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Functional traits of selected mangrove species in Brazil as biological indicators of different environmental conditions

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HIGHLIGHTS

- We investigated adaptive modifications in plants in response to differences among three estuaries.
- We used pattern recognition methods to match differences among estuaries with plant adaptations.
- *A. schaueriana* and *L. racemosa* are good bioindicators of differences among studied estuaries.
- Dry mass per leaf area (LMA) in *A. schaueriana* was the better indicator of adaptive modifications.

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ABSTRACT

Ecological studies on phenotypic plasticity illustrate the relevance of this phenomenon in nature. Conditions of biota reflect environmental changes, highlighting the adaptability of resident species that can be used as bioindicators of such changes. We report the morpho-anatomical plasticity of leaves of *Avicennia schaueriana* Stapf & Leechm. ex Moldenke, *Laguncularia racemosa* (L.) C.F.Gaertn. and *Rhizophora mangle* L., evaluated in three estuaries (Vitória bay, Santa Cruz and Itaúnas River; state of Espírito Santo, Brazil), considering five areas of mangrove ecosystems with diverse environmental issues. Two sampling sites are part of the Ecological Station Lameirão Island in Vitória bay, close to a harbor. A third sampling site in Cariacica (Vitória bay) is inside the Vitória harbor and also is influenced by domestic sewage. The fourth studied area (Santa Cruz) is part of Piraquê Mangrove Ecological Reservation, while the fifth (Itaúnas River) is a small mangrove, with sandy sediment and greater photosynthetically active radiation, also not strongly influenced by anthropic activity. Results pointed out the morpho-anatomical plasticity in studied species, showing that *A. schaueriana* and *L. racemosa* might be considered the most appropriate bioindicators to indicate different settings and environmental conditions. Particularly, the dry mass per leaf area (LMA) of *A. schaueriana* was the main biomarker measured. In our study, LMA of *A. schaueriana* was positively correlated with salinity (Spearman 0.71), Mn content (0.81) and pH (0.82) but negatively correlated with phosphorus content (−0.63). Thus, the evaluation of modification in LMA of *A. schaueriana* pointed out changes among five studied sites, suggesting its use to reflect changes in the environment, which could be also useful in the future to evaluate the climate change.

1. Introduction

Phenotypic plasticity includes all types of environmentally induced phenotypic variation (Stearns, 1989), and when beneficial to an individual, it is referred to as adaptive phenotypic plasticity (Pigliucci, 2001). Two approaches are given in the study of phenotypic plasticity: 1) traditional, *sensu stricto* (*e.g.*, Pigliucci, 2005; Richards et al., 2006), focusing on the evolution or on the mechanisms underlying the plastic response, and 2) ecological (*e.g.*, Bell and Galloway, 2008; González and Gianoli, 2004), focusing on the patterns of population differentiation in plasticity along an environmental gradient. Because it includes a broader range of study systems, the latter approach contributes to the understanding of the ecological significance of phenotypic plasticity in addition to providing a comprehensive view of its relevance in nature (Gianoli and Valladares, 2012).

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In mangrove swamps, variation in forest structure along an environmental gradient partly depends on the capacity of each plant species to exhibit adaptive phenotypic plasticity to physical and chemical conditions (Ball, 1996, 2002; Christian, 2005; Lovelock et al., 2005). Usually, mangrove ecosystems are characterized by the presence of waterlogged, clayey, saline sediments with high levels of organic matter. However, these factors can vary widely between areas. Plants can be established in various substrates, including sand, peat, volcanic lava and carbonate sediments (Woodroffe, 1992). Plants living in mangrove ecosystems may be subjected to broad salinity fluctuations (Ball, 1998). This characteristic can be influenced by the distance of the mangrove forests from the sea (Bernini et al., 2010), by the distance of individuals from the edge of the forest (Sam and Ridd, 1998), rainfall patterns and overland freshwater input (Semeniuk, 1983). Furthermore, pollution, produced by organic waste or heavy metals, may change the concentration of certain elements and modify the bioavailability of nutrients (Laing et al., 2009; Pan and Wang, 2012). In addition to edaphic factors, solar radiation also varies, primarily due to the spatial distribution of vegetation and the characteristics of the canopy (Lima and Galvani, 2013). Variation in environmental factors can thus lead to adaptive responses by the plants (Feller et al., 2010).

Many anatomical studies including *Avicennia schaueriana* Stapf & Leechm. ex Moldenke, *Laguncularia racemosa* (L.) C.F.Gaertn. and *Rhizophora mangle* L. deal with descriptive aspects of their organs (*e.g.* Evans and Bromberg, 2010; Francisco et al., 2009; Menezes, 2006; Tomlinson, 1994; Tomlinson and Cox, 2000). Studies on the influence of environmental factors on the anatomical or morphological characteristics of leaves have been reported by Ellison and Farnsworth (1997), Farnsworth et al. (1996), Sobrado (2005, 2007) and Werner and Stelzer (1990). However, reports analyzing these plants *in situ* are still scarce (Camilleri and Ribi, 1983; Farnsworth and Ellison, 1996; Feller, 1996).

Therefore, this study aimed to assess the morpho-anatomical plasticity of leaves of *A. schaueriana*, *L. racemosa* and *R. mangle* in five areas of mangrove ecosystem in Brazil, which are affected by different environmental conditions. The hypothesis was that differences in environmental conditions trigger adaptive modifications in leaves, which can be used to evidence alterations in their environments.

Thus, we looked to assess which plant could be used as a good bioindicator of differences in environmental issues as well as identifying adaptive features that could be used as biomarkers of such differences. What sets this study apart from other studies is that we investigated not only 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, looking to identify the better bioindicator and also to point out suitable biomarkers.

2. Materials and methods

2.1. Study area

Five mangrove ecosystem sites located in four municipalities belonging to the state of Espírito Santo were selected for this study: Vitória, Cariacica, Aracruz, and Conceição da Barra. Three different estuaries are located within this area: Vitória bay, Santa Cruz and Itaúnas River (Fig. 1). Two sampling sites were chosen within the municipality of Vitória, which are part of the Ecological Station Lameirão Island: one located at the Passagem Channel (Fig. 1, site 1) (20°17′35.7″S and 40°19′12.8″W) and the other is on Lameirão Island (Fig. 1, site 2) (20°15′00.6″S and 40°19′08.6″W). The sampling site in Cariacica (Fig. 1, site 3) (20°19′35.8″S and 40°22′13.0″W) is influenced by a direct input of domestic sewage. This last site, along with those on Lameirão Island and at the Passagem (exchange) Channel, is part of the estuary system of the island of Vitória, which covers an approximate area of 18 km^2. The mangrove ecosystem in Aracruz covers approximately 12 km^2 and contains the Piraquê-Açu and Piraquê-Mirim River estuaries. This sampling site (Fig. 1, site 4) (19°56′26.2″ S and 40°13′27.0″ W) is in the Piraquê-mirim River estuary, which is part of Piraquê-Açu and Piraquê-Mirim Mangrove Ecological Reservation. In Conceição da Barra, the site chosen (Fig. 1, site 5) (18°33′55.2″S and 39°44′38.1″W) is at the mouth of the Itaúnas River, known as Guaxindiba Beach. It is a small mangrove wood, approximately 30 m from the sea, on sandy sediment and with less dense vegetation than other studied sites (sites 1–4). All sites sampled have a tidal amplitude lesser than 2 m (Marinha do Brasil, 2010), classified as microtidal.

According to Kottek et al. (2006), the climate classification in the state of Espírito Santo, Brazil, is equatorial (A); equatorial savannah with dry winter – Aw (precipitation of the driest month less than 60 mm in winter) in Vitória, Cariacica and Aracruz, and equatorial

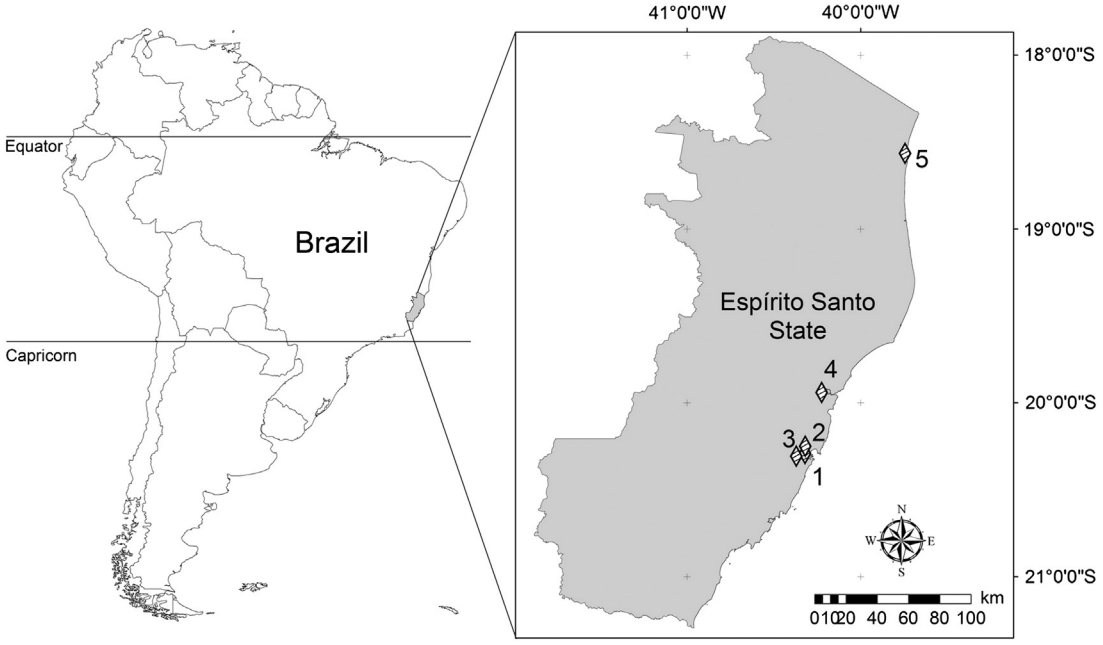

Fig. 1. Map of Brazil and Espírito Santo with the location of the sampling sites in this study (1 = Passagem Channel; 2 = Lameirão Island; 3 = Cariacica; 4 = Aracruz; 5 = Conceição da Barra).

Table 1
Physical and chemical parameters evaluated in each studied mangrove area. Values represent means ± standard deviation. Significant differences between study areas for each species are indicated with different letters within the same row (Kruskal–Wallis's test, $p < 0.05$). (OM = organic matter, PAR = photosynthetically active radiation).

Parameters evaluated			Study area				
			Site 1	Site 2	Site 3	Site 4	Site 5
Fisical parameters	Solar radiation	PAR (μmol m^{-2} s^{-1})	41 ± 32 b	50 ± 19 b	112 ± 179 b	39 ± 30 b	1856 ± 136 a
	Inorganic fractions	Coarse sand (%)	54 ± 7 a	40 ± 2 ab	25 ± 15 bc	6 ± 2 c	32 ± 6 abc
		Fine sand (%)	24.6 ± 5.4 ab	6.5 ± 2.0 bc	7.8 ± 5.1 bc	1.7 ± 0.5 c	56.9 ± 4.3 a
		Silt (%)	13 ± 4 bc	46 ± 5 ab	43 ± 13 ab	51 ± 6 a	6 ± 1 c
		Clay (%)	8 ± 2 bc	7 ± 1 bc	25 ± 6 ab	41 ± 2 a	5 ± 1 c
		Texture classification	Sandy-loam	Sandy-loam	Loam	Silty-clay	Sand
	Interstitial water	Salinity (psu)	25.2 ± 0.4 b	22.8 ± 2.4 b	29.9 ± 3.3 ab	34.7 ± 4.8 a	46.8 ± 15.4 a
		pH	4.2 ± 0.2 c	4.4 ± 0.2 bc	6.0 ± 0.4 abc	6.7 ± 0.4 ab	8.1 ± 0.2 a
		OM (g dm^{-3})	69 ± 10 abc	168 ± 24 a	63 ± 5 bc	111 ± 8 ab	7 ± 3 c
Chemical parameters	Sediment	P (mg dm^{-3})	51 ± 10 ab	20 ± 1 abc	220 ± 91 a	9 ± 3 c	13 ± 2 bc
		K (mg dm^{-3})	213 ± 40 bc	587 ± 45 ab	523 ± 167 abc	833 ± 58 a	147 ± 12 c
		S (mg dm^{-3})	769 ± 156 bc	1064 ± 161 ab	1028 ± 89 ab	1281 ± 224 a	111 ± 24 c
		Ca (mg dm^{-3})	767 ± 114 bc	2053 ± 117 a	953 ± 182 abc	1927 ± 225 ab	480 ± 20 c
		Mg (mg dm^{-3})	844.0 ± 174.9 bc	1964.0 ± 6.9 a	1708.0 ± 226.5 ab	1832.0 ± 38.6 ab	228.0 ± 0.2 c
		Fe (mg dm^{-3})	1189 ± 206 c	2656 ± 655 ab	1258 ± 330 bc	2970 ± 70 a	1216 ± 197 bc
		Na (mg dm^{-3})	2240 ± 211 bc	4747 ± 201 ab	5150 ± 1609 ab	8900 ± 200 a	1830 ± 90 c
		Zn (mg dm^{-3})	5.9 ± 1.8 bc	11.4 ± 0.3 ab	36.4 ± 19.4 a	11.1 ± 0.7 ab	2.9 ± 0.4 c
		Cu (mg dm^{-3})	1.5 ± 0.1 a	1.4 ± 0.1 ab	0.9 ± 0.5 abc	0.4 ± 0.1 c	0.7 ± 0.1 bc
		Mn (mg dm^{-3})	7 ± 2 b	19 ± 6 ab	16 ± 3 ab	66 ± 29 a	73 ± 28 a
		B (mg dm^{-3})	13.2 ± 2.4 b	30.1 ± 2.9 a	15.9 ± 0.3 ab	36.3 ± 11.3 a	2.5 ± 0.2 b

monsoon – Am (accumulated annual precipitation greater than or equal to 25 (100 — the precipitation of the driest month)) in Conceição da Barra.

All sampling and measurements in the studied sites were made in February 2010, in the summer, during the rainy season.

2.2. Analysis of physical parameters

Twenty measurements of photosynthetically active radiation (μmol m^{-2} s^{-1}) were carried out at each studied area using a Field Scout Quantum Light Meter (Plainfield, USA). Measurements were made close to leaves sampled, which were located on the outside of the canopy. All measurements were made between 10:00 and 11:00 am. Measured values obtained were averaged for each study area.

Three sediment samples were collected (0–20 cm deep) from each study site (3 replicates × 3 species × 5 sites). Sediment was sampled between the roots of each species and at the edge of the mangrove during low tide. Samples were stored in labeled plastic bags and sent to the Agronomic Analysis and Consulting Lab — Fullin (Linhares, Espírito Santo, Brazil) for analysis. The sediment granulometry was determined by densitometry (Embrapa, 1997). The texture classification was carried out in compliance with the criteria of the Sociedade Brasileira de Ciência do Solo (Brazilian Society of Soil Science). The pH was determined using a pHmeter DM-22 (Digimed, São Paulo, Brazil), while the organic matter content (OM) was extracted with $Na_2Cr_2O_7 \cdot 2H_2O$ 4 mol L^{-1} + H_2SO_4 10 mol L^{-1} oxidation and determined by atomic absorption spectrometer (Model 210 VGP, Buck Scientific, East Norwalk, USA) according to Raij et al. (2001). The salinity was measured through the electrical conductivity (EC) values of the interstitial water (bioavailable fraction) by an EC electrode tetracon 325 (WTW, Multiline P4, Germany).

2.3. Analysis of chemical parameters

Concentrations of chemical elements (P, K, S, Ca, Mg, Fe, Na, Zn, Cu, Mn and B) were determined by Agronomic Analysis and Consulting Lab — Fullin (Linhares, Espírito Santo, Brazil) according to EMBRAPA (1997). Fe, Zn, Cu, Mn, P, K, and Na were extracted with HCl 0.05 mol L^{-1} + H_2SO_4 0.0125 mol L^{-1}, while Ca and Mg were extracted with KCl 1 mol L^{-1}. Fe, Zn, Cu, Mn, Ca and Mg were determined by atomic absorption spectrometer (Model 210 VGP, Buck Scientific, East Norwalk, USA), P was determined by spectrometer (Model B542, Micronal, São Paulo, Brazil), and K and Na were determined by flame photometer (Model B462, Micronal, São Paulo, Brazil). All samples were prepared and analyzed by Agronomic Analysis and Consulting Lab — Fullin (Linhares, Espírito Santo, Brazil) according to EMBRAPA (1997) standardized methods.

2.4. Analysis of biological parameters

Fully expanded leaves, located between the third and fourth nodes from the apical bud, were collected from the first lateral branch of adult samples of *A. schaueriana* Stapf & Leechm. ex Moldenke (Acanthaceae), *L. racemosa* (L.) C.F.Gaertn. (Combretaceae) and *R. mangle* L. (Rhizophoraceae). All samples were collected at the edge of the mangrove. Exsiccates of studied species were deposited at the VIES herbarium at the Federal University of Espírito Santo, identified with numbers 19649, 19650, and 19651.

For anatomical analysis, four leaves from four individuals were collected. Samples of the middle third of the leaf blade were fixed in FAA 50 (a mixture of formaldehyde, ethanol and acetic acid) (Johansen, 1940) and stored in 70% ethanol. The material was dehydrated in a graded ethanol series and embedded in methacrylate historesin (Leica®) according to instructions from the manufacturer. Cross sections (8 μm thick) were obtained using a rotary microtome. All cuts were later stained with 0.05% toluidine blue (O'Brien et al., 1964) and mounted in Canada balsam. A quantitative anatomical analysis was carried out by measuring the thickness (μm) of the leaf blade, cuticle, epidermis, water storage hypodermis, and chlorenchyma. To this end, eight measurements for each leaf were performed, with a total of 32 measurements per species from each studied area. The stomatal and glandular density were also determined for both surfaces of the leaves through the printing technique, using a drop of cyanoacrylate ester adhesive (Super-Bonder®) on a histological blade. Six random optical fields were analyzed from each individual, totaling twenty-four optical fields. The measurements were carried out using an image capture system coupled to a Nikon E200 microscope (Tokyo, Japan), using the software Tsview v.6.1.3.2 (Tucsen Imaging Technology Co. Limited). Results were documented using photomicrographs.

For morphological analysis, 10 leaves from 10 independent individuals were collected to determine the leaf area (cm^2), dry mass (g), and dry mass per area (LMA g cm^{-2}). The leaf area was measured using an Area Meter LI-COR 3100 (Lincoln, USA), and the dry mass was obtained

Table 2
Biological parameters (anatomical and morphological) evaluated in *Avicennia schaueriana*, *Laguncularia racemosa* and *Rhizophora mangle* leaves in each studied mangrove area. Values represent means ± standard deviation. Significant differences between study areas for each species are indicated with different letters within the same row (Kruskal–Wallis's test, $p < 0.05$).

Biological parameters	Site 1			Site 2		
	A. schaueriana	*L. racemosa*	*R. mangle*	*A. schaueriana*	*L. racemosa*	*R. mangle*
Cuticle (μm)	2.8 ± 0.6^{b}	4.5 ± 0.2^{ab}	4.5 ± 0.5^{bc}	4.3 ± 0.6^{ab}	4.4 ± 0.5^{abc}	6.1 ± 1.8^{ab}
Adaxial surface epidermis (μm)	12.3 ± 0.8^{bc}	14.6 ± 1.8^{a}	9.7 ± 1.3^{c}	11.6 ± 1.3^{c}	15.2 ± 1.7^{a}	12.8 ± 0.8^{ab}
Abaxial surface epidermis (μm)	16.2 ± 1.4^{a}	10.9 ± 0.8^{b}	9.0 ± 0.7^{ab}	16.1 ± 1.6^{a}	11.2 ± 0.8^{ab}	9.8 ± 0.6^{a}
Water storage parenchyma (μm)	62 ± 2^{c}	237 ± 87^{a}	72 ± 5^{a}	72 ± 5^{bc}	187 ± 43^{a}	87 ± 21^{a}
Chlorophyll parenchyma (μm)	263 ± 22^{a}	209 ± 29^{ab}	296 ± 17^{abc}	289 ± 11^{a}	141 ± 23^{c}	315 ± 25^{ab}
Palisade parenchyma adaxial surface (Pad) (μm)	96 ± 20^{ab}	119 ± 19^{ab}	135 ± 13^{b}	115 ± 11^{b}	85 ± 17^{c}	147 ± 23^{ab}
Palisade parenchyma abaxial surface (Pab) (μm)	na	90 ± 11^{ab}	na	na	56 ± 11^{c}	na
Spongy parenchyma (Sp) (μm)	167 ± 9^{a}	na	162 ± 8^{a}	174 ± 6^{a}	na	168 ± 14^{a}
Ratio Pad/Sp	0.58 ± 0.12^{b}	na	0.84 ± 0.08^{a}	0.67 ± 0.07^{b}	na	0.89 ± 0.16^{a}
Ratio Pad/Pab	na	1.31 ± 0.07^{a}	na	na	1.53 ± 0.39^{a}	na
Leaf blade (μm)	354 ± 16^{a}	471 ± 118^{ab}	376 ± 28^{a}	389 ± 22^{a}	354 ± 68^{b}	416 ± 39^{a}
Density of stomata (abaxial surface) (n mm^{-2})	106 ± 16^{c}	71 ± 13^{a}	63 ± 6^{a}	145 ± 20^{ab}	101 ± 22^{a}	49 ± 3^{b}
Density of stomata (adaxial surface) (n mm^{-2})	na	116 ± 16^{a}	na	na	164 ± 31^{a}	na
Density of salt gland (abaxial surface) (n mm^{-2})	15.1 ± 4.0^{a}	1.6 ± 0.3^{a}	na	18.4 ± 10.5^{a}	1.8 ± 0.6^{a}	na
Density of salt gland (adaxial surface) (n mm^{-2})	51.4 ± 14.5^{b}	1.5 ± 0.2^{ab}	na	63.8 ± 16.3^{ab}	1.4 ± 0.4^{b}	na
Leaf area (cm^2)	37.1 ± 3.8^{a}	38.2 ± 8.7^{ab}	62.5 ± 6.7^{a}	26.3 ± 3.7^{b}	40.0 ± 5.0^{a}	57.6 ± 12.3^{a}
Dry mass (g)	0.36 ± 0.04^{a}	0.52 ± 0.16^{a}	0.79 ± 0.12^{a}	0.30 ± 0.04^{b}	0.60 ± 0.10^{a}	0.86 ± 0.18^{a}
LMA (g cm^2)	0.010 ± 0.001^{b}	0.014 ± 0.002^{c}	0.013 ± 0.001^{d}	0.012 ± 0.001^{b}	0.015 ± 0.001^{bc}	0.015 ± 0.001^{bc}

by weighing the leaves after drying in an oven at 60 °C until constant weight.

2.5. Statistical analysis

Data are reported as means ± standard deviations. The statistical packages, STATISTICA 7.1 from StatSoft Inc. and Infostat (Di Rienzo et al., 2010) were used for the statistical analysis. All data were tested for normal distributions. Kruskal–Wallis analysis of variance was applied with significance $p < 0.05$ when data were not normally distributed.

Multivariate statistical methods were applied to different datasets (physical parameters, chemical parameters, biological parameters, combined parameters): linear discriminant analysis (LDA), factor analysis (FA) and Spearman's rank correlation coefficient. Multivariate statistical methods evidenced the contribution of diverse variables to the model, and their capacity to discriminate one category from another (Wunderlin et al., 2001). Factor analysis (FA) was performed with the correlation matrix, in which the variables were auto-scaled using the normalized varimax procedure. The main factors were extracted using the Kaiser's criterion, which only considered eigenvalues greater than one. The cutoff for selecting the variables included in these factor loadings was 0.7. 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 parameter measurement at sites and biological interaction considering 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). LDA was carried out using those parameters showing high loadings during FA. Spearman test was also applied for assessing the correlation between the data matrix (biological, chemical and physical) using a more formal mathematical approach (Di Paola-Naranjo et al., 2011).

3. Results and discussion

3.1. Physical parameters

The values of photosynthetically active radiation showed a clear distinction between site 5 and the other sampling areas (1–4), which showed lower radiation values (Table 1). The difference observed in site 5 can be attributed to the lower tree density and to the proximity to the equator (2° less than sites 1–4), resulting in higher levels of irradiance to the leaves.

The sediment granulometry showed variation among studied areas, with higher sand content at site 5 and higher clay content at site 4. The salinity ranged from 22.8 to 46.8 psu and was the lowest at sites 1 and 2 and highest at sites 4 and 5. The pH varied from acidic at sites 1 (4.2) and 2 (4.4) to basic at site 5 (8.1). The sediment at site 5 also showed lower levels of organic matter (7 g dm^{-3}), while the highest of these levels was found at site 2 (167 g dm^{-3}) (Table 1). Sandy characteristics of the sediment, in general, contribute to the availability of metals (Machado et al., 2005). Moreover, higher pH values make metals less bioavailable to absorption by plants (Neumann and Römheld, 2012). Thus, it is likely that site 5 contained less soluble metals in the sediment because of high pH, but more bioavailable because of sandy characteristics and the absence of clay at this area (Table 1). On the other hand, metals from sites 1 and 2 should be more soluble because of low pH but less bioavailable considering the clay and silt content (Table 1). Moreover, coarse sandy sediments contained generally lower levels of metals (Liu et al., 2006), which is the characteristic of sediments from site 5 (Table 1).

3.2. Chemical parameters

Table 1 shows values measured for several chemical elements in sediments from 5 studied areas. Highest concentrations of P and Zn were found at site 3, while site 4 showed a higher concentration of K, with sites 2 and 4 showing uppermost concentrations of Ca and Fe. Conversely, site 5 showed lowest concentrations for most elements assessed (Table 1). In general, the accumulation of metals and other elements in sediments is correlated with the content of organic matter due to its high adsorption capacity (Zhou et al., 2010). In our study, K (0.747), S (0.709), Ca (0.909), Mg (0.864), Fe (0.739), Zn (0.419), B (0.843) and Na (0.690) were positively correlated with the organic matter content (Spearman's test), reinforcing this trend. Thus, the lower content of most chemical elements at site 5 appeared to occur due to the lower organic matter content in addition to the absence of clay in this area. Moreover, coarse sandy sediments contained generally lower levels of metals (Liu et al., 2006), which is the characteristic of sediments from site 5 (Table 1).

3.3. Biological parameters

The anatomical and morphological characteristics of plants are commonly correlated with a particular environmental condition where they are growing. Table 2 shows both anatomical and morphological characteristics of three studied plants from five studied sites. The three studied

Site 3			Site 4			Site 5		
A. schaueriana	*L. racemosa*	*R. mangle*	*A. schaueriana*	*L. racemosa*	*R. mangle*	*A. schaueriana*	*L. racemosa*	*R. mangle*
2.8 ± 0.7^{b}	3.1 ± 0.6^{c}	3.7 ± 0.4^{c}	3.3 ± 0.8^{b}	4.1 ± 0.5^{bc}	5.2 ± 1.1^{abc}	6.2 ± 1.2^{a}	6.4 ± 0.5^{a}	7.1 ± 1.7^{a}
11.9 ± 2.7^{bc}	15.9 ± 1.8^{a}	10.3 ± 1.9^{bc}	15.3 ± 1.3^{ab}	13.6 ± 1.2^{a}	10.8 ± 0.8^{bc}	16.0 ± 0.9^{a}	14.7 ± 3.0^{a}	13.5 ± 0.5^{a}
14.7 ± 3.5^{a}	12.9 ± 0.7^{a}	7.8 ± 0.7^{b}	16.8 ± 2.4^{a}	10.4 ± 0.8^{b}	8.8 ± 1.2^{ab}	16.8 ± 0.7^{a}	11.1 ± 0.5^{b}	10.7 ± 1.4^{a}
73 ± 16^{bc}	193 ± 34^{a}	79 ± 14^{a}	85 ± 15^{ab}	256 ± 116^{a}	76 ± 4^{a}	100 ± 12^{a}	427 ± 124^{a}	83 ± 10^{a}
275 ± 33^{a}	157 ± 26^{bc}	262 ± 36^{c}	293 ± 34^{a}	161 ± 27^{bc}	282 ± 7^{bc}	290 ± 32^{a}	233 ± 14^{a}	332 ± 19^{a}
100 ± 22^{b}	91 ± 12^{bc}	123 ± 14^{b}	122 ± 23^{ab}	89 ± 16^{bc}	133 ± 7^{b}	149 ± 23^{a}	136 ± 10^{a}	166 ± 8^{a}
na	67 ± 14^{bc}	na	na	71 ± 11^{bc}	na	na	97 ± 6^{a}	na
175 ± 27^{a}	na	139 ± 23^{a}	171 ± 19^{a}	na	149 ± 11^{a}	142 ± 11^{a}	na	166 ± 12^{a}
0.59 ± 0.16^{b}	na	0.91 ± 0.09^{a}	0.71 ± 0.13^{ab}	na	0.91 ± 0.12^{a}	1.05 ± 0.11^{a}	na	1.02 ± 0.05^{a}
na	1.36 ± 0.15^{a}	na	na	1.25 ± 0.06^{a}	na	na	1.39 ± 0.09^{a}	na
375 ± 46^{a}	377 ± 49^{b}	375 ± 49^{a}	409 ± 43^{a}	441 ± 142^{ab}	365 ± 12^{a}	423 ± 43^{a}	686 ± 126^{a}	417 ± 20^{a}
94 ± 9^{c}	71 ± 11^{a}	49 ± 4^{b}	109 ± 12^{bc}	81 ± 13^{a}	59 ± 9^{ab}	160 ± 27^{a}	65 ± 7^{a}	76 ± 18^{a}
na	124 ± 22^{a}	na	na	133 ± 18^{a}	na	na	112 ± 14^{a}	na
15.1 ± 3.6^{a}	1.2 ± 0.4^{a}	na	18.2 ± 4.4^{a}	1.3 ± 0.4^{a}	na	23.1 ± 3.4^{a}	2.3 ± 0.8^{a}	na
30.2 ± 12.1^{b}	1.0 ± 0.3^{b}	na	161.0 ± 74.4^{ab}	1.4 ± 0.2^{b}	na	162.2 ± 23.4^{a}	2.3 ± 0.3^{a}	na
26.9 ± 5.5^{b}	35.7 ± 4.8^{abc}	40.0 ± 3.9^{b}	30.6 ± 3.6^{b}	31.3 ± 3.6^{c}	51.5 ± 0.1^{a}	17.5 ± 2.9^{c}	33.7 ± 3.9^{bc}	35.0 ± 4.8^{b}
0.30 ± 0.04^{b}	0.54 ± 0.07^{a}	0.56 ± 0.10^{b}	0.42 ± 0.05^{a}	0.53 ± 0.08^{a}	0.81 ± 0.13^{a}	0.29 ± 0.05^{b}	0.63 ± 0.10^{a}	0.71 ± 0.10^{a}
0.012 ± 0.002^{b}	0.015 ± 0.002^{bc}	0.014 ± 0.001^{cd}	0.014 ± 0.001^{a}	0.016 ± 0.001^{b}	0.016 ± 0.001^{b}	0.017 ± 0.001^{a}	0.019 ± 0.002^{a}	0.020 ± 0.001^{a}

species showed leaves with a thicker cuticle and palisade parenchyma at site 5. Also at this site, *A. schaueriana* showed a thicker water storage parenchyma, while *L. racemosa* showed a thicker leaf blade. As far as the epidermis is concerned, *R. mangle* and *A. schaueriana* showed higher values on the adaxial leaf surface at site 5, while the adaxial leaf surface of *L. racemosa* was thicker at site 3, with the corresponding to *R. mangle* being thicker at sites 3 and 5. No significant difference in spongy parenchyma thickness was found among *A. schaueriana* and *R. mangle* collected at different sites in this study. Concerning the palisade parenchyma/spongy parenchyma ratio, the highest values were observed among *A. schaueriana* individuals from site 5 (Table 2).

A higher stomatal density was found for *A. schaueriana* from site 5 and also in *R. mangle* from sites 1 and 5, whereas no significant difference was found for *L. racemosa*. The density of salt glands on the adaxial leaf surface of *A. schaueriana* and *L. racemosa* was higher among individuals from site 5. No significant differences among studied areas were observed concerning the density of glands on the abaxial surface (Table 2). The salt glands present in leaves of some mangrove species are adaptive structures bestowing salt tolerance (Sobrado, 2004), with salt secretion levels varying among species (Ye et al., 2005). The higher density of salt glands found during this study in the most saline mangrove area (site 5) corroborates other studies reporting that individuals who are subject to higher salinity present higher gland density than those habiting less saline environments (Barhoumi et al., 2007; Marcum, 2006).

The smallest leaf area was found for *R. mangle* individuals from sites 3 and 5. Considering *A. schaueriana*, the lowest leaf area was found among individuals from site 5. Conversely, *L. racemosa* individuals from site 4 showed leaves with smaller areas. *R. mangle* individuals from site 3 showed lower dry mass values, while *A. schaueriana* individuals from sites 2, 3, and 5 showed the lowest values. No significant difference between the studied areas was observed in dry mass among *L. racemosa* leaves. For the three species studied, the LMA was higher among individuals of *L. racemosa* and *R. mangle* from site 5, and higher among *A. schaueriana* individuals from sites 5 and 1 (Table 2).

3.4. Multivariate statistics

Interpreting the data from biological variation *in situ* is complex, particularly when multiple variables are present. Thus, we decided to apply multivariate analysis looking to get an integrated view of the overall situation (physical, chemical and biological changes), indicating which variables are most relevant to differentiate among studied areas (Wunderlin et al., 2001; Monferrán et al., 2011).

Factor analysis (FA) was carried out first using the entire data matrix, including 23 morpho-anatomical, 10 chemicals and 7 physical parameters. FA extracted six factors (F1 to F6) accounting for 84.95% of the accumulated variance. The first factor (F1), accounted for 42.68% of the variance, showing high loadings for LMA of *A. schaueriana*, Cu, Mn, coarse sand, pH and salinity. F1 included parameters from the three different compartments (biological, chemical and physical), explaining almost half of the observed variance. The second principal component (F2), with 16.27% of retained information, presented high loadings for palisade parenchyma of abaxial leaf surface from *L. racemosa*, in addition to K, S, Mg, Fe, B, Na, fine sand, silt and organic matter. The third principal component (F3), with 11.56% of retained information, presented high loadings for the thickness of the epidermis of the adaxial leaf surface from *R. mangle*, in addition to the thickness of the leaf blade from *R. mangle* and the thickness of the cuticle from *A. schaueriana*. The fourth, fifth and sixth principal components (F4, F5 and F6), with 7.22%, 4.04%, and 3.18% of retained information, respectively, presented high loadings for P and Zn (F4); for both leaf area and leaf dry mass of *L. racemosa* (F5), and for the ratio palisade parenchyma/spongy parenchyma of *R. mangle* (F6). Altogether, F1 to F6 account for 85% of the observed variability, considering 24 out of 54 studied parameters. These parameters should point out differences between studied sites.

Table 3 presents classification functions from stepwise LDA considering five mangrove areas studied. So far, stepwise LDA required 12 out of 24 parameters pointed out by FA to distinguish between studied sites with 100% correct classification (classification matrix, data not shown). Noteworthy is that parameters pointed out by LDA included three physical parameters (pH, salinity and coarse sand), seven chemical parameters (Mg, B, K, S, Fe, Mn and Cu) and two biological parameters, being one morphological (LMA of *A. schaueriana*) and one anatomical (palisade parenchyma of the abaxial leaf surface – Pab – of *L. racemosa*).

Thus, the complex data matrix obtained by physical, chemical and biological study at estuaries, could be reduced to only 12 parameters, which were enough to perform a spatial differentiation among five studied sites. Physical and chemical were the most important for such differentiation. Also, it is worthy to remark that parameters selected by LDA did not include variables from *R. mangle*. Thus, considering three studied species, it may be that *R. mangle* is not as efficient bioindicator as *A. schaueriana* and *L. racemosa*.

Box plots showing patterns representing 6 parameters pointed out by both FA and LDA out of 63 starting parameters are shown in Fig. 3.

The LDA indicated that Mg, pH and B were the parameters showing the maximal discriminating power (Table 3). Thus, these estuaries can

be primarily differentiated on the basis of chemical and physical parameters. Site 5 is clearly different from sites 1–4, probably because of the artificial diversion done at the river mouth, which affects the normal flow of surface (less saline) water at this area. In mangroves, Mg is an element generally associated with salinity (Cohen et al., 1999). However, in our current study, these two parameters were not correlated; from Table 1 it can be seen that site 5 contained high salinity but low levels of Mg. One factor that may have contributed to this result is that site 5, unlike other sites, does not receive direct influence of the sea, and Mg is one of the major cationic constituents of the seawater. Conversely, pH, pointed in LDA, shows high correlation with salinity (Spearman 0.9107) (Fig. 3B and C).

The use of physical and chemical analysis integrated with biomarkers provided precise data about environmental effects on the biota (Souza et al., 2013), enabling efficient control for the preservation of the environment and its inhabiting organisms. In this context, plants have been used as bioindicators of environmental variations, while their morphological and anatomical parameters have shown good performance as biomarkers (Ribas et al., 2005; Talukdar, 2013).

Among biological parameters used during this study, LMA of *A. schaueriana* presented the highest capacity to differentiate between studied areas, as pointed out by FA and LDA (Fig. 3A). LMA is a composite variable of leaf density and thickness (Niinemets, 2001). In general, the highest values observed among species located at site 5 (Table 2) are partly due to an increase in the thickness of leaf tissues, especially palisade and water storage parenchyma. Some causes for increased LMA include water deficit, high solar radiation and low soil nutrient content (Poorter et al., 2009), especially that of nitrogen or phosphorus (Read et al., 2006). In our study, LMA of *A. schaueriana* was positively correlated with the salinity (Spearman 0.71), Mn content (Spearman 0.81) and pH (Spearman 0.82) (Fig. 3A–C) but negatively correlated with P content (Spearman −0.63). With respect to salinity, current results corroborate other studies with mangrove species that showed higher LMA values with increased salinity (Suárez, 2005), increased drought (Suárez, 2003, 2005) and lower rainfall (Méndez-Alonzo et al., 2008); thus, reflecting the influence of water availability on this attribute of the leaf. Furthermore, the P content appeared to influence LMA of *R. mangle* (Feller, 1995) which is also in good agreement with the results observed in our current study.

Box plots showing differences among anatomical and morphological parameters in *A. schaueriana* (Fig. 3A, D–F), in addition to the leaf anatomy of this species occurring at site 5 (Fig. 2B, D and F) in comparison to other studied areas (Fig. 2A, C and E), highlight differences from site 5.

When analyzing the results of the Spearman's test, it was possible to observe some correlations between anatomical and morphological characteristics of the species and their surrounding environments. For instance, the salinity, pointed out in FA and LDA, showed positive correlation with the water storage parenchyma of *A. schaueriana* (Spearman 0.64) (Fig. 3B and D), corroborating other studies that have also observed this increased thickness under high salinity. The increased water storage parenchyma results in higher leaf succulence (Tomlinson, 1994), which allows the plant to concentrate extra ions in the leaf cells, maintaining osmotic adjustment (Clough et al., 1982), as observed among *Avicennia germinans* (Suárez and Sobrado, 2000), *L. racemosa* (Cram et al., 2002; Sobrado, 2005) and *R. mangle* (Werner and Stelzer, 1990). A thicker water storage parenchyma also can result from higher levels of phosphorus in mangrove sediments, as observed by Feller (1996) for *R. mangle*. However, in our study, these variables were not correlated for *R. mangle* and *L. racemosa* (Spearman −0.16 and −0.37, respectively), while *A. schaueriana* presented negative correlation (Spearman −0.53). Moreover, a positive correlation was observed between the water storage parenchyma and the leaf blade of *A. schaueriana* (Spearman 0.84), *L. racemosa* (Spearman 0.94) and *R. mangle* (Spearman 0.67). Among mangrove species, an increased leaf blade thickness is often associated with an increased development of water storage tissue (Camilleri and Ribi, 1983; Cram et al., 2002; Sobrado, 2007; Werner and Stelzer, 1990), which agrees with our current results for the three species studied (Fig. 3D and E).

There was a negative correlation of pH and PAR with the leaf area of *A. schaueriana* (Spearman −0.64 and −0.51, respectively), and also of *R. mangle* (Spearman −0.69 and −0.57, respectively) (Fig. 3C and F). A decrease in the leaf area among mangrove species, in response to higher luminosity, is an important issue for the adaptation of plants (Farnsworth and Ellison, 1996), as it serves to decrease the transpiration surface (Taiz and Zeiger, 2009). Some mangrove species can also show an increase in the leaf area when exposed to higher levels of soil nutrients (McKee, 1995); of these nutrients, phosphorus is important with regard to this characteristic (Feller, 1995; Lovelock et al., 2004). In our present study, however, we did not find correlation between these variables for any of the three studied species.

The cuticle thickness of *A. schaueriana* and epidermal thickness of the adaxial surface of *R. mangle*, pointed out by FA, showed a positive correlation with PAR (Spearman 0.52 and 0.49, respectively). The higher leaf cuticle thickness observed in individuals from site 5, an area with high sun radiation, represents an important characteristic in this environment because it reflects part of the radiation incidence, which protects the photosynthetic tissue (Solovchenko and Merzlyak, 2003) and minimizes transpiration (Nandy (Datta) et al., 2005). Furthermore, higher development of the epidermis can help in photoprotection (Marques et al., 1999; Rossatto and Kolb, 2010). Evaluation of cuticle

Table 3
Classification functions corresponding to LDA of biological, physical and chemical parameters.

Sites	Site 1	Site 2	Site 3	Site 4	Site 5
	p = .20000	p = .20000	p = .20000	p = .20000	p = .20000
Classification functions, LDA					
Mg	−0.123732092	0.558861305	0.516091436	0.146471563	0.044754143
pH	1442.074495	1439.811818	2277.269099	2697.829082	4179.43971
B	41.81352551	39.87643032	62.56769425	79.18533184	121.0629135
K	−3.465871682	−4.381401603	−7.18621279	−7.893359985	−13.04491866
S	−0.900969101	−1.083833348	−1.767653741	−2.04117159	−3.381811631
Coarse sand	−1.932243273	−1.814186215	−3.61532505	−4.587387662	−7.944308682
Fe	0.435423604	0.486422764	0.77626188	0.898612085	1.418036158
Mn	−9.816252099	−10.0941253	−16.03215297	−18.145712	−28.63884966
Salinity	−8.919471793	−10.46718717	−14.88313833	−15.66638378	−22.89630573
Cu	117.4689473	−28.81899264	−104.2487425	0.628035861	−125.7432519
LMA — *A. schaueriana*	−7483.067978	2127.141216	−3623.52373	−10137.58946	−17984.26495
PAb — *L. racemosa*	1.846819749	0.289128417	0.41515425	0.915429707	1.343388424
Constant	−2223.439069	−2464.59566	−4570.727467	−6255.612566	−13679.79568

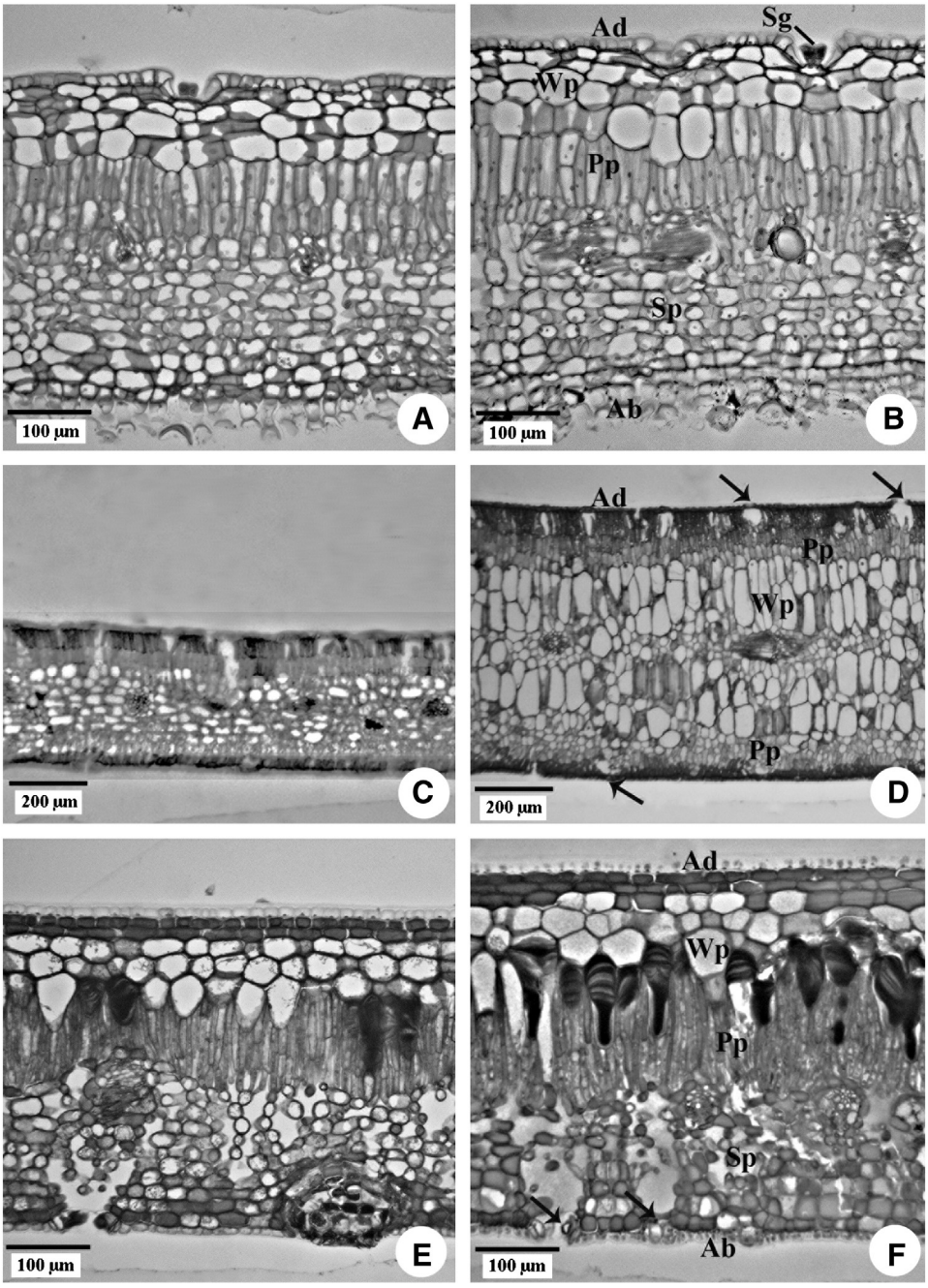

Fig. 2. Leaf blade cross sections of *Avicennia schaueriana* (A and B), *Laguncularia racemosa* (C and D), and *Rhizophora mangle* (E and F) collected in the mangrove areas located at sites 1 (A and E), 2 (C), and 5 (B, D and F). (Ab = abaxial surface of epidermis; Ad = adaxial surface of epidermis; Pp = palisade parenchyma; Sg = salt gland; Sp = spongy parenchyma; Wp = water storage parenchyma). The arrows indicate the stomata.

thickness may be important when considering the effects of climate change, greenhouse effect, *etc.*, mainly because exposure to an excess of irradiance can lead to photoinhibition in mangrove plants (Christian, 2005).

4. Conclusions

Our current results demonstrated the leaf morpho-anatomical plasticity of *A. schaueriana*, *L. racemosa* and *R. mangle* to different environmental conditions, showing the adaptive values of the features assessed, which reflects the wide adaptive geographic distribution of these species. According to multivariate statistics, *A. schaueriana* and *L. racemosa* may be the better species to reflect differences in physical and chemical conditions of mangrove ecosystems, with LMA in *A. schaueriana* emerging as a suitable biomarker to point out such differences. Noteworthy is that multivariate statistics help to extract conclusive results from complex data matrixes, combining physical, chemical and biological parameters. This multivariate approach may be especially important when analyzing neotropical estuaries, considering variations in soil/sediment structure, salinity, nutrients, and solar radiation, which influence inhabiting biota.

Conflicts of interest

No evident conflicts of interest were detected for the present work.

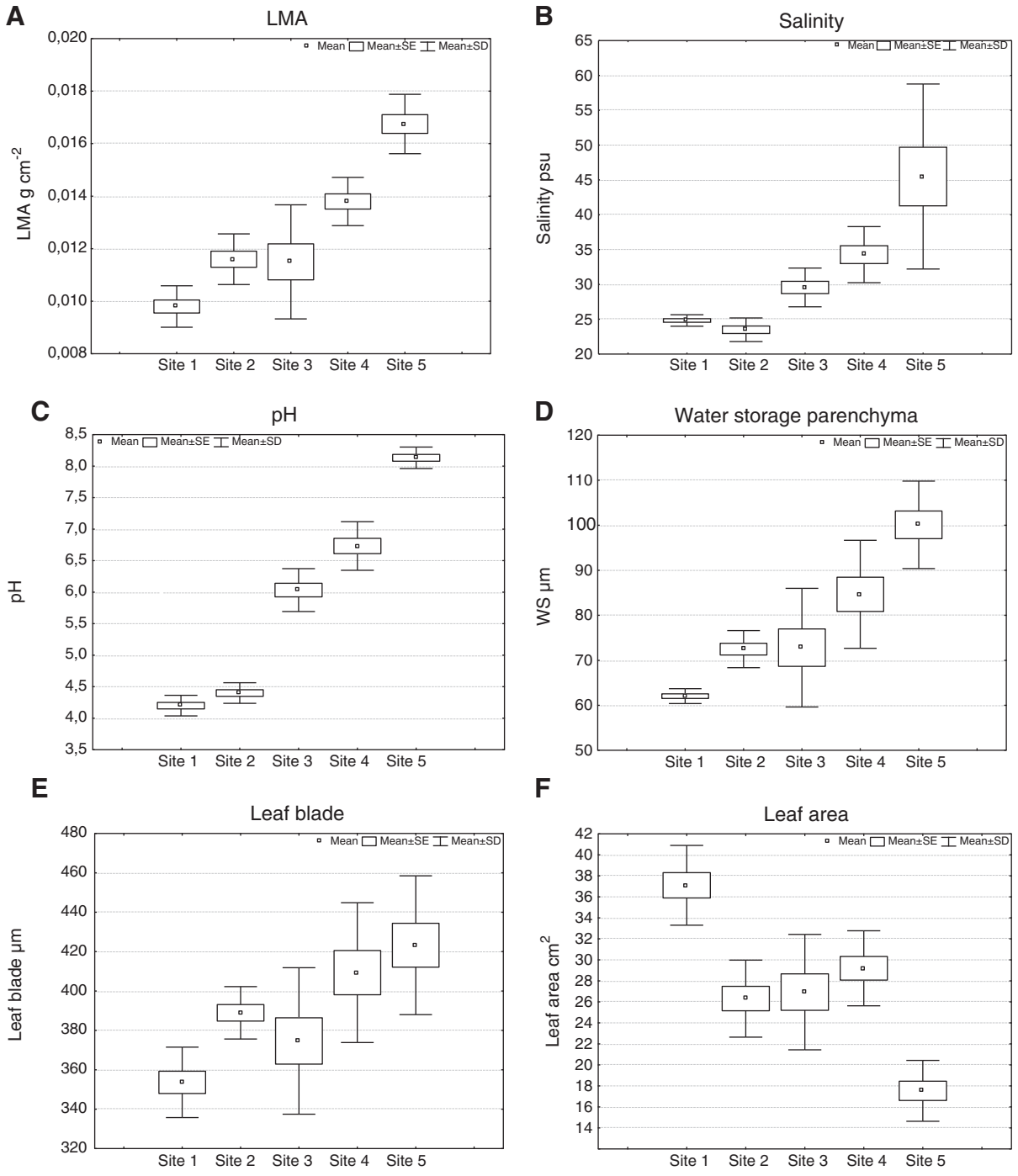

Fig. 3. Box & whisker plots from some selected biological and physical parameters measured. Values are reported as mean ± SD and SE.

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