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Matching pollution with adaptive changes in mangrove plants by multivariate statistics. A case study, *Rhizophora mangle* from four neotropical mangroves in Brazil

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HIGHLIGHTS

- *Rhizophora mangle* is a good bioindicator of environmental changes in mangrove.
- Chemometrics helps integrating physical, chemical and biological parameters.
- Anatomical analysis responded quite well to different pollution scenarios.
- *R. mangle* plasticity is an adaptive strategy to regulate the input of toxics to the root.

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ABSTRACT

Roots of mangrove trees have an important role in depurating water and sediments by retaining metals that may accumulate in different plant tissues, affecting physiological processes and anatomy. The present study aimed to evaluate adaptive changes in root of *Rhizophora mangle* in response to different levels of chemical elements (metals/metalloids) in interstitial water and sediments from four neotropical mangroves in Brazil. What sets this study apart from other studies is that we not only investigate adaptive modifications in *R. mangle* but also changes in environments where this plant grows, evaluating correspondence between physical, chemical and biological issues by a combined set of multivariate statistical methods (pattern recognition). Thus, we looked to match changes in the environment with adaptations in plants. Multivariate statistics highlighted that the lignified periderm and the air gaps are directly related to the environmental contamination. Current results provide new evidences of root anatomical strategies to deal with contaminated environments. Multivariate statistics greatly contributes to extrapolate results from complex data matrixes obtained when analyzing environmental issues, pointing out parameters involved in environmental changes and also evidencing the adaptive response of the exposed biota.

1. Introduction

Mangrove is a coastal saline woodland, transitional between terrestrial and marine environments, characteristic of tropical and subtropical regions and submitted to the tidal regime of estuaries. This area constitutes one of the most productive ecosystems, playing a vital role as a major primary producer within estuarine systems. Sediments in this environment are typically anoxic, clayey and rich in organic matter (MacFarlane et al., 2007; Bodin et al., 2011), acting as a chelating matrix for trace metals favoring they retention from tidal, freshwater and storm water runoff (Tam and Wong, 2000; Zhou et al., 2010).

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Human activities promote modifications on the physical and chemical characteristics of estuaries, reducing the water quality in these environments and, consequently, in mangrove areas (Borja et al., 2012). In last decades, estuaries of the State of Espírito Santo, Brazil, have been occupied by metallurgical and mining complexes, including harbors for iron exportation, which results in increasing pollution of water and sediments with metals and other compounds, which are dangerous for the resident biota (Souza et al., 2013).

Rhizophora mangle L. is a typical mangrove plant, presenting high tolerance to the salinity in addition to morphological plasticity (Boizard and Mitchell, 2011). This species presents aerenchyma in its roots, which permits root respiration in flooded mangrove soil that has low oxygen availability. Besides, its root morphology changes in response to a variety of other edaphic stresses (Postma and Lynch, 2011).

The roots of mangrove trees have an important role in depurating the water and sediment by retaining trace metals which may accumulate in the root or be translocated to aerial parts, such as leaves, where they may affect the cellular physiological processes and tissue anatomy (Liu et al., 2009; Pi et al., 2009; Pinheiro et al., 2012; Cheng et al., 2012a). The success of mangrove plants in these environments is directly related to the anatomical adaptations and permeability of roots (Pi et al., 2009; Cheng et al., 2012b). Nowadays, the mangrove trees, especially species from the genus Rhizophora, are used for evaluating environmental metal contamination (Aksoy and Öztürk, 1997; Ramos and Geraldo, 2007; Lewis et al., 2011). Studies of metal distribution in the sediment and in plant compartments of mangrove areas have great importance for biomonitoring such environments, looking for the conservation of these coastal areas. What sets this study apart from other studies is that we not only investigated adaptive modifications in plants but also differences in the corresponding environments, using a combined set of multivariate methods (pattern recognition) to match differences in the environment with adaptations in plants.

In this context, the main goal of this study was to evaluate the response of *R. mangle* when submitted to impacted Brazilian mangrove areas, trying to verify links between environmental contamination levels and changes in the root anatomy of this plant by applying multivariate statistics to challenge traditional methods for the evaluation of plant plasticity.

2. Material 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 Mass Spectrometer Inductively Coupled Plasma (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).

2.1. Study areas

This study was conducted in four neotropical mangrove areas in the State of Espirito Santo, Brazil: Vitoria bay (composed by Santa Maria, Serra and Lameirão) and Santa Cruz which were selected because they represent areas affected by different pollution sources and ocean influence (Fig. 1). Vitoria bay 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. Three sites were selected in Vitoria bay: Santa Maria (S 20°14′31.5″ and W 40°19′84.7″) where major continental water influence can be observed; Serra (S 20°14′19.6″ and W 40°18′48.7″) representing an area impacted by industrial and sanitary effluents; Lameirão (S 20°14′60.6″ and W 40°18′68.6″), which is a legally protected environment. The second sampling area, Santa Cruz (S 19°56′26.2″ and W 40°12′87″) is an estuary formed by two rivers having an extensive mangrove area (Souza et al., 2013), which add complexity to this ecosystem (Fig. 1). Both studied areas were geo-referenced during field sampling, using a portable GPS 368 (Garmin Vista, USA).

2.2. Water, sediment and plant sampling

Water dissolved oxygen (DO) and conductivity were determined in the field, using a multiparametric probe (YSI model 85, USA), operated 20 cm above the sediment surface.

Sediment samples were taken throughout the experiment simultaneously with plant 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). Sediment and *R. mangle* plant (leaves and roots) samples were collected from all sites along two seasons (winter-2009 and summer-2010). Sediment samples (approximately 20 cm depth interval from the sediment–water interface) were collected close to areas used for sampling *R. mangle* using a plastic spoon. Sediment samples were quickly transferred into clean plastic containers (1 L) without head space, for metal analyses. Subsequently they were dried at room temperature and sieved through nylon meshes (63 μm) with an acrylic frame to avoid the transfer of metals from metallic meshes during sieving. 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. Interstitial water samples were acidified with ultrapure HNO_3 (sub-boiling) and stored at 4 °C until analysis. Prior to measurement, the samples were filtered using 0.45 μm nitrocellulose.

Adventitious roots and leaves samples were collected from five individuals at each site and immediately washed with distilled water. Roots and leaves were also sampled, dried at 40 °C until constant weight and stored at 4 °C until analysis. For histochemistry staining, fresh collected root samples were fixed, in the field, in FAA 50 (a mixture of formaldehyde, ethanol 50% and acetic acid) (Johansen, 1940) and stored in 70% ethanol.

For metal analyses, dryed biological samples were ground and homogenized with a mortar. Sediment and plant 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 Paar Multiwave 3000, Austria). Controls were prepared using the same protocol without sample (only reagents). The assay of organic matter in the sediment was performed 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 7500 cx, 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

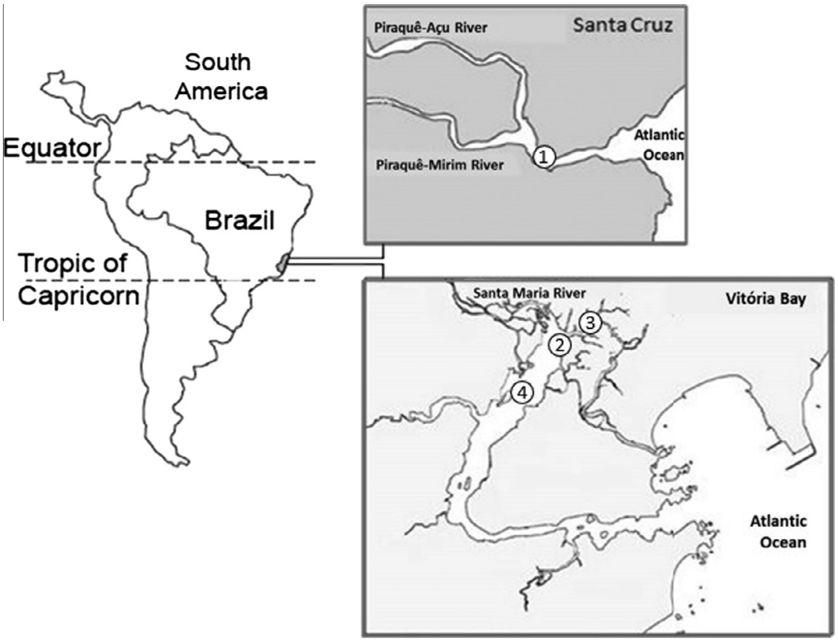

Fig. 1. Localization of the State of Espírito Santo (South America, Brazil), showing sampling sites. 1 – Santa Cruz (S 19°56′26.2″; W 40°12′87″); 2 – Lameirão (S 20°14′60.6″; W 40°18′68.6″); 3 – Serra (S 20°14′19.6″; W 40°18′48.7″); and 4 – Santa Maria (S 20°14′31.5″; W 40°19′84.7″).

generally ⩾97%. Quality assurance (QA) and quality control (QC) were done using certified reference materials (CRMs): NIST SRM 1547 (peach leaves) and NIST 1573a (sediment sludge). Recoveries from CRMs were 87.00 ± 14.30% and 95.90 ± 15.46%, 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 roots, leaves 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 95.17 ± 12.62%.

2.4. Biological analyses

Adventitious roots were separated in two groups: first order (origin from the shoot) and second order (origin of adventitious first order). In both groups, the thickness of the total root, cortex, air gap area and vascular cylinder were measured (30 measurements/root section); the cortex/vascular cylinder ratio was calculated. The thickness of the periderm was also measured in first order roots. Then, roots were dehydrated in ethanol series, embedded in historesin (Leica®, Germany), cross sectioned (8–10 μm in thickness) using a rotary microtome, and stained with 0.05% toluidine blue, pH 4.7 (O'Brien et al., 1964). Roots sections were photodocumented using a Nikon Eclipse 50i microscope (Tokyo, Japan), analyzing the anatomical structure. The measures were done (30 measurements/root section) using the Nikon NIS-Elements software (Tokyo, Japan).

To determine the chemical nature of the cell wall, hand-free sections were obtained and stained with phloroglucinol (1,3,5-trihydroxybenzene) reagent in acid medium (Johansen, 1940) to detect lignified walls. Positive staining indicates the presence of lignin (pink color owing to the presence of coniferaldehyde groups in the lignin) and iron (yellow color) in the plant cell. For negative control, samples without staining were also analyzed. Sections were documented in photomicroscope Nikon Eclipse 50i (Tokyo, Japan).

Fresh root samples were immersed in Prussian blue staining (4% potassium ferrocyanide and 4% hydrochloric acid) (Churukian, 2008) for detecting iron in the surface. After 24 h, samples were washed and photographed with a FujiFilm FinePix S100FS camera (Shanghai, China). Positive staining is indicated by blue color.

2.5. 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 Bonferroni'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 interstitial water elemental data to the corresponding sediment and plant data (both elemental and anatomical). CCA was also applied for assessing the relationship between diverse data matrix (interstitial water, sediment and plant) using a more formal mathematical approach (Di Paola-Naranjo et al., 2011).

3. Results and discussion

3.1. Environmental physical and chemical variables

Organic matter in sediment was significantly higher in three sites of Vitoria Bay (Santa Maria, Serra and Lameirão, with no differences between them) in comparison with Santa Cruz (Table 1). Water conductivity was significantly higher in Santa Cruz in comparison with Santa Maria. Results of dissolved oxygen showed that Santa Cruz also had significant difference compared to the other sites. In this case, Santa Cruz showed higher values of dissolved oxygen. No seasonal differences were observed in the analyzed parameters (Table 1).

The oxygen level in the environment determines the nature and velocity of chemical and biochemical reactions. The reduction and oxidation processes of metals and metalloids can elevate or decrease its toxicity, where high dissolved oxygen values result in a higher metal bioavailability due to an increase of the redox potential (Khalid et al., 1978; Chasin and Azevedo, 2003).

3.2. Metals and metalloids

Many metals and metalloids analyzed in the interstitial water showed values below the LOD. Among the quantifiable ones, higher concentrations of aluminum were observed in Santa Maria (during both sampling periods) and Lameirão (in winter season). For iron, lower concentrations were observed in Serra. The winter season exhibited higher concentrations of Al and Fe when each sampling site was separately analyzed. On the other hand, higher concentrations of Mn were observed at each sampling site in the summer (Table 2).

The multielemental analysis in sediment samples demonstrated that Serra site showed higher concentrations of Cr, Ni, As, Se and Ag compared with other areas of this study. Additionally, Santa Maria had Fe and Pb concentrations greater than other studied stations (Table 2). In both sites, higher levels of analyzed elements were found in the winter season. These results indicate combined effects from different pollution sources at these sites, probably associated with diverse anthropic activities related to those areas. According to Nascimento et al. (2006), high levels of Fe and Pb in sediments can be associated with activities of steel industry, due to the inputs used in the siderurgical process. Conversely, levels of Cd, Zn and Hg were below LODs in four sampled areas during both sampling periods (Table 2).

The lower concentrations of the chemicals analyzed were observed in Santa Cruz. Despite this result, metals and metalloids can be more bioavailable in this site, when correlate with organic matter and dissolved oxygen. Moreover, the lowest levels of dissolved oxygen (DO), associated with the highest concentration of organic matter in Vitoria Bay, probably decreases the bioavailability of metals and metalloids in this environment.

When comparing the concentrations of measured elements in sediment, root and leaf, the highest concentrations were found in the sediment for all elements with concentrations above the LOD and LOQ, except for Mn (Table 2). When comparing the concentrations of metals in root and leaf, Al, Cr, Fe, As and Pb showed always higher concentrations in the root, whereas B and Mn showed higher concentrations in leaves (Table 2). In general, Se, Ag, Cd and Hg were below the LOD or LOQ. It is worthy to mention that translocation of B, Mn and Cu from the root to the leaf was observed in all studies areas (Table 2).

The boron concentrations found during this work indicate accumulation of this element in leaves. Boron plays an important role in the vegetative growth, affecting the cell wall composition (90% of boron cell allocation goes to cell wall) and plasma membrane, changing its mechanical and biochemical properties. It has also been reported that boron has an important physiological role on the cell wall (Malavolta, 2006). In this work, we observed a negative correlation between boron levels and air gap areas. So far, this negative correlation can be explained by translocation of boron from root to leaf (Tables 1 and 2).

The concentrations of metals in the roots were much higher than those in leaf tissue, indicating that only a small part of metals absorbed by roots can be transported to leaves (Table 2). Moreover, our current results indicate that root can uptake Zinc from sediment, without evidences of translocation of this metal to leaves. On the other hand, Mn was accumulated in leaves. The different behavior observed between diverse metals may be related to the formation of iron plates in roots, which is a quite usual mechanism found in plants living in wetlands. For instance, Machado et al. (2005) found that the preferential accumulation of Fe and Zn in root tissue can partially suppress its translocation to the leaves, which was not observed with Mn in seedlings of *R. mangle*. Previous studies also showed low translocation of most trace metals (Walsh et al., 1979; Chiu et al., 1995; MacFarlane and Burchett,

Table 1
Physical, chemical and biological parameters, measured in *Rhizophora mangle* L., (n = 9 in each site) captured in the estuaries Santa Cruz, Lameirão, Serra and Santa Maria in summer and winter. Values are expressed as mean ± SD. Equal letter in the same line data do not differ significantly (Tukey test; $P < 0.05$).

Physical and chemical parameters	Santa Cruz		Lameirão		Serra		Santa Maria	
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
OM (mg g^{-1} sediment)	19.41 ± 0.28^{a}	19.48 ± 0.28^{a}	$25.60 \pm 0.28^{c,d}$	26.44 ± 0.28^{d}	$24.47 \pm 0.28^{c,d}$	$24.47 \pm 0.28^{c,d}$	23.52 ± 0.28^{b}	$25.49 \pm 0.28^{c,d}$
Cond (mS)	72.4 ± 0.35^{f}	72.47 ± 0.35^{f}	50.40 ± 0.35^{c}	$54.6 \pm 0.35^{d,e}$	47.43 ± 0.35^{b}	56.0 ± 0.35^{e}	40.43 ± 0.35^{a}	53.57 ± 0.35^{d}
DO (mg L^{-1})	4.77 ± 0.08^{c}	4.74 ± 0.08^{c}	1.25 ± 0.08^{b}	1.25 ± 0.08^{b}	$0.85 \pm 0.08^{a,b}$	0.74 ± 0.08^{a}	0.76 ± 0.08^{a}	0.80 ± 0.08^{a}
Adventitious roots								
Primary order								
Root thickness (mm)	$8.50 \pm 0.43^{a,c}$		$10.40 \pm 1.52^{b,c}$		12.13 ± 1.82^{b}		7.50 ± 0.55^{a}	
Vascular cylinder diameter (mm)	1.10 ± 0.00^{a}		0.98 ± 0.12^{a}		1.02 ± 0.04^{b}		0.90 ± 0.33^{b}	
Cortex thickness (mm)	7.29 ± 0.43^{a}		9.24 ± 1.61^{a}		10.91 ± 1.83^{a}		6.37 ± 0.25^{b}	
Periderm thickness (mm)	0.11 ± 0.01^{a}		0.18 ± 0.02^{b}		$0.21 \pm 0.01^{b,c}$		0.23 ± 0.05^{c}	
Air gap area (µm^2)	4906 ± 705^{a}		11838 ± 3275^{b}		15839 ± 1606^{b}		24659 ± 4439^{c}	
Cortex/vascular cylinder ratio	6.62 ± 0.40^{a}		$9.67 \pm 2.66^{a,b}$		10.75 ± 1.98^{b}		$7.87 \pm 2.60^{a,b}$	
Secondary order								
Root thickness (mm)	1.36 ± 0.37^{a}		1.09 ± 0.31^{a}		1.99 ± 0.57^{b}		2.07 ± 0.25^{b}	
Vascular cylinder diameter (mm)	0.28 ± 0.07^{a}		0.30 ± 0.12^{a}		0.39 ± 0.10^{a}		0.54 ± 0.05^{b}	
Cortex thickness (mm)	$0.91 \pm 0.28^{a,b}$		0.59 ± 0.10^{a}		1.15 ± 0.42^{b}		1.31 ± 0.10^{b}	
Air gap area (µm^2)	4725 ± 1476^{a}		9717 ± 5138^{b}		$5187 \pm 847^{a,b}$		$6380 \pm 2355^{a,b}$	
Cortex/vascular cylinder ratio	3.20 ± 0.24^{a}		2.09 ± 0.43^{b}		$2.93 \pm 0.63^{a,c}$		$2.42 \pm 0.06^{b,c}$	

Table 2
Chemical characterization of Santa Cruz, Lameirão, Serra and Santa Maria. Metal concentrations in interstitial water, sediment and in *Rhizophora mangle* L. roots and leaves. ($n = 9$ in each site). Values are expressed as mean ± SD. IW: interstitial water (mg L^{-1}), SE: sediment (µg g^{-1} dry mass), RO: root (µg g^{-1} dry mass), LE: leaf (µg g^{-1} dry mass).

	Metals													
	B	Al	Cr	Mn	Fe	Ni	Cu	Zn	As	Se	Ag	Cd	Hg	Pb
Santa Cruz														
Winter														
IW	*NA	2613 ± 479^{b}	<LOD	1324.25 ± 183.49^{d}	13113.3 ± 2033.0^{e}	38.68 ± 10.94^{c}	23.71 ± 6.95^{c}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
SE	<LOD	31617 ± 1541^{f}	27.11 ± 1.20^{b,c}	96.15 ± 3.99^{e}	19173.8 ± 741.4^{a}	10.24 ± 0.53^{d}	3.73 ± 0.25^{a}	<LOD	7.68 ± 0.44^{c}	<LOD	<LOD	<LOD	<LOD	6.15 ± 0.14^{a}
RO	21.3 ± 2.1^{c}	2913 ± 355^{b}	2.52 ± 0.12^{b,c}	27.86 ± 1.36^{d}	3521.5 ± 124.4^{b}	<LOQ	<LOQ	0.63 ± 0.36^{a}	2.45 ± 0.16^{b}	<LOQ	<LOD	<LOD	<LOD	0.33 ± 0.03^{b}
LE	44.7 ± 0.5^{f}	127 ± 4^{d}	0.15 ± 0.01^{b}	254.66 ± 10.80^{f}	102.2 ± 4.4^{c}	0.07 ± 0.02^{a}	0.51 ± 0.02^{b}	3.21 ± 0.23^{b,c}	0.05 ± 0.00^{b}	<LOD	<LOD	<LOD	<LOQ	0.07 ± 0.00^{d}
Summer														
IW	*NA	2472 ± 368^{b}	<LOD	2359.75 ± 247.84^{e}	10841.8 ± 1109.2^{d}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
SE	<LOD	29359 ± 1548^{e,f}	29.75 ± 1.48^{c,d}	81.92 ± 3.84^{d}	22338.8 ± 1106.1^{b,c}	7.82 ± 0.44^{c}	3.40 ± 0.24^{a}	<LOD	9.55 ± 0.72^{d}	<LOD	<LOD	<LOD	<LOD	5.87 ± 0.26^{a}
RO	10.8 ± 1.3^{b}	4893 ± 502^{d}	4.41 ± 0.47^{f}	64.29 ± 6.59^{f}	13477.5 ± 1294.5^{d}	0.21 ± 0.20^{a,b}	<LOQ	16.84 ± 2.96^{e}	12.11 ± 1.08^{c}	<LOQ	<LOD	<LOD	<LOD	1.42 ± 0.05^{e}
LE	17.3 ± 0.2^{a}	216 ± 26^{e}	0.17 ± 0.03^{b,c}	200.55 ± 25.18^{e}	131.7 ± 15.6^{d}	<LOQ	0.22 ± 0.05^{a}	2.69 ± 0.53^{a,b}	0.07 ± 0.00^{b}	<LOD	<LOD	<LOD	<LOQ	0.03 ± 0.00^{a}
Lameirão														
Winter														
IW	*NA	5465 ± 295^{e}	11.84 ± 1.39^{b}	126.38 ± 6.47^{a,b}	11549.7 ± 704.7^{d,e}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	9.39 ± 4.50^{a}	<LOD
SE	<LOD	27029 ± 707^{d,e}	31.01 ± 0.68^{d}	48.32 ± 0.76^{d}	22080.4 ± 418.8^{b,c}	5.68 ± 0.13^{a}	3.58 ± 0.11^{a}	<LOD	3.69 ± 0.15^{a}	<LOD	<LOD	<LOD	<LOD	11.97 ± 0.10^{c}
RO	<LOQ	1157 ± 185^{a}	1.08 ± 0.25^{a}	4.88 ± 0.64^{a}	804.2 ± 149.3^{a}	0.23 ± 0.27^{b}	<LOQ	3.02 ± 2.36^{a,b}	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ
LE	25.8 ± 0.3^{e}	47 ± 3^{a,b}	0.09 ± 0.00^{a}	110.36 ± 3.38^{b,c}	83.6 ± 2.3^{b,c}	0.52 ± 0.02^{b}	1.16 ± 0.04^{e}	3.91 ± 0.18^{c,d}	<LOQ	0.05 ± 0.03^{a}	<LOQ	<LOD	0.11 ± 0.01^{c}	0.06 ± 0.00^{c}
Summer														
IW	*NA	3465 ± 75^{c}	<LOD	340.25 ± 3.54^{b,c}	8164.4 ± 0.1^{c}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
SE	<LOD	24761 ± 529^{c,d}	22.40 ± 0.36^{a}	37.31 ± 0.75^{a}	21935.4 ± 490.8^{b}	5.84 ± 0.19^{a,b}	4.34 ± 0.15^{a}	<LOD	3.97 ± 0.14^{a}	<LOD	<LOD	<LOD	<LOD	10.44 ± 0.16^{d}
RO	<LOQ	3869 ± 49^{c}	3.18 ± 0.04^{c,d,e}	14.08 ± 0.12^{b,c}	3750.2 ± 37.1^{b}	0.20 ± 0.03^{a,b}	<LOQ	17.92 ± 0.47^{e}	1.00 ± 0.21^{a}	<LOQ	<LOQ	<LOD	<LOD	1.16 ± 0.02^{d}
LE	23.2 ± 0.6^{c,d}	54 ± 2^{a,b}	0.07 ± 0.00^{a}	92.55 ± 2.46^{b}	71.2 ± 2.2^{b}	0.03 ± 0.01^{a}	0.53 ± 0.03^{b,c}	3.31 ± 0.09^{b,c}	<LOD	<LOD	<LOD	<LOD	<LOQ	0.04 ± 0.00^{b}
Serra														
Winter														
IW	*NA	3735 ± 74^{c}	23.92 ± 11.46^{c}	58.79 ± 22.68^{a}	6216.1 ± 102.8^{d}	20.20 ± 0.75^{b}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
SE	140.5 ± 6.1^{b}	19842 ± 495^{a}	107.43 ± 2.20^{f}	86.08 ± 0.93^{d}	18058.8 ± 263.0^{a}	29.28 ± 0.53^{e}	7.51 ± 1.59^{a}	<LOD	11.23 ± 0.39^{e}	3.66 ± 0.38^{b}	0.12 ± 0.02^{b}	<LOD	<LOD	8.61 ± 0.08^{b}
RO	<LOQ	1888 ± 160^{a}	1.97 ± 0.21^{b}	10.18 ± 0.85^{a,b}	4297.0 ± 298.4^{b}	0.01 ± 0.01^{a}	<LOQ	11.06 ± 1.30^{d}	0.67 ± 0.06^{a}	<LOQ	<LOQ	<LOD	<LOD	1.22 ± 0.08^{d}
LE	22.0 ± 0.2^{b,c}	33 ± 2^{a}	0.05 ± 0.00^{a}	50.94 ± 2.15^{a}	45.7 ± 1.6^{a}	2.25 ± 0.12^{c}	0.43 ± 0.02^{b}	2.33 ± 0.12^{a}	<LOD	<LOD	<LOD	<LOD	<LOD	0.02 ± 0.00^{a}
Summer														
IW	*NA	1842 ± 81^{a}	<LOD	88.18 ± 4.38^{a}	2928.2 ± 151.1^{a}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
SE	4.2 ± 0.7^{a}	22494 ± 556^{a,b,c}	40.43 ± 0.52^{e}	49.72 ± 0.42^{d}	23940.4 ± 306.7^{c}	11.08 ± 0.08^{d}	4.83 ± 0.06^{a}	<LOD	7.20 ± 0.17^{c}	<LOD	<LOD	<LOD	<LOD	13.89 ± 0.28^{e}
RO	<LOQ	3270 ± 105^{b,c}	2.78 ± 0.10^{c,d}	9.88 ± 0.40^{a,b}	4095.4 ± 175.1^{b}	0.18 ± 0.05^{a,b}	<LOQ	6.21 ± 0.73^{b,c}	0.09 ± 0.08^{a}	<LOD	<LOD	<LOD	<LOD	0.96 ± 0.02^{c}
LE	24.0 ± 0.3^{d}	65 ± 1^{b,c}	0.73 ± 0.03^{d}	139.81 ± 5.10^{c,d}	97.5 ± 3.8^{c}	0.04 ± 0.01^{a}	1.20 ± 0.04^{e}	4.73 ± 0.32^{d}	<LOD	<LOQ	<LOQ	<LOD	<LOQ	0.09 ± 0.00^{e}
Santa Maria														
Winter														
IW	*NA	5764 ± 8^{e}	8.41 ± 0.01^{a,b}	58.91 ± 0.62^{a}	13339.9 ± 63.6^{e}	<LOD	6.84 ± 0.08^{b}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
SE	<LOD	22857 ± 493^{b,c}	24.29 ± 0.59^{a,b}	61.45 ± 1.41^{c}	27535.4 ± 943.8^{d}	6.81 ± 0.25^{b,c}	3.63 ± 0.13^{a}	<LOD	4.34 ± 0.08^{a}	<LOD	<LOD	<LOD	<LOD	15.90 ± 0.17^{g}
RO	<LOQ	5446 ± 179^{d}	3.67 ± 0.13^{e}	45.87 ± 0.91^{e}	3622.9 ± 110.9^{b}	0.55 ± 0.05^{b}	<LOQ	8.71 ± 0.46^{c,d}	<LOQ	<LOQ	<LOD	<LOD	<LOD	1.21 ± 0.04^{d}
LE	21.1 ± 0.5^{b}	210 ± 10^{e}	0.21 ± 0.01^{c}	152.45 ± 5.31^{d}	182.9 ± 7.6^{e}	<LOQ	0.68 ± 0.04^{d}	3.02 ± 0.26^{a,b}	<LOQ	<LOD	<LOD	<LOD	<LOQ	0.11 ± 0.00^{f}
Summer														
IW	*NA	4498 ± 280^{d}	<LOD	367.00 ± 16.62^{c}	9993.6 ± 456.9^{c,d}	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
SE	<LOD	20671 ± 420^{a,b}	25.23 ± 0.30^{a,b}	63.40 ± 0.94^{c}	26140.4 ± 369.8^{d}	7.47 ± 0.16^{c}	4.35 ± 0.06^{a}	<LOD	5.77 ± 0.27^{b}	<LOD	<LOD	<LOD	<LOD	14.61 ± 0.23^{f}
RO	<LOQ	3849 ± 22^{c}	3.42 ± 0.05^{d,e}	20.31 ± 0.03^{c}	6379.2 ± 25.7^{c}	0.13 ± 0.04^{a}	<LOQ	8.67 ± 0.33^{c,d}	0.93 ± 0.05^{a}	<LOQ	<LOD	<LOD	<LOQ	1.19 ± 0.02^{d}
LE	22.5 ± 0.7^{c}	89 ± 6^{c}	0.08 ± 0.01^{a}	107.35 ± 7.89^{b}	91.2 ± 7.2^{b,c}	<LOQ	0.64 ± 0.06^{c,d}	2.19 ± 0.31^{a}	<LOQ	<LOD	<LOD	<LOD	0.07 ± 0.00^{b}	0.09 ± 0.00^{e}

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

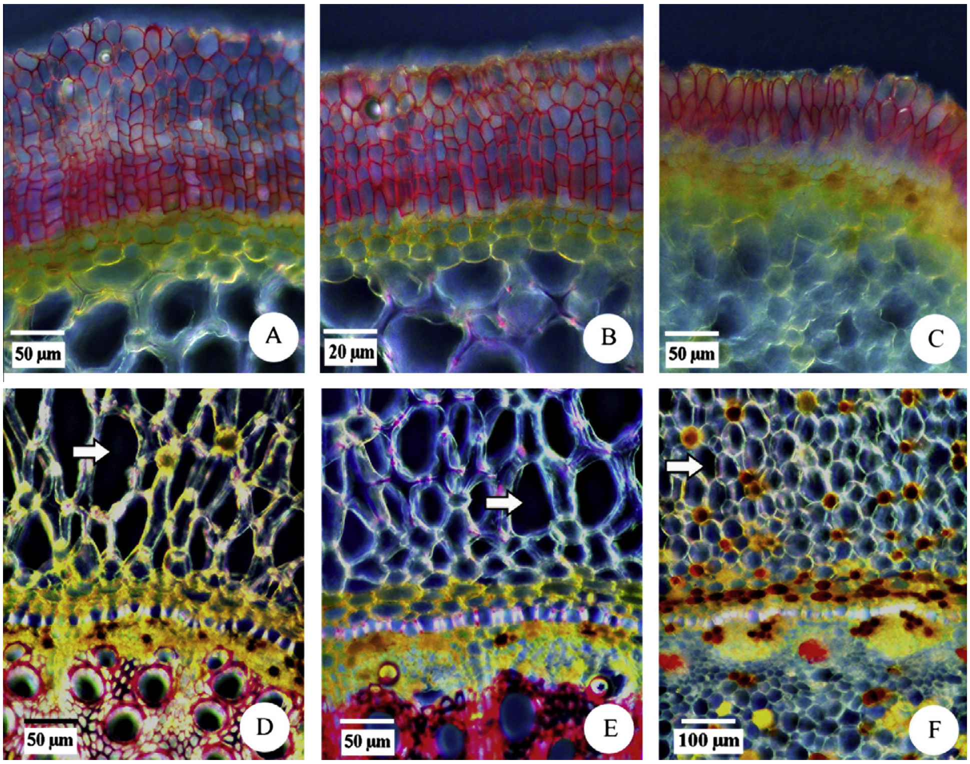

Fig. 2. Histochemical analysis in *Rhizophora mangle*. Sections were stained with phloroglucinol–HCl. (A and D) Santa Maria, (B and E) Serra, (C and F) Santa Cruz; * = Air gap.

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

Sites	Santa Cruz $p = .2500$	Lameirão $p = .2500$	Serra $p = .2500$	Santa Maria $p = .2500$
Classification functions LDA				
Air gap area – rad2	26632	13987	1074.3	6017.5
Periderm – rad1	−60698	−32008	12076.8	5194.5
Air gap area – rad1	−410763	−217362	142276.7	105953.2
As – Sediment	−1046	−638	1233.5	1120.9
Mn – Leaves	5	0	3.2	3.8
Pb – Leaves	−87631	−49784	76444.1	67078
Al – Sediment	1	0	−0.4	−0.3
Ni – Root	−1247	−812	1628.8	1507.8
Conductivity	457	258	38.1	72.1
Root thickness – rad2	748	356	−96.1	−50.5
Cr – Interstitial water	145	81	10.8	22.3
Hg – Root	−4308	−2477	−706.6	−997.9
Cu – Sediment	20	12	−3.3	−1.9
Constant	−16558	−5608	−7095.8	−7580.2

2000), but also reported translocation of Mn (Tam and Wong, 1997) from roots to leaves of mangrove seedlings.

3.3. Biological parameter

For first and second order adventitious roots, values of air gap area (μm^2) were higher in Santa Maria, with the lowest result found in Santa Cruz (Table 1). Several studies have shown that aerenchyma development, mainly in roots, is related to the amount of oxygen available in the environment (Bona and Morretes, 2003; Rodrigues and Estelita, 2004; Batista et al., 2008).

Oxygen deficiency stimulates ethylene production by inducing the formation of aerenchyma through cell disintegration (Taiz and Zeiger, 2006). In this context, lower levels of oxygen imply further development of aerenchyma. All studied sites showed negative correlation between levels of DO and air gap area of root, with Santa Cruz showing the highest value of DO and, therefore, smaller air gap areas (Table 1).

R. mangle presents morphophysiological mechanisms such as iron plates, which made it a salt excluding specie by root ultrafiltration (Patel et al., 2010). Iron plate is recognized as a mechanism involved in the regulation of metal absorption, increasing mangrove plants tolerance to different estuarine conditions (Silva et al., 1990; Lacerda et al., 1993; Ong Che, 1999). Also wetland plants eventually present precipitated metals over both cell walls and intercellular gaps of radicular tissue, indicating analogous function to iron plates (Ye et al., 1998). Smolders and Roelofs (1996) indicated that, apparently, the iron plate is a mechanism developed to avoid toxicity, caused by this element, inside the stele.

Histochemical test using Prussian blue revealed the presence of iron in root surface at all studied sites (data not shown). The iron plate is present in a variety of wetland plants (Smolders and Roelofs, 1996; Moller and Sand-Jensen, 2008). In these places, plants exposed to anoxic sediment can tolerate flooding by releasing oxygen from the roots (Pi et al., 2009), which causes precipitation of metals in the sediment and on the root surface. This precipitate on the root, rich in iron, has been associated with the tolerance of mangrove species to heavy metals (Machado et al., 2005; Pi et al., 2009), forming a physical barrier to the absorption of elements.

The highest and lowest values of periderm thickness (mm) were found in Santa Maria and Santa Cruz, respectively. Phloroglucinol in acid medium (Johansen, 1940) identified a lignified cell wall in the periderm. Previous studies (Cheng et al., 2010, 2012a) showed that lignification could avoid the excessive input of metals to roots. Lignin and suberin contribute significantly to the formation of an apoplastic barrier that influences the radial transport of water, gas and ions, playing an important role in the protection of

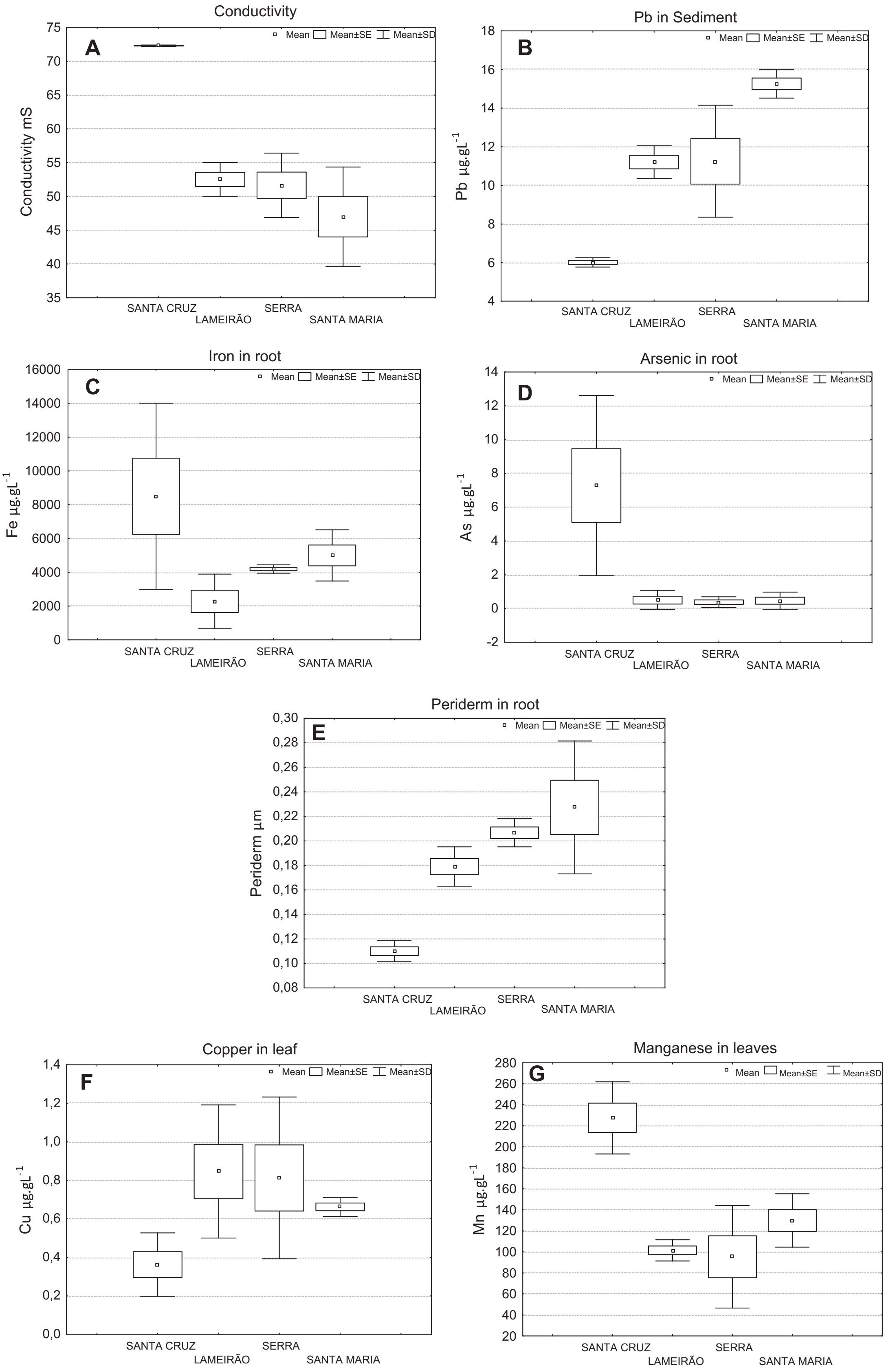

Fig. 3. Box and whisker plots from some selected parameters measured in sediment, root and leaves. Values are reported as mean ± SD and SE.

exposed plants to biotic and abiotic stresses. Deng et al. (2009) and Cheng et al. (2012b) reported that plants with lignified periderm tend to accumulate less metal than plants with a relatively high permeability in the periderm.

Also, phloroglucinol in acid medium was used to identify the presence of iron in underlying layers of the periderm, in aerenchyma rounded cells, in layers close to the endodermis and in phloem cells. Our current results show that, when absorbed, part of the iron is retained in root cells before reaching the vascular cylinder, while other part of this metal is translocated to aerial parts of the plant (Fig. 2 and Table 2). According to several studies (Koch and Mendelssohn, 1989; Otte et al., 1989; Lacerda et al., 1992; Doyle and Otte, 1997; Sundby et al., 1998), the formation of iron plates occurs due to high concentration of Fe, as a consequence of oxygen release processes performed by the roots to increase the tolerance to flooding conditions. These processes relate to the oxidation of reduced constituents in the interstitial water, which results in partial precipitation of trace metals in their oxidized forms in rhizosphere sediments as well as in the root.

This study provides new evidences of anatomical strategies used by roots of *R. mangle* to deal with flooded environments. The plasticity of periderm, aerenchyma and oxidized iron layers in distinct locations of the root became an adaptive strategy to regulate the flow of oxygen, nutrients and toxins to the root from the sediment or interstitial water.

3.4. Overall discussion using multivariate statistics

Interpreting data from environmental biomonitoring is complex, particularly when multiple pollution sources are present. The absence of statistically significant differences between areas or seasons (measured at univariate level using ANOVA and similar tests) does not necessary implies lack of differences, or the absence of effects on organisms. 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 our case, we started carrying out stepwise LDA looking to point out which parameters may explain spatial and temporal differences between studied areas. LDA was performed including chemical parameters analyzed in interstitial water, sediment, root and leaves in addition to anatomical root parameters as independent variables. Discriminant functions from LDA are shown in Table 3. Thirteen out of 69 starting parameters were necessary to distinguish between studied areas with 100% correct classification (classification matrix, data not shown). No significant differences were observed between summer and winter (data not shown); thus, reported LDA includes only spatial but not temporal variations.

Noteworthy is that parameters pointed out by LDA include conductivity in interstitial the water, one metal (Cr) measured in interstitial water, three elements measured in sediments (As, Al and Cu), two metals measured in root (Ni and Hg), two metals measured in leaves (Mn and Pb) and four anatomical root parameters. Considering that 8 out of 13 parameters pointed out by LDA belong to the plant (4 metals and 4 anatomical root parameters), it is clear that plant condition can evidence differences between studied areas.

Box plots with patterns representing seven out of 13 parameters pointed out by LDA are shown in Fig. 3. The conductivity in interstitial water (Fig. 3A) and the concentration of Pb in sediment (Fig. 3B) evidence the spatial difference between areas, showing that Santa Cruz has more marine influence than other sites located in a different estuary (Fig. 3A). It also seems that the pollution level at Santa Cruz is lower, evidenced by the level of Pb in sediment, which is coincident with higher levels of other toxic metals in sediments observed between both estuaries, namely Cr, Ni and Cu (Table 2), and the amount of some of these metals present in leaves (Cu, Fig. 3F). On the other hand, levels of Mn in leaves seems to be better related to the amount of this element in the interstitial water (Fig. 3G and Table 2). Also the root is evidencing adaptive differences between both estuaries, with higher levels of Fe and As in Santa Cruz with relation to Lameirao, Serra and Santa Cruz (Fig. 3C and D). Also the root periderm shows this adaptive response, being thin in plants from Santa Cruz and dicker as the level of toxic compounds rose in the sediment (e.g. Pb) (Fig. 3B and E). Thus, it is clear that mangrove plants responded quite well to different pollution conditions. It is also worthy to remark the usefulness of LDA to extract valuable information from a complex data matrix (Table 2).

These results are in agreement with Souza et al. (2013), reporting concentrations of several elements in surface and interstitial waters at both estuaries (Vitoria and Santa Cruz bays), highlighting a higher pollution degree at Vitoria Bay. Thereafter, a generalized procrustes analysis (GPA) was performed, looking to demonstrate matching between parameters analyzed in interstitial water, sediment root and leaves, considering only spatial differences (no temporal differences were observed during this work). Fig. 4 shows a graphical representation of GPA, showing the correspondence between interstitial water, sediment root (metal and anatomical root parameters) and leaves, considering spatial differences. Significant differences between studied areas were described primarily by the

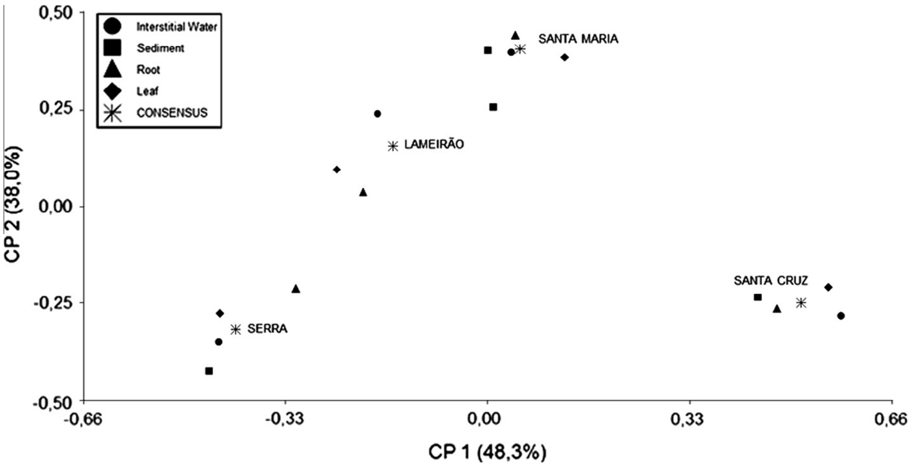

Fig. 4. Generalized procrustes analysis (GPA) of studied parameters from each sampling site.

first axis, which explained 48.3% of the total variance, while the second function, described by the second axis (CP2), accounts for additional 38%. Also from Fig. 4 it can be observed that CP1 contributes to the spatial differentiation between both studied areas (Santa Cruz and Vitoria bay), while both CP1 and CP2 clearly differentiate between studied sites at Vitoria bay (Lameirão, Serra and Santa Maria).

Although both LDA and GPA point out differences between studied areas, none of them indicate which environmental compartment is affecting plant to a bigger extend. To elucidate it, we carried out CCA considering paired data matrix; namely, levels of metals in: sediment – root; sediment – leaves and root – leaves. Results from CCA show that interstitial water, sediment and root presented the same correlation with levels of metals in leaves ($R^2 = 0.99$; $P = 0.0$).

Multivariate statistical analyses help to indicate that plants from each studied site exhibited a distinctly response, pointing out that the bioaccumulation of selected elements significantly contribute to discriminate among studied areas (Fig. 3C, D, G and H), 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 use of anatomical root analysis, in addition to the evaluation of bioaccumulation in different plant compartments (roots and leaves in this case), responded quite well to different pollution scenarios, matching their response to the physical and chemical parameters measured at different partitions within the estuary. The use of multivariate statistics greatly contributes to extrapolate results from field and laboratory measurements, pointing out those parameters that help to differentiate sites with different contamination sources and, consequently, different risks for the aquatic environment. 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.

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