 2.94^{a}	409.66 ± 94.85^{a,b}	–	–
	VBS	9.29 ± 3.95^{a}	0.90 ± 0.22^{b}	5.97 ± 1.95^{a}	814.73 ± 579.10^{b}	–	–
	SCW	7.50 ± 3.94^{a}	0.67 ± 0.13^{a}	9.59 ± 2.30^{a}	274.81 ± 124.38^{a}	–	–
	SCS	4.68 ± 0.63^{a}	0.66 ± 0.08^{a}	9.68 ± 2.48^{a}	486.85 ± 90.52^{a,b}	–	–
		MT (μmol-SH mg Pt^{-1})		**LPO** (nmol mg Pt^{-1})	**OP** (nmol mg Pt^{-1})	**AChE** (μmol mg Pt^{-1} min^{-1})	
Hepatopancreas							
	VBW	0.30 ± 0.14^{b}		0.43 ± 0.86^{a}	7.55 ± 0.67^{b}	–	
	VBS	0.21 ± 0.02^{a,b}		0.18 ± 0.36^{a}	6.92 ± 2.27^{b}	–	
	SCW	0.11 ± 0.07^{a}		4.15 ± 1.20^{b}	2.90 ± 1.53^{a}	–	
	SCS	0.14 ± 0.12^{a,b}		2.81 ± 1.14^{b}	3.30 ± 1.00^{a}	–	
Muscle							
	VBW	0.16 ± 0.01^{b,c}		ND	1.86 ± 0.23^{a,b}	29.84 ± 2.07^{a}	
	VBS	0.26 ± 0.10^{c}		ND	2.76 ± 0.47^{b,c}	26.92 ± 1.38^{a}	
	SCW	0.13 ± 0.12^{a,b}		ND	1.81 ± 0.54^{a}	27.95 ± 1.71^{a}	
	SCS	0.03 ± 0.01^{a}		ND	2.80 ± 0.90^{c}	27.12 ± 2.78^{a}	

levels of Al and Mn in gills, Cr in gills and muscle, Cu in both hepatopancreas and kidneys, and V, Fe and Zn in kidneys. In general, metal and metalloid concentrations in fish collected in winter were significantly higher than those collected in summer in both estuaries (Table 1). Fish from Vitória Bay showed a higher Hg concentration in kidneys and Pb in gills and kidney (winter), while those from Santa Cruz had higher Hg and Pb in gills (winter and summer) and Pb in kidney (summer).

The higher values of biochemical biomarker antioxidant defences were found in hepatopancreas of fish from both sites (Table 2). In the gills, the activity of CAT and GSH (winter and summer) and GST (winter) were higher in fish from Vitória Bay; GSH (summer) and SOD, CAT and GST (winter) activities were higher in those from Santa Cruz. In the hepatopancreas, SOD, CAT, MT and OP were higher in fish from Vitória Bay (winter and summer) and GST and GSH (winter), SOD and CAT (summer) and LPO (winter and summer) in those from Santa Cruz. In the kidney, CAT (winter) and GST (winter and summer) were higher in Vitória Bay than in Santa Cruz. In muscle, CAT, GSH, MT and OP were higher in fish from Vitória Bay (winter and summer) and MT (winter) and OP (summer) were higher in Santa Cruz. AChE in muscle did not differ between sites and seasons.

At both sites, the H^+-ATPase activity did not differ in gills but, the activity of this enzyme in kidney was higher (in winter); Na^+/K^+-ATPase activity in kidney was higher than gills and the highest values were found in winter.

3.2. Integrated analysis between metals and metalloids and biochemical biomarkers

Metal accumulation and the biomarker responses differ in each organ between winter and summer. All data in each site/season and each fish organ, independent of sites, were integrated by PCA complemented by Pearson analyses. The first and second principal components of the PCA analyses explained more than 80% of total data variability for all tissues, showing the main associations between metal and metalloid concentrations and studied biomarkers in each organ, highlighting clear differentiation between sampling sites and seasons (Fig. 2, Tables 1S–4S). Comparing Vitória Bay and Santa Cruz, most correlations were found in the former, in winter, independent of fish organ (Fig. 2).

In the gills, significant correlations were detected between B, A, Se, Al, Mn and GSH (winter) and Cu and CAT activity (summer) in Vitória Bay. In Santa Cruz there was no correlation between metals and metalloids and enzymatic activity in the gills during winter. Conversely, in the summer, Mn and Fe were correlated with SOD, while Mn correlated with HAT (Fig. 2A, Table 1S).

In the hepatopancreas, fishes from Vitória Bay showed positive correlations between levels of Fe, Se, Zn, Mn and As with MT. Additionally Fe, Se, Zn, Mn, As correlated with SOD activity. Also Fe, Se, Zn, Mn, As correlated with CAT, in the winter in Vitória Bay. Most summer samples showed higher enzyme activity and significant correlations between B and CAT, in addition to enzymatic correlations between SOD and CAT, SOD and OP, SOD and LPO, CAT and LPO (Fig. 2B, Table 2S). In contrast, there were no significant correlations in Santa Cruz between metals and biomarkers.

In the kidney, correlations between metals and metalloids and biomarkers occurred in the winter in Vitória Bay. Thus, Cr, As and Se correlated with GST; Cr, Se and Cu correlated with NKA, while Se, Cr, B, Cu correlated with HAT (Fig. 2C, Table 3S). Enzyme correlations were found between HAT and NKA, NKA and GST, HAT and GST. On the

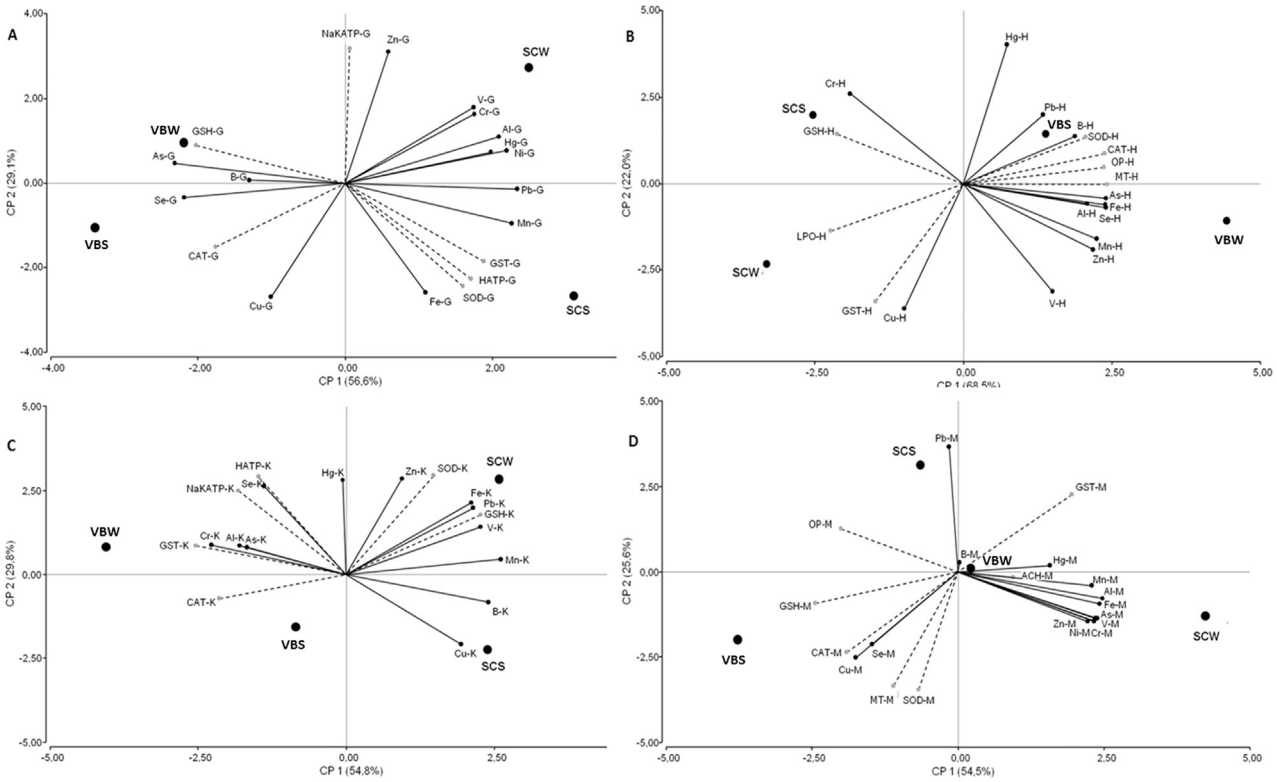

Fig. 2. Graphical representation of Principal Component Analysis (PCA). A: Gill (G); B: Hepatopancreas (H); C. Kidney (K); M. Muscle (M). VBS: Vitória Bay- Summer; VBW: Vitória Bay- Winter; SCS: Santa Cruz- Summer; SCW: Santa Cruz- Winter.━ Metals; —— Biomarkers.

other hand, samples from Santa Cruz showed correlations between Fe, V and Mn with SOD, while V and Mn correlated with GSH.

In muscle, the correlations occurred only in Vitória Bay (Fig. 2D). In winter the correlation was between Hg and AChE, although the absolute values of Hg and AChE did not differ significantly between sites. In summer, correlations were between Se and CAT, Se and MT, Cu and MT, Hg and GST, Se and GST (Fig. 2D, Table 4S).

3.3. Integrated analysis between metals and metalloids in the environment, accumulation and biomarkers in fish organs

The environmental metal and metalloid data in water and sediment reported by Souza et al. (2013), and those accumulated in each fish organ from the present study, show that Al, Mn, Fe and Pb were present in both water and sediment. Additionally, Ag was present in water, while Cr and As were present in the sediment at both sites and seasons. Furthermore, Ni and Cu were present in the water and sediment in Santa Cruz, but only in the water in Vitória Bay. Moreover, Zn was higher in the water from Vitória Bay than in water from Santa Cruz. In addition, Cd and Hg were detected in the water, but were below the quantification limit in both sites and seasons.

GPA analyses were carried out using metal accumulation and biomarker data in each fish organ from our current study whereas environmental data (metal and metalloid in water and sediment) were taken from Souza et al. (2013). These combined data showed a consensual configuration of four data sets, discriminating the seasons and sites. This configuration, defined by its first (CP1 − 54.9%, which indicate the site segregation) and second (CP2 − 27.3%, which indicates to seasonal differences) principal axes, explained > 80% of the variability in the combined data, indicating that the difference of metal and metalloid concentrations and their bioavailability between sites were more important than the season (Fig. 3).

4. Discussion

The study showed a complex set of relationships between metal and metalloid accumulation, the activity of enzymes (SOD, CAT, GST) and the content of GSH and MT related to antioxidant defences in gills, hepatopancreas, kidneys and muscles, in addition to those enzymes related to the osmoregulation processes (NKA and HAT) in gills and kidney. The enzymatic responses depended on both the metal and metalloid accumulation and the organ sensitivity. In general, metal and metalloid availability in estuarine water is highly variable, depending on the tide, the season and the chemical-physical characteristics of the water (Souza et al., 2013; Wolanski and Elliott, 2015). Therefore, the bioaccumulation of metals and metalloids in the fish reflect their bioavailability in the environment. However, the higher metal and metalloid accumulation in fish during the winter could be related to the higher salinity in this season (Souza et al., 2013) and fish osmotic and ionic regulation. At high salinity, fish drink water with excess of ions and consequently soluble (Marshall and Grosell, 2006)

Liver and kidney are the organs that accumulate the highest levels of essential and non-essential metals and metalloids as they are the main detoxification and excretion organs (Elliott et al., 1988; Lawrence and Hemingway, 2003). However, in juvenile *C. parallelus* most metals and metalloids were distributed in all organs. In general, some metals and metalloids were higher in a particular organ, which is the case for B, Al, Cr, Mn and Ni in gills; V and Zn in kidney; Cu and As in the hepatopancreas, and Fe in the hepatopancreas and kidney, showing differential organ capability for storage, detoxification and excretion such elements (see also Carvalho et al., 2015). Environmental chronic exposure, in addition to the exposure route (via gills or gut), alter tissue-specific accumulation patterns, resulting in different blood perfusion rates among organs. Thus, metal and metalloid bioaccumulation in gills is expected to be higher following waterborne exposure, while higher bioaccumulation in liver is followed by gut metal uptake (Wood, 2012).

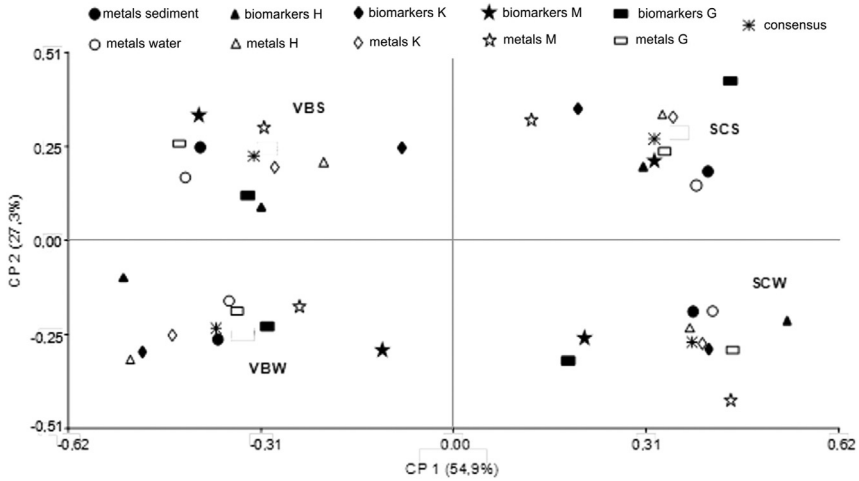

Fig. 3. Graphical representation of Generalized Procrustes Analysis (GPA). VBS: Vitória Bay Summer; VBW: Vitória Bay Winter; SCS: Santa Cruz Summer; SCW: Santa Cruz Winter; G: Gill; H: Hepatopancreas; K: Kidney; M: Muscle.

Respiration requires a large amount of water moving across the lamellar epithelium, and the high blood perfusion rate in these organs. The tide dynamics in the estuaries implies a more intense osmoregulation activity in resident fish, favouring the metal and metalloid uptake by gills (Bianchini et al., 2008), which, at least in part, may explain the presence of all metals in these organs. Gills are also potential excretory organs. For instance, aluminium accumulates in the gills at higher levels than in other organs (Jarić et al., 2011; Wood, 2002), but it may return to the water as aluminium hydroxide, or discarded as mucus-bound metal (Playle and Wood, 1991), reducing its accumulation into the fish body. In contrast, the small water-blood diffusion distance in gills favours the metal diffusion into the blood, and then to other organs via the bloodstream, indicating the metal and metalloid burden clearance into the blood, *e.g.* Cu (Grosell et al., 1997; Wood et al., 2002).

Despite the high metal and metalloid bioaccumulation in gills, in this study this organ showed low sensitivity to most studied elements. Higher Al levels were found in Santa Cruz although there were negative correlations between Al and Mn with GSH at this area, which may explain the lower content of GSH in the gills of fish. Al and Mn have strong affinity for reduced GSH, producing oxidized glutathione (GSSG) and the formation of the glutathione-aluminium (GS-Al) conjugate (Khan et al., 2012). Hence, the contaminants are kept biologically inactive in lysosomes (Luoma and Rainbow, 2008; Nikinmaa, 2014). Conversely, B and As showed positive correlation with GSH, which may induce GSH production related to their strong interaction with sulfhydryl groups, favouring the formation of a metal complex and subsequent excretion. With regard to the enzymatic detoxification processes, SOD is a first-line metalloenzyme defence that uses Mn as a cofactor, converting the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) while CAT uses Fe as cofactor and neutralizes H_2O_2 to O_2 and H_2O (Pereira et al., 2010). Copper, even at low concentration in the gill, may induce ROS formation, via the Fenton reaction, which may explain the positive correlation with CAT in the gills (Collén et al., 2003; Carvalho et al., 2015). The activation of such antioxidant systems by Mn and Fe, and the Cu positive correlation with SOD and CAT, respectively, represents an important biochemical defence response against metals and metalloid, avoiding the oxidative damage of lipids, proteins and nucleic acids (Collén et al., 2003). The morphological integrity of gills in this species living in these estuarine areas reinforces the gill tolerance to metals (Souza et al., 2013).

The kidneys are important organs for metal excretion even in fish where urine volume is lower when marine and estuarine fish are in high salinity (Marshall and Grosell, 2006). The high accumulations of Cu in the kidney of fish from Vitória Bay and Fe and Zn in fish from Santa Cruz, are probably due to the high bioavailability of these metals in these estuarine areas. Such high accumulation of Zn in this organ was unexpected, even considering that some seawater fish as the tuna *Thunnus albacares*, accumulate high levels of Zn in the kidney (Kojadinovic et al., 2007). However, MT is the main Zn binding protein in the cells, small molecules such as glutathione, cysteine, and histidine may act as Zn binders (Hogstrand, 2012). This phenomenon may explain the high Zn accumulation in the kidney, since this metal was not correlated with any antioxidant defence system in this organ. The bioaccumulation of Fe in the kidney may bind to intracellular proteins, such as ferritin, keeping Fe in a soluble bioavailable non-toxic form in the cytoplasm until excretion (Arosio and Levy, 2010). Fe is mainly eliminated via the liver (biliary routes) (LeSage et al., 1986), while the kidney enables, at a low level, Fe excretion (Ferguson et al., 2003). V and Fe are required for the production of hydroxyl radical ($HO^{\bullet}$) from H_2O_2 via the Fenton reaction, and by conversion of O_2- to $HO^{\bullet}$, via catalysis in the Haber-Weiss reaction (Stohs and Bagchi, 1995). The positive correlation between V, Fe and Mn with SOD activity may relate to $HO^{\bullet}$ and H_2O_2 production; Mn can also catalyse these reactions.

There was a positive correlation between Mn and HAT in fish gills of Santa Cruz (summer), but a negative correlation between Cr as well as Se with NKA in fish kidneys of Vitória Bay (winter). These enzymes are directly related to the ionic and acid-base regulation in fish gills and kidneys, but little is known about the direct action of metals on their activities, except for many reports of NKA inhibition by Cu (Dang et al., 2000; Atli and Canli, 2007; Li et al., 1996, 2009). It is of note that observed correlations may be an indirect effect of Mn, Cr and Se on both enzymes. For instance, high Cu concentration inhibits NKA activity but increases plasma cortisol, which, in turn, increases NKA activity (Dang et al., 2000; McCormick, 1995); thus, the net NKA activity is the result of the Cu-inhibition and cortisol up-regulation. Thus, the correlation between NKA and HAT activity in *C. parallelus* reflects the role of gills and kidney to keep an electrical gradient and ionic regulation.

The hepatopancreas (liver region) is usually the main detoxification organ, biotransforming or storing contaminants, and so reducing the biological damage (Pacheco and Santos, 2002). The positive correlation between SOD and CAT observed in the fish hepatopancreas can be expected when a high level of H_2O_2 is produced, as shown by the activation of this first-line antioxidant system in fish from Vitória Bay (winter). Furthermore, the positive correlation of SOD and CAT with their metal cofactors (Se, Mn, Fe and Zn), Al and As in the fish hepatopancreas reinforce this hypothesis, showing an effective protection role against non-essential metals and metalloids, given by the negative

correlation with LPO in *C. parallelus*. The possible peroxisome proliferation, containing high levels of CAT, has an important role in the degradation of peroxides (Lawrence and Hemingway, 2003). Moreover, the positive correlations of MT with Fe, Se, Zn, Mn and As show a non-enzymatic antioxidant response that helps to maintain metal and metalloid homeostasis in Vitória Bay, although OP was higher than in Santa Cruz. Metallothioneins regulate the free concentrations of essential metals, quenching non-essential ones to protect cells against their toxic effect (Luoma and Rainbow, 2008).

Muscle is the ultimate soft tissue for metal accumulation and, in general, indicates that the liver and other excretion organs (gills and kidney) were exceeded in their capacity to remove metals and metalloids. The exception is Hg, which preferentially accumulates in muscle (see also Elliott et al., 1988). The positive correlation between Se with CAT and MT, Cu with MT, and Hg with GST in the muscle suggest that, even at low levels, these metals may stimulate the activity of these antioxidant defences, protecting the cells.

The differences between the metal and metalloid bioaccumulation and the activity and/or levels of biological biomarkers observed in both sampling areas highlight the importance of assessing the responses of estuarine fishes at different sites, instead of evaluating seasonal changes. Metal accumulation in fishes can even indicate the migratory habitats of estuarine fishes and the relationships between their home ranges and environmental contaminant levels (e.g. Elliott et al., 1988). Overall, biological responses to metal accumulation were observed at both sites although such responses were more evident in Vitória Bay in which metal concentration in the surface water were higher. Although, the physicochemical properties of the water at each site (Vitória Bay and Santa Cruz Bay) differ (Souza et al., 2013), metal bioaccumulation was observed in both areas.

5. Conclusions

The evaluation of metals and metalloids accumulation in different organs of *C. parallelus* showed that the responses (and their absence) in a given organ show organ and tissue-specific responses suggesting that, at least to some extent, *C. parallelus* is able to tolerate such environmental conditions. The high metal and metalloid levels in gills indicate water contamination as well as the low sensitivity of this organ to most studied metals and metalloids. Moreover, biochemical responses suggest that the metal and metalloid excretion pathway of *C. paralellus* is through both gills and kidney. The hepatopancreas and kidney were found to be important detoxification organs, while the muscle was less responsive probably due to low level antioxidant defences and low accumulation. It is of note that these responses have an energetic cost, which can lead to a negative effect on the growth rate and reproduction although the long-term repercussions of contamination throughout the life or across generations have been rarely studied. Given the ecological and economic importance of *C. parallelus*, the level of toxic metals and metalloids in juvenile fish can be proposed as an important early-warning for the future management, conservation and commercial value of this species.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.05.068.

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