

Multidimensional approach to evaluate *Limonium brasiliense* as source of early biomarkers for lead pollution monitoring under different saline conditions



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ABSTRACT

The search for early indicators of stress (biomarkers) with low cost of determination (both economic and logistic) is a prominent issue in environmental studies. Consequently, an experimental study was conducted with the aim of validating the leaf shape variations as an early biomarker in plants growing in contaminated soils. We manipulated the Pb (0, 45 and 90 μM) and salt (0 and 598 mM NaCl) concentrations to evaluate their accumulation pattern, and their effect in biomass, leaf shape, photosynthetic pigments, phenolic contents, antioxidant enzyme activities, and lipid peroxidation in *Limonium brasiliense*. Results showed that growth and chlorophylls pigments synthesis in *L. brasiliense* are mainly impacted by Pb rise. Furthermore, the activities of the antioxidant enzymes and the lipid peroxidation both were increased by the Pb and salt addition; whereas phenolic content appeared to be more sensitive to salinity rise. On the other hand, changes in leaf shape were induced with Pb principally, and salt rise. The plants growing under stress conditions (Pb and salt treatments) showed expanded petioles and lanceolate limbos. Pb concentrations were always higher in roots than in aerial structures, suggesting that *L. brasiliense* could be a good candidate to the phytostabilization of Pb in polluted soils. The agreement in the changes of the several response variables measured as a product of the manipulated stressful factors suggests that *L. brasiliense* is a suitable bioindicator and their phenotypic plasticity is an early biomarker of stress by contamination. Leaf shape resulted to be a useful, practical and low-cost biomarker of stress on plants growing on polluted environmental conditions.

1. Introduction

Lead is a natural element of the Earth's Crust which is naturally present in soils, water, and living organisms; however, sometimes its concentration in the environment is increased by human activities, reaching levels which result toxic for both plants or animals, and human beings themselves (Kabata-Pendias, 2011).

Among worldwide ecosystems, salt marshes have been considered as one of the most impacted ecosystems in terms of anthropogenic-driven lead contamination (Banus et al., 1974; Fitzgerald et al., 2003; Reboreda and Caçador, 2007; Idaszkin et al., 2015, 2017). Like other heavy metals that reach the salt marshes, Pb can be accumulated in their soils or plants. Particularly in plants Pb can cause an inhibitory

effect in their metabolism, affecting several processes such as photosynthesis, respiration and water absorption (Kabata-Pendias, 2011; Khodaverdiloo and Hamzenejad, 2014), with the consequent effect on the productivity of these ecosystems, a severe problem which these environments have to face (Weis and Weis, 2004; Duarte et al., 2010; Redondo-Gómez, 2013; Idaszkin et al., 2017).

Several monitoring and metal restoration programs have been developed by the competent authorities in the last years, in order to evaluate the impact of metals on the ecosystem biota and to reduce medium metal bioavailability (Ruiz, 2001; Sáinz et al., 2002). Usually, these monitoring and remediation programs have been based on physical and chemical methods (Marques et al., 2011). However, recently biological methods have gained importance on account of their cost-

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effective and long-term applicability for a wide variety of contaminated sites (Weis and Weis, 2004; Cabrita et al., 2016; Duarte et al., 2018).

Plants inhabiting salt marshes are halophytes adapted to extreme conditions of high soil salinity, tidal influence and soil waterlogging (Khodaverdiloo and Hamzenejad, 2014). Moreover, these species have demonstrated an elevated tolerance to heavy metals excess (Redondo-Gómez, 2013; Idaszkin et al., 2017); this tolerance being related to their capacity to regulate their absorption and compartmentalization, as well as with their ability to modulate the antioxidant systems and the synthesis of organic compounds (Bankaji et al., 2016; Mesnoui et al., 2016, 2018; Pérez-Romero et al., 2016). All these adaptations make these plants possible candidates for metal remediation purposes (Mateos-Naranjo et al., 2012, 2014) as well as for long and short term monitoring tasks (Duarte et al., 2010, 2018).

Eco-physiological or morphometric techniques are very useful when characterizing the tolerance to metals of different plant species without needing to carry out costly analyses of metal concentrations, both in soils and plant tissues. In addition, they provide information on the effect that these pollutants have on the health of ecosystems and allow quick and continuous monitoring of the deleterious effects of pollution. There are several types of biomarkers that can be measured, such as modification of photosynthetic activity, hormones production, and secondary metabolites, enzymatic and non-enzymatic antioxidants synthesis (Duarte et al., 2013, 2018; Ferrat et al., 2003; Monni et al., 2001; Sivaci et al., 2007). Likewise, several studies carried out in different plant structures postulate the use of shape variations as a potential biomarker, due to the fact that they found an association between the pollutants and morphogeometric changes (Veličković, 2010; Ambo-Rappe et al., 2011; Klisarić et al., 2014; Vujić et al., 2015; Smith, 2016; Pollicelli et al., 2018). However, information is scarce on the extent to which leaf shape variations could be a useful tool in ecotoxicology and impact assessment for an extensive number of species, specific metals and in combination with other important factors characteristic of marsh ecosystems, particularly salinity.

Limonium brasiliense (Boiss.) Kuntze (Plumbaginaceae) is a perennial halophyte plant endemic to southern South America, distributed throughout Argentina, Uruguay and southern Brazil. Its natural habitats are wetlands and salt marshes where it tolerates high soil salinity (Alvarez et al., 2015). Furthermore, this species is really common in Patagonian salt marshes highly polluted with heavy metals, particularly with Pb (Idaszkin et al., 2015, 2017). These aspects make *L. brasiliense* a suitable model plant to validate the leaf shape variations as an early biomarker in *L. brasiliense* plants growing in Pb contaminated and contrasted saline medium, enlarging the tools that make it possible to monitor and predict the effects of this element, before significant changes occur in the natural structure of the communities. For this end we: (1) evaluate the leaf shape variations of *L. brasiliense* and plant tolerance in terms of growth, physiological and biochemical response variation with rise in Pb, either alone or combined with rise in salinity; and (2) ascertain the extent to which the accumulation pattern of Pb in the different structures (roots and leaves) of *L. brasiliense* determine plant leaf shape variation and tolerance. Likewise, these results could highlight the potential use of *L. brasiliense* in phytoremediation techniques, evaluating its potential capacity to extract, accumulate and

tolerate Pb under different salinity conditions considering that salinity is a key factor in the accumulation and translocation of metals in plants (Mausakaki and Kalogerakis, 2009).

2. Material and methods

2.1. Plant material and experimental design

Seedlings of *Limonium brasiliense* with the associated substrate were collected during October 2016 from Fracasso salt marsh (42° 25' S, 64° 37' W), at the Península Valdés Natural Reserve (Patagonia, Chubut, Argentina). The collected seedlings were transferred to individual pots filled with perlite and maintained in a greenhouse with a natural photoperiod and mean temperatures of 15 °C at night and 25 °C at daylight and irrigated with 20% Hoagland's solution in order to increase their biomass. After 4 weeks of growth, plants were randomly arranged in six experimental blocks of 10 plants in shallow trays and assigned to three Pb concentrations (0, 45 and 90 µM) in combination with and without NaCl addition (0 and 598 mM). At the beginning of the experiment, 3 L of each treatment solution, obtained combining 20% Hoagland's solution with the adequate amount of Pb(NO₃)₂ and NaCl, were placed in each of the trays down to a depth of 2 cm. During the experiment, trays levels were monitored to avoid Pb and NaCl concentration variation due to water evaporation. Finally, the complete solution (including Pb(NO₃)₂ and NaCl) of each specific treatment was changed weekly. Four weeks after the experiment set up, measurements of growth, leaf geometric morphometrics, photosynthetic pigment concentrations, antioxidant enzyme activity, total phenolic content, lipid peroxidation, and tissues Pb concentrations were made.

2.2. Plant growth analysis and leaf geometric morphometrics data acquisition

At the end of the experiment, all plants were harvested and separated in roots and aerial structures and dried at 60 °C until they reached constant weight to calculate the final dry mass (n = 10 per treatment).

Before plants harvest, the three first fully deployed leaves per plant from each pot were separated in order to study the shape attributes on leaves of *L. brasiliense*. To avoid the loss of turgor and consequently any type of modification of the leaf shape, each leaf was digitized immediately after being cut from the plant using a conventional Epson perfection v37 scanner, obtaining the image of the adaxial side of the leaf. Then the 3 leaves were immediately stored in liquid nitrogen and were stored in a freezer at –80 °C until the pigments, enzyme, and lipid peroxidation analysis.

The overall leaf shapes were captured by the Cartesian coordinates of a 2D configuration of 5 landmarks and 20 semi-landmarks (Fig. 1). A total of 53 leaves were digitized using the tpsDig2 module of the TPS series (Rohlf, 2017a), 7 leaves (broken outlines) were deleted. The arching effect of each leaf was removed from the uncorrected series with the “unbend specimens” module provided in tpsUtil v1.58 software (Rohlf, 2017b). Landmarks 1, 24 and 25 (Fig. 1) were used to define a longitudinal axis for correcting leaf arching. After that, landmark 25 was deleted.

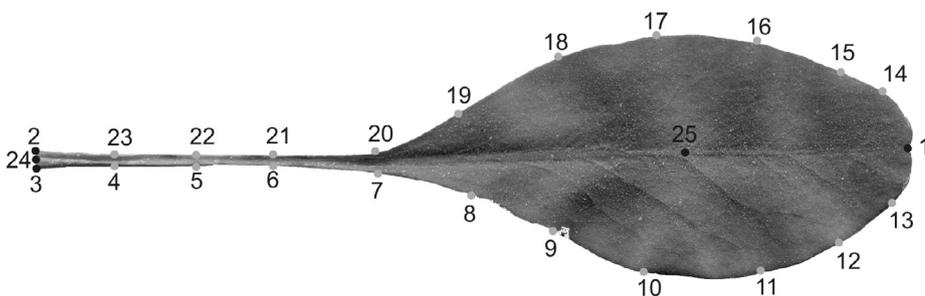


Fig. 1. Landmarks (black dots) and semi-landmarks (grey dots) configuration placements on the contour of the leaf of *Limonium brasiliense*. These landmarks include: (1) Apex, (2) Left tip of petiole, (3) Right tip of petiole, (4–13) semilandmarks placed equidistantly between landmark 1 and 3, (14–23) semi-landmarks placed equidistantly between landmark 1 and 2, (24) middle point between landmarks 2 and 3 and (25) centroid of limbo on midrib.

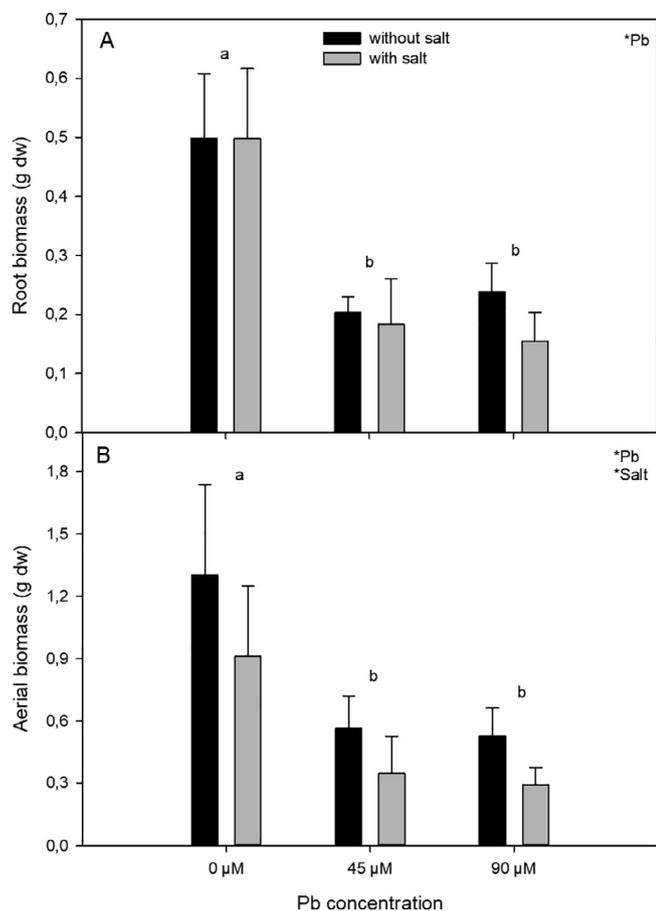


Fig. 2. Biomass of roots (A) and aerial structures (B) of *Limonium brasiliense* from each Pb and salinity treatments. Data are means + SE, n = 10. [Pb] and Salt in the corner of the panels indicate that one or both main factors were significant effects ($p < 0.05$). Different letters indicate means that are significantly different from each other (Two-way ANOVA, [Pb]; Tukey test, $P < 0.05$).

All semi-landmarks were allowed to slide along the outline profile of the leaf using the tpsRelw v 1.64 (Rohlf, 2017c) module, according to the minimum bending energy criterion (Gunz et al., 2005). Since the leaf presents object symmetry, we adjusted the aligned coordinate of the shape mean to three leaves per plant following the recommendations of Mardia et al. (2000) and Klingenberg et al. (2002). Consequently, the landmark configurations were superimposed by Generalized Procrustes Analysis with reflection (Dryden and Mardia, 1998; Klingenberg et al., 2002). After superimposition, pure shape information was preserved in the specimens' aligned landmarks, and variation around the mean shape in the sample (consensus) was decomposed into a symmetric and an asymmetric component (Klingenberg and McIntyre, 1998; Klingenberg et al., 2002). Since our interest is on symmetry, the asymmetric variation components were disregarded. Centroid size (CS) was used as a proxy to size (Zelditch et al., 2012), and it is defined as the square root of the sum of the squared distances from the landmarks to the centroid which they define.

2.3. Photosynthetic pigment analysis

To determine photosynthetic pigments leaves samples (100 mg of FW) were randomly collected at the end of the experiment and flash-frozen in liquid N_2 and stored at $-80^\circ C$ until the analyses. For pigments quantification samples were freeze-dried for 48 h in the dark to avoid photodegradation processes and crushed with 10 ml of acetone 80% and stored for 1 h at $4^\circ C$ (n = 5 per treatment). The extracts were

centrifuged at 5000 rpm for 5 min at $4^\circ C$ and the supernatants were collected and were read in a spectrophotometer at 470, 646.8 and 663.2 nm. The calculations of the concentration of chlorophyll a, b, and carotenoids were carried out applying the formulas of Lichtenthaler (1987).

2.4. Total phenolic contents

To determine the total soluble phenolic concentration, dry material (100 mg of oven dried at $45^\circ C$ by 48 h) was extracted with methanol (50% v/v; 10 ml), it was stirred for 12 h and then kept still for 1 h at $25^\circ C$ (n = 5 per treatment). Then, an aliquot of the extract (0.25 ml) was mixed with 1.25 ml of Folin–Ciocalteu reagent. This solution was left to stand at $25^\circ C$ for 5 min before adding 3.75 ml of sodium carbonate (20% w/v) and was then diluted to a final volume of 25 ml with distilled water. After 2 h at $30^\circ C$ in darkness, the absorbance of samples was measured at 760 nm. The results were expressed as $mg L^{-1}$ of tannic acid using a calibration curve obtained from tannic acid as standard (Saraví Cisneros et al., 2013).

2.5. Enzyme assays

Enzyme extraction was done following Duarte et al. (2015). For these, the leaf samples (500 mg of PF) were homogenized in 8 ml of 50 mM sodium phosphate buffer (pH 7.6) with 0.1 mM Na-EDTA and were centrifuged at 8923 rpm for 20 min at $4^\circ C$ and the supernatants were stored for the later determinations (n = 5 per treatment). Ascorbate peroxidase (APx) activity was measured by monitoring the decrease in the absorbance at 290 nm. The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.0), 12 mM of H_2O_2 , 0.25 mM L-ascorbate (Tiryakioglu et al., 2006). Molar coefficient of $2.8 mM^{-1} cm^{-1}$ was used to calculate the amount of ascorbate oxidized. Guaiacol peroxidase (GPx) activity was calculated following the Bergmeyer et al. (1974). The reaction mixture consisted of 50 mM of sodium phosphate buffer (pH 7.0), 2 mM of H_2O_2 , and 20 mM of guaiacol. The activity was measured scanning the increase in absorbance at 470 nm. For all these enzymatic enzymes, 100 μL of the vegetal extract was added to the reaction mixture to begin with the reaction. Superoxide dismutase (SOD) activity was assayed by monitoring the reduction of pyrogallol at 325 nm according to Marklund and Marklund (1974). The reaction mixture was 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA, 3 mM of pyrogallol, Mili-Q water. The reaction was started with the addition of 10 μL of enzyme extract. Catalase (CAT) activity was assessed by the decrease in absorbance at 240 nm due to the consumption of H_2O_2 according to Teranishi et al. (1974). The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA, and 100 mM of H_2O_2 . The auto-oxidation of the substrates was evaluated by control samples without the enzyme extract. Results were expressed as U per minute per gram of fresh leaves.

2.6. Lipid peroxidation

Lipid peroxidation was determined measuring thiobarbituric acid reactive substances (TBARS) according to Heath and Packer (1968). Leaf samples (n = 5 per treatment) were homogenized in 20% trichloroacetic acid (TCA), containing 0.5% thiobarbituric acid (TBA) at a ratio of 100:1 (m/v) leaf fresh weight to acid and then incubated at $95^\circ C$ for 30 min. Then the reaction was immediately stopped in ice and centrifuged at 4930 rpm for 5 min at $4^\circ C$ and the supernatant absorbance was determined at 532 and 600 nm. The malondialdehyde (MDA) concentration was estimated using an extinction coefficient of $155 nM^{-1} cm^{-1}$.

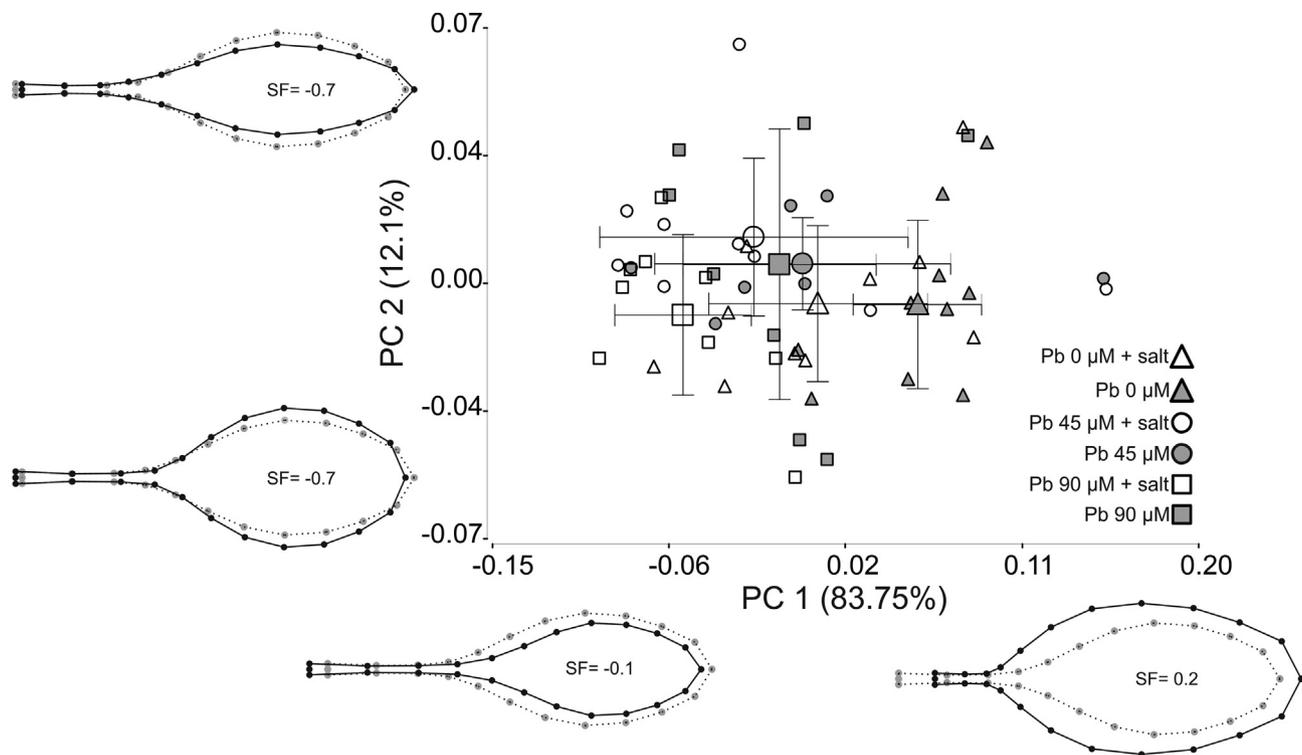


Fig. 3. Principal components showing the morphospace generated by the two first principal axes of variations. The graphs (wireframes) outside represent the transformation and displacement vectors from consensus (dashes outlines and grey dot) to the positive and negative extreme shape (solid outline and black dot). Percentages of explained variance for each axis are in parentheses. SF = scale factor.

2.7. Lead concentration in plant tissues

For lead quantification in tissues, leaves and roots samples were collected from 3 pots randomly selected from each specific treatment and meticulously washed with distilled water and dried at 60 °C for 48 h. After that sub-samples of 0.5 g were digested in 2 ml of HNO₃ ultrapure using microwave oven MARS-5, CEM Corporation, USA (2011) and were then diluted to a final volume of 15 ml with HNO₃. Lead in both matrixes was then measured as previously described by Idaszkin et al. (2017) by inductively coupled plasma (ICP-AES) spectroscopy (Shimadzu, 9000).

We calculated the bioaccumulation factor (BAF: [Pb in roots]/[Pb in solution]) and the translocation factor (TF: [Pb in aerial structure]/[Pb in roots]; Chen et al., 2011).

2.8. Statistical analysis

All the statistic tests were performed using InfoStat software (Di Rienzo et al., 2009). Two-way ANOVA of variance were used to analyze the main and the interactive effect of each Pb and Salt concentrations (as categorical factors) treatments on the main plant parameters (as dependent variables) of *L. brasiliense*. Multiple comparisons were analyzed by a Tukey (post hoc) test (Zar, 1999). Before the analyses, data were tested for normality with the Shapiro-Wilk test and for homogeneity of variance with the Levene test. To correct for non-normality and heterogeneity of variance, both root and aerial biomass variables were ln (X)-transformed (Zar, 1999).

To evaluate and control putative allometric effects (the shape change exclusively explained by variations in size), we computed the pooled-treatment multivariate regression of leaf shape (Procrustes coordinates used as dependent variables) on size as an independent variable (CS), running a permutation test with 10,000 rounds using MorphoJ v1.06d (Klingenberg, 2011). To study the magnitude and direction of leaf shape variation, a principal component analysis (PCA) of

the variance-covariance matrix (Zelditch et al., 2012) was done. Then, to display axes of among-treatment maximum discrimination in leaf shape, we performed a Canonical Variate Analysis (CVA).

3. Results

3.1. Biomass analysis

All *Limonium brasiliense* plants survived independently of Pb and NaCl treatment at the end of the experiment. However, we found that there was a significant effect of lead and salinity on plant biomass but not a significant interaction. Thus root biomass was affected by Pb increment, independently of the salt addition (Two-way ANOVA: [Pb], $p < 0.05$; Fig. 2A); while both lead and salinity augmentations had a deleterious effect in the aerial biomass (Two-way ANOVA: [Pb], Salt, $p < 0.05$; Fig. 2B). Compared with the control (i.e. treatment without Pb and without salt), root biomass reduction was between 59% and 63% for plants grown at 45 μM Pb, and between 52% and 68% for those exposed to 90 μM Pb, without and with salt addition, respectively. On the other hand, the aerial biomass reached a reduction close to 58% for both lead treatments (45 μM and 90 μM, respectively) in absence of salt supplementation. These reductions were exacerbated in those plants grown under saline conditions, with reductions between 73% and 77% by 45 μM and 90 μM Pb treatment, respectively. In addition, salt effect was evident in plants grown at 0 μM Pb with a reduction of 30% respect to the control treatment.

3.2. Leaf shape

The samples showed a significant effect of static allometry: the multivariate regression of the leaf shape onto centroid size was statistically significant ($p < 0.0001$), and accounted for 13.56% of the total shape variation. In consequence, subsequent analyses were done on the regression residuals, considered as new allometry-free shape

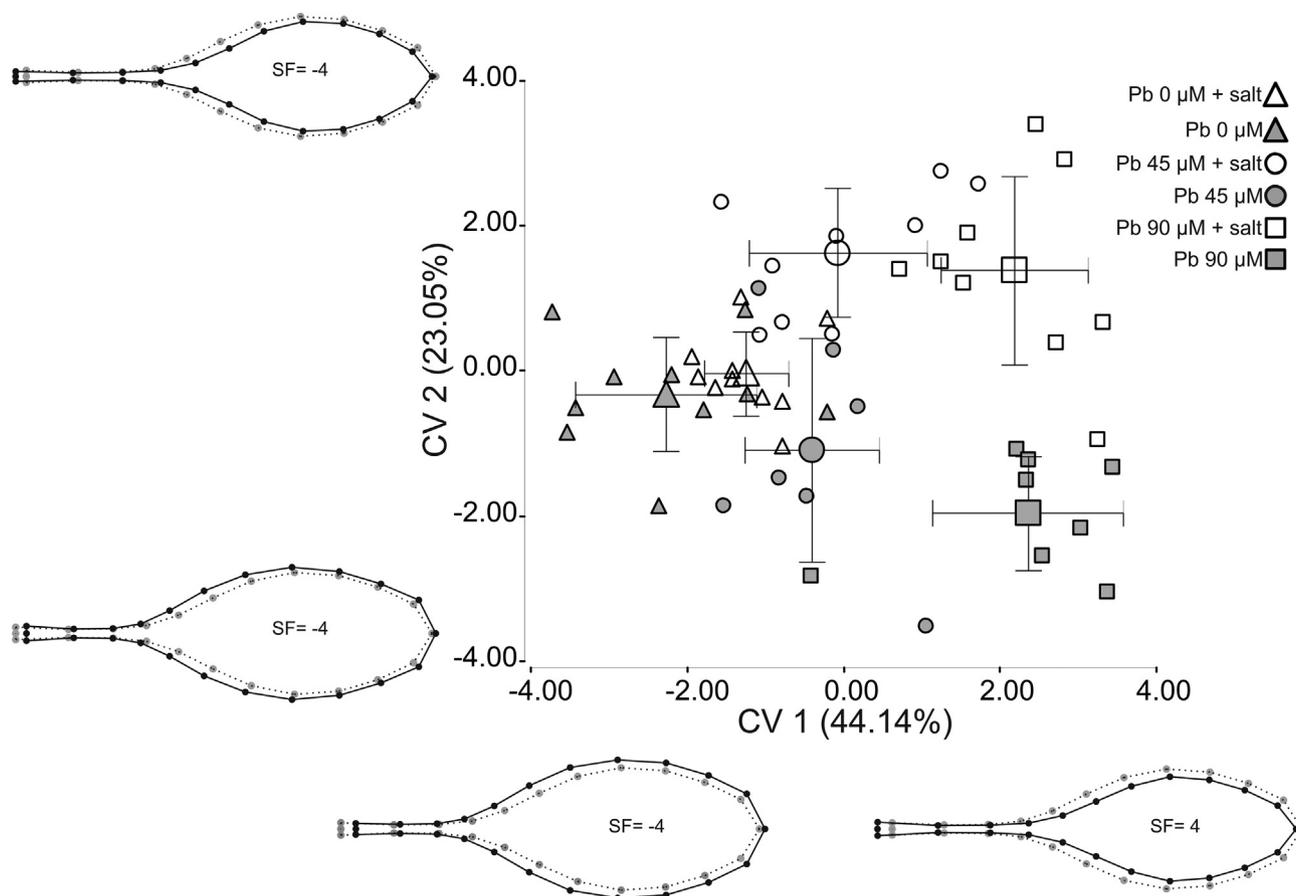


Fig. 4. Analysis of the maximum variation among treatment in leaf shape along the first two canonical axes. The wireframes show shape changes from the consensus shape to the positive and negative extreme shape along the axis. Between brackets are present amounts of the variance among-treatment differences, relative to within-treatment variation presented as a percentage of the total. SF = scale factor.

variables. The first two principal component (PC) scores accounted for 95.85% of the total leaf shape variation. PC1 (83.75%) was related with the leaf slenderness (Fig. 3). For negative values, leaves presented spatulate shape with narrowly elliptic limbos and enlarged petiole; while for the positive values, leaves presented ovate shapes. The leaf shape representations in negative values of PC2 were associated with the orbiculate shape, while lanceolate shapes were represented in the positive values of this second PC (Fig. 3).

Concerning the canonical discriminant analysis, the most conspicuous trait separating shapes along the CV1 (44.14% of the variance) were the petiole expansion and the lanceolate limbo towards the positive values located by plant under lead treatments (45 μM and 90 μM , respectively) with and without salt (Fig. 4). The plants located in the direction of the negative values (under lead treatments without salt) of the CV2 presented the petiole less developed with limbo expansion. As a general pattern, the CV1 was represented with the increment of stress, while CV2 was associated principally with the salinity addition.

3.3. Photosynthetic pigment

There were significant effects of lead rises on chlorophylls concentrations after 30 d of treatment (Two-way ANOVA: [Pb], $p < 0.05$; Fig. 5A–C). Thus, overall Pb excess decreased pigments concentration in plants grown at 90 μM Pb.

3.4. Total phenolic contents

The total phenolic content did not vary with Pb excess but overall increased with salt supplementation in the grown medium (Two-way

ANOVA: Salt, $p < 0.05$; Fig. 6).

3.5. Enzyme assays

APx enzyme activity tended to increase with Pb concentration and salt rises (Two-way ANOVA: [Pb], Salt, $p < 0.05$; Fig. 7A). On the other hand, GPx and SOD enzyme activities were significantly affected by Pb, independently of the salt addition (Two-way ANOVA: [Pb], $p < 0.05$; Fig. 7B, C). Finally, CAT enzyme activity did not vary with Pb and salinity showed in all cases mean values c. 40 $\text{U min}^{-1} \text{g}^{-1}$ fresh weight (Fig. 7D).

3.6. Lipid peroxidation

Overall MDA concentration increased in presence of Pb in growth medium; this being increment more accused under saline conditions. In addition, MDA concentration was greater with salt supplementation in plants grown at 90 μM Pb compared with their non-Pb treated counterparts (Two-way ANOVA: [Pb], Salt, $p < 0.05$; Fig. 8).

3.7. Lead concentration on plant tissues

Pb concentration in *L. brasiliense* tissues was greater in roots than in leaves and increased markedly with the Pb concentration in the growth medium (Table 1). However, this augmentation was in a certain degree ameliorated with medium NaCl supplementation.

Concerning the factors evaluated, BAF values were always higher than 1 for both Pb enrichment treatments, but overall its values were lower under saline conditions (Table 1). Contrarily TF values were

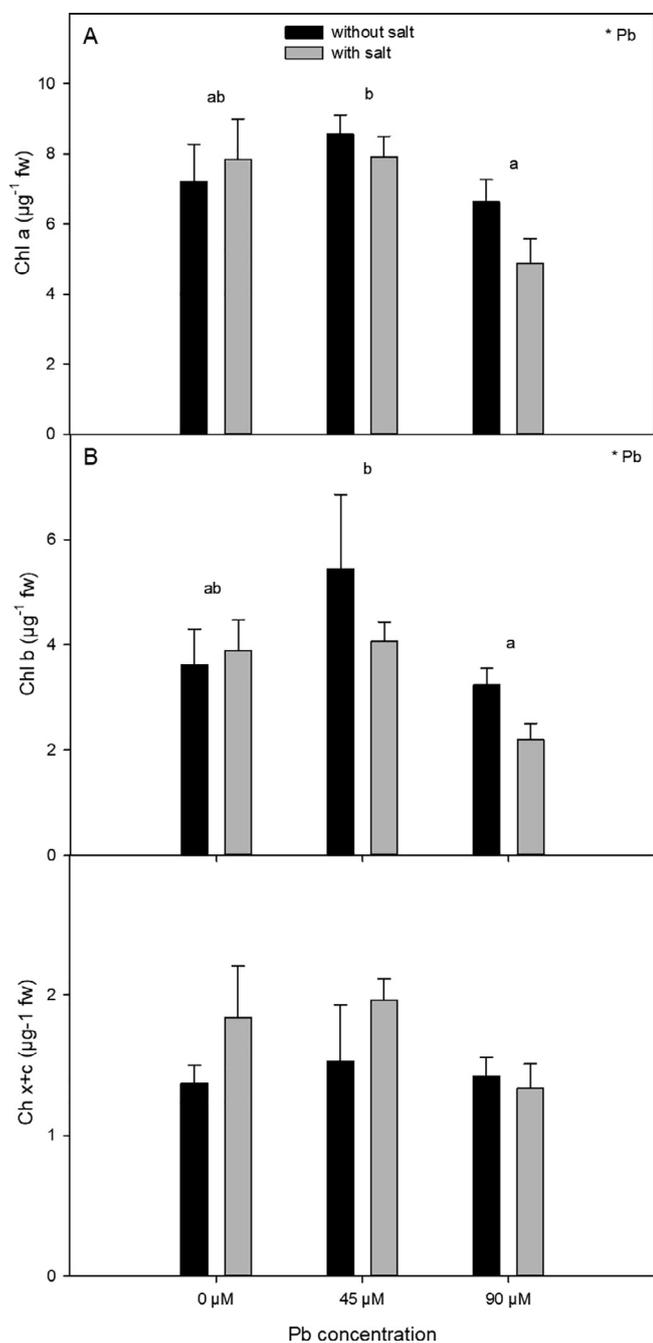


Fig. 5. (A) Chlorophyll *a* (Chl *a*), (B) chlorophyll *b* (Chl *b*), and (C) carotenoid (Cx + c) concentrations in leaves of *Limonium brasiliense* from each Pb and salinity treatments. Data are means + SE, n = 5. [Pb] in the corner of the panels indicate that one or both main factors were significant effects ($p < 0.05$). Different letters indicate means that are significantly different from each other (Two-way ANOVA, [Pb]; Tukey test, $P < 0.05$).

always lower than 1 for all Pb treatments and decreased considerably with Pb supplementation to the growth medium independently of saline levels (Table 1).

4. Discussion

In this work, we achieve to validate experimentally the use of the leaf shape as an earlier biomarker, providing compelling analytical and graphical evidence of the effect of Pb and salt rises through a different approach related to morphological, biochemical, and physiological responses in *Limonium brasiliense*. Nowadays, soil pollution is a serious

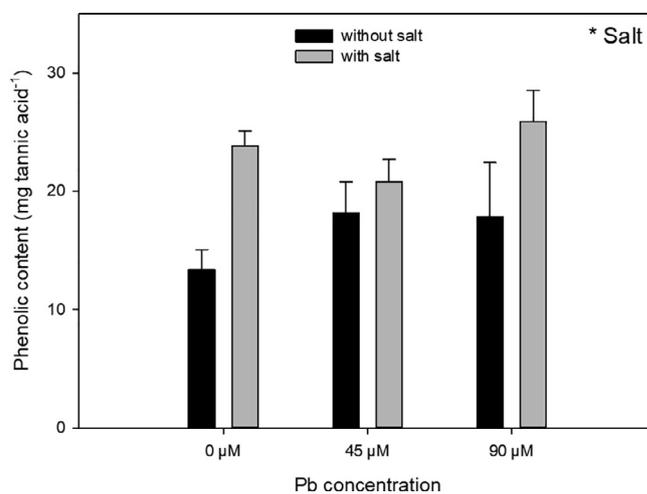


Fig. 6. Total phenolic content in leaves of *Limonium brasiliense* from each Pb and salinity treatments. Data are means + SE, n = 5. Salt in the corner of the panels indicate that one or both main factors were significant effects ($p < 0.05$).

environmental problem worldwide, which requires an intense research effort to establish management and remediation guidelines. In this sense, we highlight the importance of studies that contribute to enlarge our knowledge about the potential use of different biomarkers that allow us to detect the contamination effect early and easily, as well as those which contribute to finding species potentially usable in phytoremediation techniques. We address both issues by evaluating the ability of *L. brasiliense* to accumulate and tolerate high levels of Pb, alone or in combination with salinity rise, and by evaluating its effect through different morphological, biochemical, and physiological responses, as potential biomarkers of the stress generated.

Our results showed that although *L. brasiliense* seemed to be tolerant to Pb and salinity increment, since all plants survived throughout the whole experimental period, we noted that both manipulated factors had some negative effect on the development of *L. brasiliense*, as biomass reduction indicated when Pb was added. In several species, e.g. *Zea mays*, *Oryza sativa*, *Iris lacteal* var. *chinensis*, *Iris tectorum*, this same pattern was observed, with growth reduction when plants were exposed to a Pb enrichment solution (Gupta et al., 2009; Han et al., 2008; Verma and Dubey, 2003). In addition, even though *L. brasiliense* is a halophyte species, this aerial biomass reduction was more marked when salt was added. However, no plants from the treatments without Pb showed any signs of salt toxicity, while leaves of plants from the treatments with salt (regardless of the Pb addition) presented salt deposition on their surface, evidencing salt excretion through the glands (Lutts and Lefèvre, 2015). These processes of salt excretions could represent an effort to the plants, which could be in detriment of their growth. In other halophyte species, a decrease in growth was also seen when exposed to rise in salinity, which in some cases was compensated for with the passing of time (Zurayk et al., 2001; Alhdad et al., 2013).

Regardless of the leaf shape variation, the geometric morphometrics allowed us to detect shape change of *L. brasiliense* growing with Pb rise, either alone or combined with salt addition. The morphospace showed the presence of leaf phenotypic plasticity changing the pattern of variation according to the increment of the stress. The most conspicuous traits separating leaf shapes exposed to Pb with and without salt were the petiole expansion and the lanceolate limbo, while the plants without salt and Pb presented ovate shapes. This finding supports the results of Pollicelli et al. (2018) who observed in the halophyte *Cressa truxilensis*, more lanceolate leaf shape in the point nearest to the pollutant's source, and an oval leaf shape in the unpolluted site. Therefore, our results are in agreement with the idea that the leaf slenderness is associated with the presence of stress generated by the medium-metal

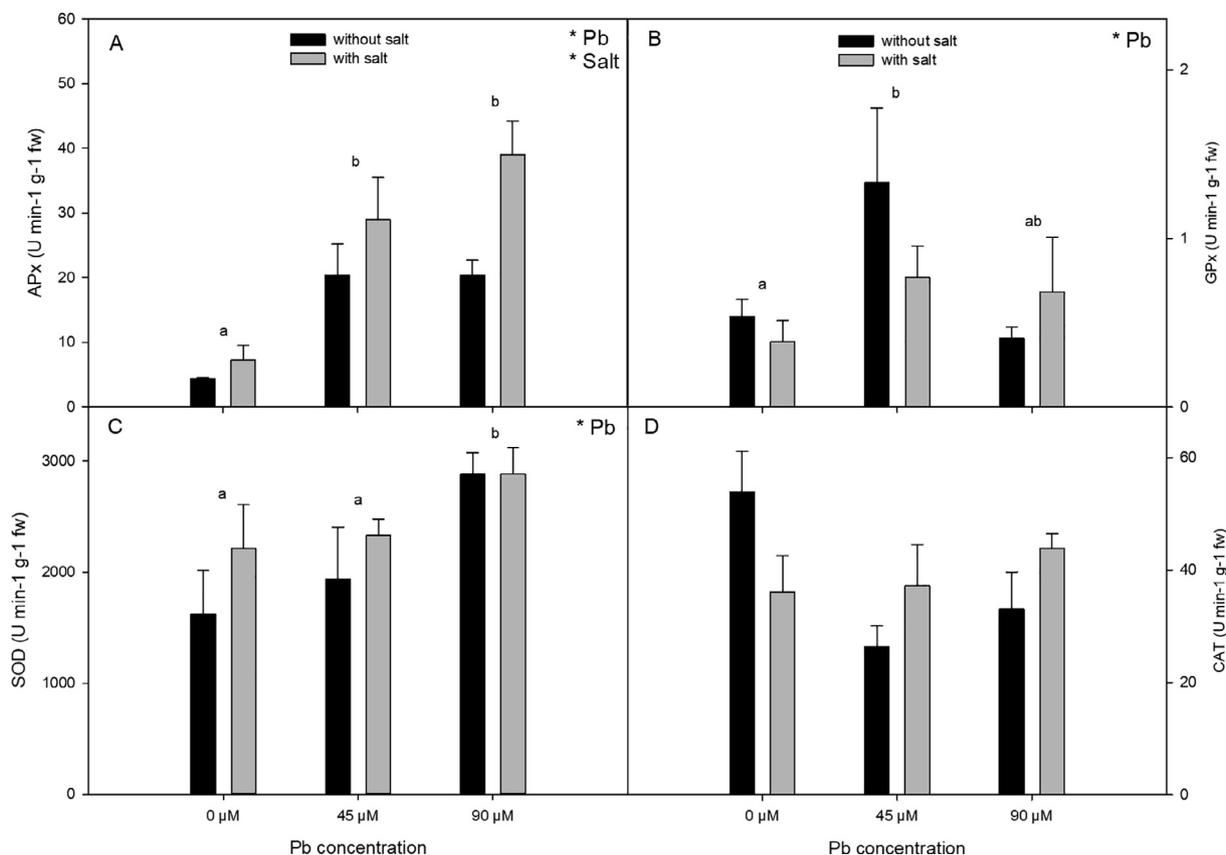


Fig. 7. Enzymatic activities in leaves of *Limonium brasiliense* from each Pb and salinity treatments. (A) Guaiacol peroxidase (GPx), (B) Ascorbate peroxidase, (C) Superoxide dismutase (SOD), and (D) Catalase (CAT). Data are means + SE, n = 5. Different letters indicate means that are significantly different from each other (Two-way ANOVA, [Pb] × Salt; Tukey test, P < 0.05). [Pb] and Salt in the corner of the panels indicate that one or both main factors were significant effects (p < 0.05).

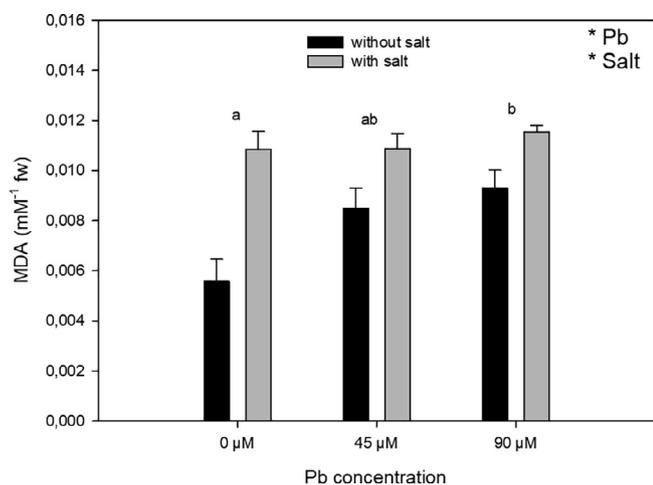


Fig. 8. Malondialdehyde (MDA) concentrations in leaves of *Limonium brasiliense* from each Pb and salinity treatments. Data are means + SE, n = 5. Different letters indicate means that are significantly different from each other (Two-way ANOVA, [Pb] × Salt; Tukey test, P < 0.05). [Pb] and Salt in the corner of the panels indicate that one or both main factors were significant effects (p < 0.05).

concentration.

On the other hand, these leaf shape variations in response to Pb and salinity increments were accompanied by modifications in the other eco-physiological and biochemical parameters tested, which have been frequently used as stress biomarkers. Thus, we found that chlorophylls concentrations in *L. brasiliense* leaves decrease at the highest Pb

Table 1

Lead concentrations in roots and aerial structures, bioaccumulation factor (BAF) for roots and translocation factor (TF) of lead uptake in *Limonium brasiliense* (mean ± S.D of three replicates, n = 3).

Pb treatment (μM)	Salinity	Pb in roots (μg.g ⁻¹)	Pb in aerial structures (μg.g ⁻¹)	BAF	TF
0	Without salt	2.6 ± 1.5	1.1 ± 0.4	–	0.6 ± 0.4
45	Without salt	93.3 ± 28.9	8.0 ± 1.3	6.2 ± 1.9	0.1 ± 0.0
90	Without salt	148.7 ± 28.9	9.1 ± 4.0	4.9 ± 2.1	0.1 ± 0.0
0	With salt	1.68 ± 0.4	1.0 ± 0.1	–	0.6 ± 0.3
45	With salt	48.3 ± 28.6	4.5 ± 2.5	3.2 ± 1.9	0.1 ± 0.1
90	With salt	123.6 ± 126.7	7.1 ± 1.9	4.1 ± 4.2	0.1 ± 0.1

concentration treatment. This may be because Pb excess can cause a decrease in the chlorophyll synthesis due to deficit in Mn or Fe uptake or induce rise in its degradation by increment in the chlorophyllase activity (Sharma and Dubey, 2005). On the other hand, there are many studies where this does not occur. For example, the synthesis of total chlorophyll in *Sarcocornia fruticosa* leaves did not change when the plants were grown at high levels of Pb and Zn (Bankaji et al., 2016). While, in *Atriplex halimus*, chlorophyll content was not affected when only Cd and Pb were added, it was affected when these metals were combined with salinity increment (Manausaki and Kalogerakis, 2009), but this not occurred in *L. brasiliense* leaves, due to the fact that the pigments content did not change under saline conditions.

In addition to the photosynthetic pigments responses to the stress induced by Pb aforementioned, the increment of the reactive oxygen species (ROS) is another important phytotoxic effect in plants exposed

to stressful environmental and specific to metal pollution and salt excess. In fact, under these conditions plants produce and accumulate specific compounds, such as phenolic compounds and MDA, and modulate their specific antioxidant enzyme machinery to cope with the increment of oxidative stress produced with the presence of metals (Merlin et al., 2012; Mesnoui et al., 2018) and salt (Alhdad et al., 2013). In *L. brasiliense*, salt addition seems to have an effect in their leaves phenolic content which increased significantly together with the APx enzyme activity and MDA; although this enzymatic activity and MDA were also affected by Pb addition. In addition, Pb increment also affected the SOD and GPx enzymatic activities. As several authors reported, halophyte species are well adapted to highly saline environments therefore they are well prepared to face ROS increments (Flowers and Colmer, 2008; Alhdad et al., 2013; Bose et al., 2014), in accordance with the increments in phenolic content, MDA and enzyme activities found in *L. brasiliense* leaves. On the other hand, regarding Pb increment, our results coincide with what was observed in other species, such as *Triticum aestivum*, *Oryza sativa*, *Raphanus sativus*, in which SOD, APx and GPx enzyme activities increased when Pb was added (Dey et al., 2007; Gopal and Rizvi, 2008; Verma and Dubey, 2003). However, the absence of Pb effect on phenolic compounds could be due to an encrypted response, since we measured their total content of soluble, this might result from the fact that some of them could be increasing and others decreasing (Kisa et al., 2016). Further studies should be delineated in order to understand these results in more depth, as the identification and quantification of the different phenolic compounds.

Finally, *L. brasiliense* leaf shape, eco-physiological and biochemical variations in response to Pb and salinity increments were highly associated to lead concentration in plant tissues. Our results indicated that *L. brasiliense* had a great capacity to accumulate Pb in their tissues, but its concentration was higher in roots than in shoots. Thus, Pb concentrations in plant tissues rose according to the increase of the Pb solution concentration, independent of medium salinity level, a similar response has been previously recorded in other species, such as *Brassica juncea*, *Iris lactea* var. *chinensis* and *Iris tectorum* (Jiang et al., 2000; Han et al., 2008). As is common in halophyte species, the TF were significantly lower when Pb was added for both salt treatments (Redondo-Gomez et al., 2010; Idaszkin et al., 2017). In fact, in most plant species, the major fraction of Pb absorbed is retained in roots, and only a small proportion is translocated to aerial structures (Pourrut et al., 2011). Unlike most other metals, Pb appears to be blocked in the endodermis cells, limiting its translocation from the roots to the aerial parts (Pourrut et al., 2011). Even *Zea mays*, which is able to absorb very high amounts of Pb from the solution, only translocated a small fraction from its roots to its shoots (Gupta et al., 2009). Also, seedlings of two rice cultivars (*Oryza sativa* L.) accumulated more Pb in their roots than in shoots when they were experimentally exposed to higher Pb concentration (Verma and Dubey, 2003). In addition, the fact that the Pb concentration in *L. brasiliense* leaves was similar for both Pb treatments may be due to the fact that the plants actively excreted Pb through the excretory glands of salts potentially present in their leaves (Zurayk et al., 2001; Weis and Weis, 2004).

In conclusion, the presence of multidimensional variable changes (i.e.: leaf shape, aerial biomass, chlorophylls, APx, GPx and SOD activities, and MDA) as a product of different environmental stress measured, indicates that *L. brasiliense* is a useful monitoring tool in environmental and ecotoxicology salt marsh studies, suitable as a source of easy, early and cheap quantifiable biomarkers of stress. Furthermore, the high tolerance and Pb accumulation capacity, mainly in their roots demonstrated by *L. brasiliense* highlighted the potential of this species also as a source for Pb remediation programs.

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