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THE INFLUENCE OF CADMIUM CONTAMINATION AND SALINITY ON THE SURVIVAL, GROWTH AND PHYTOREMEDIATION CAPACITY OF THE SALTMARSH PLANT *SALICORNIA RAMOSISSIMA*

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ABSTRACT

The major aim of this study was to evaluate the capacity of *Salicornia ramosissima* on Cadmium phytoremediation under distinct salinities and, consequently, the toxic effects on the plant's development. A greenhouse experiment was performed, using two Cd concentrations (50 and 100 $\mu g.l^{-1}$) in different salinities (0, 5 and 10). Mortality and weight variation, observed at the end of the experiment, showed significant differences between some treatments, meaning that these variables were affected by the salinity and Cd concentrations. The highest Cd accumulation was detected in the roots, and decreased with the increase of salinity and Cd concentration. *Salicornia ramosissima* is a potential candidate for Cd phytoremediation at salinities close to 0 and its capabilities in Cd phytoaccumulation and phytoestabilization proved to be quite interesting. The optimization of phytoremediation processes by *S. ramosissima* could turn possible the use of this plant in the recovery of contaminated ecosystems.

Keywords: Cadmium, salinity, halophytes, trace metals, phytoremediation, saltmarsh.

1. INTRODUCTION

Pollution is one of the main threats to marine environments and may affect both the biotic and abiotic components of the ecosystem. Research efforts have focused primarily on estuarine and coastal environments as these highly productive and sensitive areas are often directly and most seriously exposed and affected by urban runoff, industrial and agricultural effluents and domestic discharges (Cohen *et al.*, 2001). These ecosystems are often considered sinks for pollutants, especially for metal(loid) pollutants (Doyle and Otte, 1997), whose accumulation can cause severe environmental problems, due to their high degree of toxicity.

Metal(loid)s, present in sediments, pore water and water column, can occur in different forms, depending on many factors, such as redox potential, pH, organic matter and plant species, which may control their bioavailability and toxicity (Ololade and Ologundudu, 2007). Trace metals tend to be absorbed onto colloids suspended in water and removed from the water column into sediments (Monterroso *et al.*, 2003). Sediments in coastal systems may contain high quantities of metals that become available to benthic organisms and eventually become transferred to upper trophic levels, affecting the marine food chains (Warwick *et al*., 1998), or that are remobilized when sediments are dredged and disposed into the water bodies (Monterroso *et al*., 2003).

Cadmium is a non-essential element, recognized as an extremely significant pollutant due to its large solubility in water and high toxicity, persistent to most organisms, being the fourth most toxic to vascular plants (Ghosh and Singh, 2005). Total Cd levels exceeding 8 $mg.kg^{-1}$, or soluble levels exceeding 0.001 $mg.kg^{-1}$, are considered toxic to plants (Kabata-Pendias, 1993). Metal accumulator plants, however, as is the case of the genus *Salicornia* (Sharma *et al*., 2010), are capable of accumulating and tolerating higher pollutant concentrations in their above-ground tissues (Gosh & Singh, 2005). In the aquatic environment, Cd can be present in different physico-chemical forms, depending on abiotic factors, such as salinity, temperature and dissolved organic matter. The free ionic form (Cd^{2+}) is the most bioavailable and consequently more toxic for aquatic organisms, but it can complex with oxides and organic compounds and it is not soluble above pH 7.5.

Cadmium is considered a priority pollutant by the European Community, within the Water Framework Directive (EC, 2001), USA Environmental Protection Agency (USA EPA, 2001), UNEP – United Nations Environment Programme, 2010, being also included in the OSPAR List of Chemicals for Priority Action (OSPAR, 2004). Although it occurs naturally in the environment, Cd concentrations can be largely increased as a consequence of human activities, such as mining, smelting and refining activities. Important sources of Cd input to the marine environment include atmospheric deposition, domestic waste water and industrial discharges (Benavides *et al*., 2005). Cadmium effects can be

observed at both the organism and population levels, but it may also enter in food chains, get biomagnified and pose a potential threat to community and ecosystem health (Sugiyama, 1994 *in* Hu *et al*., 2010). In the case of plants, Cd interferes with the nutrients uptake, transport and the use of water (Benavides *et al*, 2005). The ingestion by humans and animals of contaminated plants can lead to a series of clinical manifestations, such as emphysema of the lungs and destruction of red blood cells (Bowen, 1966; Bryce Smith, 1977 *in* Ololade and Ologundudu, 2007).

The genus *Salicornia* (Chenopodiaceae family), is composed by halophyte and mostly pioneer plants, growing in periodically wet saline coastal or inland habitats, and distributed by Eurasia, North America and South Africa (Teege *et al*., 2011). The interest on these plants for their versatile commercial products is growing, making them promising candidates for the development of novel halophytes as crop species (Lu *et al.,* 2010; Ventura *et al*., 2011). Interest has also aroused in phytoremediation, in the removal of nutrients (Brown *et al.,* 1999) and the accumulation of trace metals (Sharma *et al.,* 2010). Halophyte crops, especially the accumulator type, that can eliminate excess organic compounds, that become toxic, and remove elements (especially Se, Pb, Cr, Cd, Zn, As), petroleum products or radio nucleides via phytoremediation and bioremediation are actually in development. A previous study on the salt tolerance of *Salicornia europaea* L. (1753) showed its great capacity to accumulate Na^+, K^+, Mg^{2+}, and Ca^{2+} in the shoot (Ozawa *et al.,* 2009).

Salicornia ramosissima J. Woods is a common and widespread plant on the European coastline (Davy *et al.*, 2001), namely on the saltmarshes of the Iberian Peninsula (Castroviejo *et al.*, 1990). It usually occupies the higher reaches of the salt marsh, where the salinity is lower, and is a pioneer species in the colonization of the intertidal zones of such habitats (Davy *et al.*, 2001). The optimum growth is at low salinity (Silva *et al.*, 2007), but it can also tolerate high salinity and water potentials (Rubio-Casal *et al*., 2003). These features, coupled with the geographic distribution and the potential for bioremediation of the genus *Salicornia* (see for instance Ozawa *et al*., 2009 and Sharma *et al.,* 2010), make *Salicornia ramosissima* an excellent candidate for phytoremediation.

Salinity is one of the most important environmental parameters that influence the distribution, abundance and physiology of estuarine organisms. Also, in transitional systems, trace metal pollution could only be remediated by using plant species capable of growing in saline conditions. It would, therefore, be beneficial to explore the potential of trace metal tolerance and bioaccumulation by various halotolerant species. Although the life cycle and the population biology of *S. ramosissima* are well known (e.g. Rubio-Casal *et al*., 2003; Silva *et al*., 2007), the trace metal accumulation ability of this plant under distinct salinities has not yet been studied. Experimental essays with the aim of understanding the effects of the interaction between salinity and trace metal contamination on the plants' survival, development and bioaccumulation ability are needed. These

essays, namely with highly toxic elements like cadmium, may contribute for the development of a biotechnological tool capable of reducing the effects of trace metal pollution on salt marsh habitats.

The main objective of this study is to evaluate the capability of *S. ramosissima* in the bioaccumulation of Cd, when submitted to different salinities (0, 5 and 10, simulating distinct natural conditions) and Cd concentrations (0, 50 and 100 $\mu g.l^{-1}$), in a greenhouse experiment. Therefore the following specific objectives are proposed: (i) to analyze the effects of different Cd concentrations and salinities on the plants survival and growth parameters; (ii) to assess the effects of distinct Cd concentrations and salinities in the plant bioaccumulation capacity of Cd; (iii) and to evaluate the plants phytoremediation potential (Transportation Index and Bioaccumulation Factor).

2. MATERIALS AND METHODS

2.1. Sampling procedure (plants)

Green juvenile plants of *S. ramosissima* without a senescent appearance and with similar size (11.0 cm ± 2.141), were collected in June 2011, at low tide, from Óbidos Lagoon (39º24'N, 9º17'W), one of the most extended coastal lagoons in Continental Portugal, with a mean area of 7 km^2 and a mean depth of 3 meters (Costa *et al,* 2009). The lagoon is permanently connected to the Atlantic Ocean and the tides are semidiurnal (tidal range between 0.5 to 4.0 m) extending their influence to the entire lagoon, without pronounced longitudinal variation of salinity or stratification (Malhadas *et al.,* 2009). This lagoon is characterized by two distinct regions, with different hydromorphological and sedimentary characteristics: the lower lagoon and the upper lagoon, which is characterized by low velocities, muddy sediments and high residence time (Malhadas *et al.*, 2009). *Salicornia ramosissima* develops itself on the higher reaches of the upper lagoon, where the salinity is lower. More detailed information on this lagoon can be found elsewhere (e.g. Malhadas *et al.*, 2009).

Plants were carefully washed in lagoon water to remove sediments, placed in plastic buckets, and carried to the laboratory within 30 minutes. During the collection of the plants, a portable multiparameter probe was used to register values of salinity.

At the laboratory, plants were carefully washed again using tap water and then distilled water, to remove slurry, green algae's and other adherent particles. Fresh weights of the plants were registered and their lengths (total, roots and aerial part) were measured. The plants looking healthy and of similar age and size were chosen for the experiment. Some of the plants were used as

reference plants and, therefore, their Cd concentrations were immediately determined, following the same procedures, described later for plants used in the experiment.

2.2. Experiment design

All the glass and plastic materials were washed by immersion in 3% Derquim for 24 h, then in 25% HNO_3 for 24 h and finally rinsed with distilled water and dried. All the standard solutions were daily prepared with ultra-pure water for metal analysis, from stock solutions. All the procedures of the experiment were conducted in a climate-controlled room at the School of Tourism and Maritime Technology, Polytechnic Institute of Leiria (ESTM - IPL), in Peniche (Portugal).

The plants collected were transplanted into perforated plastic containers (3 in each container), containing 320 g (dry weight) of gravel that covered entirely the roots, and functioned as sediment. The gravel was previously washed with 10% hydrochloric acid solution (HCl 37%) for 12 h and burned at 500 ºC, 3 h in a muffle, in order to eliminate the organic matter (Lillebø *et al.*, 2003). Each of those containers (about 8 cm∅) was placed within a bigger plastic container (about 14 cm∅) containing 500 ml of artificial seawater, so that the gravel was completely immersed. The artificial seawater was prepared by dissolving synthetic Sea Salt Tropic Marine ® in aerated ultra-pure water, according to the manufacturer's instruction, set for a salinity of 2 (the same registered on the collection day).

All the containers (total of 54) were placed in a climate-controlled room for an acclimation period of 15 days, with an air temperature of 20 $\pm$ 1 ºC. Artificial lights were used to create a light intensity of 11.5 $\pm$ 12.5 µmol photons.cm^{-2} s^{-1} for a daily light period of 14 h. The containers were watered twice a week with distilled water and nutrient solution, alternately, to replace evapotranspiration losses and ensure the survival of the plants. To prepare the nutrient solution a source of N (620 mg N/l) ($Ca(NO_3)_2.4H_2O$) and a source of P (94 mg P/l) (KH_2PO_4) were used (Lillebø *et al.,* 2003). The plants showing visible stress symptoms (e.g. wilting, chlorosis) were eliminated from the experiment.

After the acclimation period, the water solutions were replaced by treatment solutions to study the effects of salinity on the trace metal uptake of *S. ramosissima*. Three solutions with different salinities were prepared (0, 5 and 10) by adding synthetic the Sea Salt to aerated ultra-pure water. Those salinities were chosen considering the most frequently observed in the Óbidos Lagoon, at the sampling site throughout the year.

To prepare the trace metal treatments, cadmium ($Cd(NO_3)_2.HNO_3$ 0.5 mol/l) was added to each salinized solution, from a 1000 µg.l^{-1} stock solution, in order to produce the final

concentrations of 0, 50 and 100 $\mu g.l^{-1}$. Therefore, 9 different treatments where tested: 3 salinities (0, 5 and 10) x 3 cadmium concentrations (0, 50 and 100 $\mu g.l^{-1}$) (table 1), with the treatments without cadmium (S0Cd0, S5Cd0 and S10Cd0) as the controls since, according to Silva *et al* (2007), the optimum salinity for the development of *S. ramosissima* is between 0 and 11.7. The cadmium concentrations were selected considering the Portuguese legislation (Decree Law 236/98), that states Cd 50 $\mu g.l^{-1}$ as the Maximum Allowed Concentration (MAC) for irrigation waters, Cd 200 $\mu g.l^{-1}$ as the Emission Limit Value (ELV) for residual water discharges, and that some plants are affected when submitted to Cd 100 $\mu g.l^{-1}$. Therefore, the treatment Cd 100 $\mu g.l^{-1}$ was used in order to simulate a discharge with high concentration of cadmium, without risking extreme toxicity for the plants.

For each treatment 3 sets of 3 containers, each containing 3 plants of similar size, number of branches and weight and uniform health, were exposed to 500 ml of treatment solution, in a total of 27 containers, and placed in the climate-controlled room. The volume of the solution in each container was carefully monitored and kept at a constant level during the entire experiment to avoid changes in concentration due to water loss from evapotranspiration. Therefore, the containers were watered following the same method used in the acclimation period.

Throughout the treatments, which ran for one month, the plants were monitored. During the experiment, a few plants were infected with a phytoparasite, whose presence was not detected during the acclimation. Despite not having proceeded to the identification of the phytoparasite, according to Davy *et al.* (2001), larvae of *Coleophora salicorniae* Wocke have been recorded specifically on *S. ramosissima*, boring and feeding off the plant tissues.

The phytoparasites (and their droppings) were removed, whenever possible, without disturbing the plants. Moreover, plants showing signs of advanced senescence, or extensive damage, were eliminated, following the procedure adopted by Rosso *et al.* (2005). After one month of treatment, the plants were washed with distilled water and growth parameters such as fresh length and weight of the plants (total, roots and aerial portions) were measured.

2.3. Samples treatment (plant, water and sediment)

The plants were carefully washed using distilled water, measured and weighted, and the dry weight was determined after 48 h of desiccation in an oven at 80ºC (Ghnaya *et al.*, 2005). Dried roots and aerial parts were separated and individually weighted and then ground to a fine powder using a mortar. The powder was afterwards acid digested by using approximately 0.1 g of dried material with two times 3 ml of 69% nitric acid. The digested samples were dried on a hot plate at 150 ºC, until 1 ml solution remained (Sharma *et al.*, 2010). After cooling, 3 ml of 1% HNO_3 was

added to the samples and filtered. The filtered samples were then diluted with ultra-pure water to make up the final volume of 50 ml. Due to the large number of samples for analyzes (the number of samples matches the number of portions of individual plants), and the impossibility of doing so in a timely manner, without risking the modification of the sample's properties, the filtered and diluted samples were then transferred to 50 ml plastic containers and frozen (- 18 ºC) until analysis.

The treatment solutions were filtered, under vacuum conditions, for the analysis of dissolved and suspended cadmium. For the analysis of dissolved cadmium, the samples of filtered water were acidified (69% HNO_3), to a pH<2, and then transferred to 50 ml plastic containers. For the reasons previously mentioned, the samples were then frozen (-18 ºC) until analyses. Regarding suspended cadmium, the membrane filter of each sample was digested in 6 ml of 69% HNO_3, using a hot plate at 200 ºC. Once more, after the digestion, the samples were filtered, diluted with ultra-pure water to the final volume of 50 ml, and frozen at -18 ºC (EPA Standard procedures).

In order to determine the organic matter, the sediment of all containers was dried in an oven at 60 ºC, during 48h, and then burned at 500 ºC, during 3h in a muffle.

2.4. Cadmium determinations

To determine the cadmium concentrations, each plant portion (root and aerial portion) was analysed individually and the average for each treatment was afterwards calculated. Those analyses were performed by Atomic Absorption Spectrometry (AAS) (Thermo Scientific ICE 3500, Thermo Unicam, Portugal), with graphite furnace (SOLLAR FS95 Furnace autosampler), using a cadmium Hollow cathode lamp, magnesium nitrate as a matrix modifier and Argon. The detection limit of this technique for Cd was 0.03 $\mu g.kg^{-1}$. Standard cadmium solutions were daily prepared for metal analysis, using a cadmium stock solution (Cadmium standard solution, traceable to SRM from NIST Cd $(NO_3)_2$ in HNO 0.5 mol/l 1000 mg/l Cd CertiPUR®, © Merck KGaA, Germany). For each sample aliquot, 20µl of chemical modifier was added. As certified reference plant material was not available, cadmium concentrations were also determined in plants collected at the lagoon, in June 2011, which were immediately digested, to obtain reference values for these plants on natural conditions at the Óbidos Lagoon.

Metal concentrations were determined using the standard addition method and samples were re-analyzed when the correlation coefficient for the calibration of six standards was <0.99. Blank

solutions were prepared for each type of sample, following the respective sample treatment. Three independent replicates of each sample were prepared and analyzed, and, after blank subtraction, mean values and respective standard deviations were calculated.

2.5. Data analysis

After the experiment, the following parameters were calculated for each treatment: mortality (as the complementary parameter of survival), stem elongation (length increment), increases in weight, Cd accumulation in plants (aerial portion and roots), percentage of organic matter in substratum (burned gravel), and dissolved and suspended Cd in each solution of the treatments.

Due to the detection of larvae in some plants during the experiment, the influence of larvae's on the plants mortality was also tested for each treatment, applying the chi-square test, using the MINITAB 12.2 Software package.

To determine if the length increment of the plants during the experiment was correlated with the plants initial length, regression models between the two variables were simulated using the Curve Estimations procedure, with the display of ANOVA results, and the curve model with a better fit was selected, using the SPSS 19.0 Software package.

The transportation index (Ti) gives the leaf/root cadmium concentration and depicts the ability of the plant to the metal species from roots to leaves at different concentrations. This index was calculated for the plants of each treatment, by applying the same equation used by Ghosh and Singh (2005). The phytoremediation potential was also evaluated using the Bioaccumulation Factor (BAF), which corresponds to the ratio of a contaminant concentration in the organism tissues to its concentration in the ambient water, according to the following equation:

$$BAF = C_t/C_W$$

Where BAF is the BAF calculated using empirical data ($l.kg^{-1}$ of tissue); C_t is the concentration of Cd in the roots of plants of each treatment ($mg.kg^{-1}$, dry weight); and C_w is the concentration of dissolved Cd in the water ($mg.l^{-1}$) (USA EPA, 2000).

The organic matter present in the substratum, in which the Cd could bind itself, was determined by applying the equation used by Eleftheriou and McIntyre (2005).

Before performing any kind of statistical analysis, all variables were first tested for normality using the non parametric test Kolmogorov-Smirnov, using the SPSS 19.0 Software package, and transformed whenever necessary (square root transformation for Cd in the suspended matter). When transformations did not remove heterogeneity (Cd accumulation on roots, aerial portion and plant), analyses were performed on the untransformed data since analysis of variance is quite robust to departures from their assumptions (Underwood, 1997).

To test the effects of salinity and cadmium concentrations on mortality, growth parameters (stem elongation and weight variation), cadmium accumulation on *Salicornia ramosissima* (roots and aerial portion), but also on dissolved and suspended cadmium, all these variables were tested for differences between treatments using Two-Way ANOVA's (significance level $\alpha = 0.05$). The significant effects detected were then subjected to post-hoc tests: (i) Tukey HSD and LSD tests to analyse the individual effects of the factors; (ii) Bonferroni tests to analyse the significant interactions between the factors (pairwise comparisons). Statistical analyses were performed using the SPSS 19.0 Software package.

3. RESULTS

3.1 Mortality

All the plants survived to the first fifteen days of the experiment (Figure 1, A to C). At the fifteenth day mortalities were registered at all the treatments, except for S0Cd50, where no deaths occurred during the entire experiment, and for S5Cd100, where dead plants were only observed on the third week (23 days) of treatment.

According to the Two - Way ANOVA results, the exposure of *S. ramosissima* to different salinities and Cd concentrations for one month, had a significant effect on the mortality of the plants, with significant differences observed between the treatments S0Cd0 and S0Cd50 (table 2). Although a uniform pattern in the percentage of mortality for the different treatments was not observed (table 3), mortality was highest on the treatments S0Cd0 and S10Cd50 (89% for both) and for the treatment S5Cd50 (78%). These three treatments presented also a high occurrence of larvae (table 3). Considering the concentration 0 µg $Cd.l^{-1}$, mortality decreased with the increase of salinity, while the opposite was observed for the concentration 50 µg $Cd.l^{-1}$. For the concentration 100 µg $Cd.l^{-1}$, salinity 5 presented the lowest mortality, with a value of 33% (table 3).

The presence of the larvae, detected during the experiment, had no effect on the mortality of the plants subjected to two of the three treatments with salinity 5 (χ^2 = 1.102, p = 0.294, to 0 µg $Cd.l^{-1}$; χ^2 = 0.225, p = 0,635, to 100 µg $Cd.l^{-1}$). Also for the treatments S0Cd50 and S10Cd0 µg, the test was not applied, since all the plants survived the experiment in the case of the first treatment and plants with larvae were not observed in the second treatment (table 3). As for the other treatments and according to the software, the chi-square approximation was most probably invalid or did not apply.

3.2. Growth parameters

Comparing the initial and final lengths of the aerial portion of the plants during one month of treatment, the length increased in all treatments resulting in stem elongation (Figure 2A). For the salinities 0 and 10, the growth was smallest at the highest Cd concentration (100 $\mu g.l^{-1}$).

In general, *S. ramosissima* grew more when treated with the lowest Cd concentrations (0 and 50 $\mu g\ Cd.l^{-1}$). In the treatments where no cadmium was added, the mean stem elongation reached to 4.25, 3.34 and 3.31 cm, at the salinities 0, 5 and 10, respectively (Figure 2A); mean stem elongation was only 1.27 and 1.31 cm, in the treatments S0Cd100 and S10Cd100, respectively. However, according to the Two-Way ANOVA results, significant differences in stem elongation between the treatments were not observed (factor salinity p =0.966; factor cadmium concentrations p = 0.141; interaction between the factors p = 0.771).

To check the influence of the initial length in the growth of the plants, the length increment of the aerial portion during the experiment was correlated with its initial length. Due to the number of dead plants observed in some treatments during the experiment, only the treatments with a minimum of 5 surviving plants were tested for this correlation (S5Cd0, S5Cd100 and S10Cd100). Although a negative correlation was observed between the two variables for the tested treatments, the associated ANOVA results were not significant ($p > 0.05$). Considering all the plants involved in the experiment, significant results were achieved (p = 0.006) and the cubic model presented the best fit (r =0.548; n = 38), according to the following equation:

$$\text{Length increment} = -0.038\,(\text{initial length})^3 + 1.001\,(\text{initial length})^2 - 8.807\,(\text{initial length}) + 28.295$$

Contrarily to what was observed for the length, the weight of the plants decreased during the month of experiment, for all treatments, except in S0Cd50 and S10Cd0 (Figure 2B). However the greatest loss occurred in the treatments with the highest salinity and contaminated with Cd, S10Cd50 (1.23 g) and S10Cd100 (1.27 g) (Figure 2B). Salinity and Cd concentration influenced the weight variations, exhibiting statistical significance when the Two-Way ANOVA was performed (p = 0.00) (table 2).The application of the Bonferroni test showed differences between the treatments 0 and 50 $\mu g\ Cd.l^{-1}$ (p = 0.000) and 0 and 100 $\mu g\ Cd.l^{-1}$ (p = 0.000), on salinity 10.

The organic matter present in the sediment where plants were developing during the experiment was negligible, with the highest value of 0.49% in the treatment S10Cd0 and the lowest value of 0.28% in S5Cd50 (total data not shown).

3.3. Cadmium accumulation

At the end of the experiment the Cd accumulated both in roots and aerial portions generally decreased with the increase of salinity, especially on the roots of the plants treated with the highest Cd concentration (Figure 3, A and B; Table 3). However the cadmium accumulated in the roots of the plants submitted to the cadmium solutions was always higher when compared with the results obtained for the roots of the reference plants (with a mean value of 7.50 ± 1.59 mg $Cd.kg^{-1}$). Roots accumulated more Cd when submitted to the treatment S0Cd100, with a mean value of 85.94 mg $Cd.kg^{-1}$. In the case of the aerial portions, the Cd accumulation was similar to the values recorded for the reference plants (1.45 ± 1.18 mg $Cd.kg^{-1}$), except in the treatment S0Cd50, ascertaining a mean concentration of 4.61 mg Cd. kg^{-1}. Still, salinity and Cd concentration have not influenced the Cd accumulation on plants, since no statistically significant differences were detected when the Two-Way ANOVA was performed ($p > 0.05$).

The Cd accumulation in the roots exceeded that of the aerial portions, for all treatments. In fact, in most treatments Cd concentration in aerial portions was not detectable. Maximum transport was observed at S0Cd50, with a maximum Ti that reached 21.6%. For the other treatments it was less than 2.2% (Figure 4A). The Bioaccumulation Factor assumed higher values at salinity 0, with the maximum reached at the treatment S0Cd100 (955.0 $l.kg^{-1}$), while the minimum value (188.9 $l.kg^{-1}$) was recorded at the highest salinity and Cd concentration, excluding the treatments without Cd, where dissolved Cd in water was not detectable. In fact, BAF decreased with the increase of salinity and concentration of Cd, except in the treatment with no salinity (Figure 4B).

In general, at the end of the experiment, the amount of Cd dissolved in the solution treatment was higher compared to the Cd associated with the suspended matter in these solutions, except for the treatments S0Cd0 and S0Cd50 where the opposite was observed (Figure 5). Salinity 0, with 50 μg $Cd.l^{-1}$ was the treatment with the highest Cd suspended concentration, with a mean value of 0.11 mg $Cd.l^{-1}$, followed by the treatment S5Cd100, which presented a mean value of 0.04 mg $Cd.l^{-1}$, while the other treatments presented mean values in general below 0.03 mg $Cd.l^{-1}$. For the treatments with 0 μg $Cd.l^{-1}$, the values were close to 0 mg $Cd.l^{-1}$ (0.01 mg $Cd.l^{-1}$ in salinity 0) or even not detected (salinity 5 and 10). Regarding the Cd dissolved, the highest mean value, 0.12 mg $Cd.l^{-1}$, was detected at S5Cd100 μg $Cd.l^{-1}$, while at S0Cd0, S5Cd0 and S10Cd0, Cd was not detected. Although there were no statistically significant differences on the influence of salinity and Cd concentrations in suspended Cd results, significant differences between treatments with distinct Cd concentrations were observed for the dissolved Cd when the Two-Way ANOVA was performed (p=0.001). Post-hoc tests consistently revealed differences between the treatments 0 and 50 μg $Cd.l^{-1}$ and between 0 and 100 μg $Cd.l^{-1}$ (table 2).

4. DISCUSSION

In the present study the main objective was to check the capacity of *Salicornia ramosissima* for phytoremediation of Cadmium, when exposed to different salinities and Cd concentrations.

The chi-square tests proved that the parasite larvae had no effect on the mortality of the plants subjected to two of the three treatments with salinity 5 (0 and 100 μg $Cd.l^{-1}$). Also for the treatments S0Cd50 (no presence of larvae), and S10Cd0 (about 78% of plants with larvae, but all of these plants survived to the experiment) the effect of the larvae on plants mortality is excluded. In all the treatments mentioned above, the mortalities found during the experiment, were the lowest observed, corresponding to mortalities inferior to 50%, with emphasis on the treatment S0Cd50 where the survival of the plants was 100%. For the remaining treatments, and even though the chi-square test was not conclusive, the mortality observed was, almost entirely, superior to 50% (S0Cd0, S0Cd100, S5Cd50, S10Cd50 and S10Cd100, with a mortality of 89%, 56%, 78%, 89% and 45%, respectively). Therefore it is not possible to exclude some influence of the larvae on the mortality of the plants, especially on the treatments: (i) S0Cd100; (ii) S5Cd50 and (iii) S10Cd50, since the incidence of the larvae was higher than 75% in these treatments. Also, it is our belief that the high incidence of larvae registered on the treatment S0Cd0 was determinant for the death of these plants, causing the high mortality observed in this control treatment, contrarily to our expectations, since the conditions of this treatment are among the most favorable for the development of *S. ramosissima* (see for instance Silva *et al*., 2007).

The plants used in the present study were collected from a natural population at the Óbidos Lagoon. Although the degree of tolerance to Cd of this population is not known (no published data exist) and, to our knowledge, a comparison study with plants obtained from an uncontaminated site is not available, the present study allowed drawing some relevant conclusions. Stem elongation seems to be a parameter more tolerant to Cd and salinity, when compared with weight variation, since plants showed some growth at all treatments. Although the mean values of stem elongation observed in the final of the experiment were not consistent between the treatments neither significantly different, the lowest values were registered in highest Cd concentrations, demonstrating the detrimental effect of this trace metal on plant development. Indeed, Cd has been found to cause reduction in photosynthesis and cell membrane damage (Rosso *et al.*, 2005), which might have repercussions in the plants growth parameters.

Considering only the treatments without Cd, the stem elongation observed was similar to those observed by Silva *et al*. (2007), even though in the referred study, experiment duration has

been longer. As for the effect of the increase of salinity on its own, i.e. in the absence of Cd, stem elongation tends to decrease. This is in agreement with the findings of Silva *et al.* (2007) on the effect of salinity on the growth of *S. ramosissima*, which states that some halophytes develop better under non-saline conditions, despite of their salinity tolerance.

Regarding the negative relation observed between the initial length of the plants and the growth increment, higher rates of plant growth on juvenile plants might explain these results, since their own metabolism and therefore the ability to absorb nutrients is more intense in the growth phase.

According to Vassilev *et al.* (1998) Cd conducts to a decrease in turgor potential and cell wall elasticity, which, according to Ghnaya *et al.* (2005), might result in a smaller size of leaf cells formed with smaller intercellular space area. The decrease of weight observed in *S. ramosissima*, after one month of exposure to almost all treatments considered in this study, may support the depressive action of Cd on cellular turgor. In fact, plants exposed to the higher Cd concentrations, on salinity 10, showed a substantial loss of weight, significantly different when compared with 0 μg $Cd.l^{-1}$. Salinity, in turn, may decrease biomass production, i.e. affecting weight and elongation, because it causes a lowering of plant water potentials, specific ion toxicities, or ionic imbalances (Neumann, 2001). Although *S. ramosissima* is a halophyte that develops well in low or moderate salinity (Silva et al., 2007) and despite the salinities used in this study, the synergistic effect, once more, was involved.

This study showed that Cd accumulation in roots by *S. ramosissima* generally decreased with increasing salinity, especially on the treatments with the highest Cd concentrations. The likely reason is the decreased availability of Cd in the growth medium because of the complexes formed between chloride and metals (Förstner, 1979). Plants uptake Cd into the cells mainly in the form of Cd^{+2}. The complexation of Cd^{+2} and Cl^{-} may cause the decrease in Cd concentration in plant at higher concentrations of NaCl (Ozawa *et al.*, 2009). This has been shown to depress Cd uptake in *Salicornia europaea* (Ozawa *et al.*, 2009) and in *Potamogeton pectinatus* L., *Elodea canadensis* Rich. and *Potamogeton natans* L. (Fritioff *et al.*, 2005). In addition, increasing competition with sodium ions at uptake sites, both on the plasma membrane and in apparent free space in the cell walls, may account for the decreased Cd accumulation at higher salinities (Noraho and Gaur, 1995).

The uptake of essential elements may increase during the growth of the plant and their concentration may be higher at the plant mature stage. However, Cd is not an essential element, being toxic to plants. Nevertheless, toxic metals are thought to enter root cells by means of the same uptake processes that move essential micronutrient metal ions (Ross and Kaye, 1994). For instance,

a competitive transport of Cd via voltage-gated cation (like Ca^{2+}) channels has been pointed out as a way of Cd absorption by roots (Raskin *et al.*, 1997).

Plants have a range of different mechanisms for protecting themselves against the uptake of toxic elements and for restricting their transport within the plant (Almeida *et al*., 2006). These mechanisms include the sub-cellular compartmentalization of the metal, namely in vacuoles, and the sequestration of the metal by specially produced organic compounds, like phytochelatins, concentrating metal in the plants roots (Ross and Kaye, 1994). This could explain the larger bioaccumulation of Cd on roots of *S. ramosissima* for all treatments, with emphasis on the value of 85.95 mg $Cd.kg^{-1}$ root dry weight on plants submitted to S0Cd100. Unexpectedly, the highest Cd accumulation on aerial portion, corresponding to a mean value of 4.61 mg $Cd.kg^{-1}$, was observed in S0Cd50, which does not match with the treatments where the highest or the lowest values of Cd accumulation on the roots were detected. An explanation for this result is not easy; although it could be related with the accumulation of Cd in the Óbidos lagoon by plants, the Cd analyses on the reference plants showed lowest values (an average of 2.18 mg $Cd.kg^{-1}$) and moreover that would also have been observed in the other treatments, which in fact did not happen; another possible explanation could be related with the transport of Cd from the roots to the aerial portion, which in this treatment, under those conditions, was much more efficient.

For most of the treatments it was not possible to determine the accumulation of Cd in the aerial portions, since the concentration was below the detection limit of the recording equipment used. However, the maximum transport was observed on the treatment S0Cd50, meaning that, under the scope of the salinities here tested, the salinity 0 might be the ideal one for the development of the plant, therefore, promoting translocation of cadmium in the plant. Although it was not possible to compare the BAF values obtained for *S. ramosissima* with the values of other species, salinity conditions close to 0 also seem to promote the ability of the plant to accumulate Cd, as shown in the higher BAF values registered in treatments with salinity 0.

The differential accumulation of cadmium in the roots of *S. ramosissima* and the BAF values, observed in the present study, decreases the possibility of this metal getting into the trophic webs of the ecosystem. This capacity might decrease the availability of this trace metal for the animals that feed on these plants, preventing the transfer of cadmium to the upper trophic levels. In fact, accumulation in fruits, seeds, and leaves typically creates more exposure than accumulation in roots. In this scenario, the potential of *S. ramosissima* in phytoremediation of this trace metal becomes even more relevant, considering its application in revegetation of contaminated soils and/or waters and in phytostabilization techniques.

The presence of Cd in the suspended matter presented the highest value in the treatment S0Cd50, with 0.11 mg $Cd.l^{-1}$ that correspond to 27731.97 mg $Cd.kg^{-1}$ of suspended matter. This value could be related to the development of microorganisms in the treatment solution that accumulated Cd. For the other treatments (e.g. S5Cd100 and S10Cd50) where Cd in the suspended matter assumed substantial values, the same explanation is plausible.

In regard to the dissolved Cd, it was observed that some treatments increased their Cd concentration, namely S5Cd50, S5Cd100 and S10Cd50. Without excluding the possibility that contamination has occurred (which the probability is very low, given the care taken throughout the experiment), the most plausible explanation is the release of Cd from organic matter in decomposition (Weis and Weis, 2004), deriving of any small amounts of the plants, larvae or their droppings, which might have fallen to the solution. This might have been the case for instance in the treatment S10Cd50, where a high mortality of the plants was observed (89%), and explain the similarities observed in the dissolved Cd quantifications of the treatment solution S10Cd50 and S10Cd100.

5. CONCLUSIONS

Although the comparison of the results obtained in the different treatments for each measured growth parameter did not permit to establish a common pattern of the observed variations, the analysis of the data leads to the conclusion that *S. ramosissima* develops best under non saline conditions. The same could be assumed for the Cd concentrations, which represent a stress factor for the development of the plant.

The Cd bioaccumulation capacity of *S. ramosissima* generally decreased with the increase of salinity, especially on the roots of the plants treated with the highest Cd concentration. Regarding the Cd compartmentation within the plant, the Cd accumulation occurs especially in the roots, where the concentration largely exceeds the accumulation detected in the aerial portions.

Knowing that the natural conditions of ecosystems are not possible to simulate in the laboratory, further trials will be needed, especially in the field, thereby confirming the behavior of the plant in its natural environment. However, based on these results, it can be assumed that this particular wetland species may be successfully used for phytoremediation, namely on phytoaccumulation and phytostabilization, since plants have a considerable bioaccumulation potential and were able to bioaccumulate Cd mainly in the roots, acting like a sink for this metal and preventing it from becoming available to other organisms. Even so, it should not be forgotten that the performance of this plant is more efficient when submitted to low salinities, what should be

taken into account while choosing suitable conditions during wetland system construction and management.

Considering the foregoing, there is still plenty to be known in the interaction of salt marsh organisms and their potential for biorremediation, where natural native organisms in a given ecosystem are under the complex influence of abiotic and biotic factors, and this study provides a promising start.

6. AKNOWLEDGEMENTS

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8. LEGENDS OF FIGURES

Figure 1: Variations in the percentage of mortality of *S. ramosissima* exposed to different salinities and Cd concentrations: (A) – Comparison between distinct Cd treatments, at salinity 0, over one month of treatment; (B) – Comparison between distinct Cd treatments, at salinity 5, over one month of treatment; (C) – Comparison between distinct Cd treatments, at salinity 10, over one month of treatment. Calculated for $n=9$ for each treatment.

Figure 2: Comparison of the growth parameters of *S. ramosissima* exposed to different salinities and Cd concentrations, after one month of treatment. (A) - Mean stem elongation (± standard error); (B) mean weight variation (± standard error). Calculations using the surviving plants of each treatment.

628 **Figure 3**: Final concentrations (averages) (± standard errors) of Cd in *S. ramosissima* roots (A) and
629 aerial portion (B) treated for one month with Cd concentrations of 0, 50 and 100 $\mu g.l^{-1}$ and salinities
630 of 0, 5 and 10. Calculations using the surviving plants of each treatment.
631
632 **Figure 4:** Leaf/root cadmium concentration index (Ti) (A) and comparative cadmium
633 Bioaccumulation Factor (BAF) (B), at the end of the experiment.
634
635 **Figure 5**: Mean suspended Cd (± standard errors) (A) and mean dissolved Cd (± standard errors)
636 in the treatment solutions (Cd concentrations of 0, 50 and 100 $\mu g.l^{-1}$ and salinities of 0, 5 and 10),
637 after one month of treatment.

Table 1

Experiment design: identification of the different treatments.

		Salinity		
		0	5	10
Cd concentrations ($\mu g.l^{-1}$)	0	S0Cd0	S5Cd0	S10Cd0
	50	S0Cd50	S5Cd50	S10Cd50
	100	S0Cd100	S5Cd100	S10Cd100

Table 2

ANOVA and post-hoc tests results for the mortality, growth parameters and Cd accumulation, considering the effects of salinity (0, 5 and 10) and Cd concentrations (0, 50 and 100 $\mu g.l^{-1}$) as factors. Only the variables that presented significant results are represented ($p<0.05$). *df* – degrees of freedom; MS – Mean Square.

ANOVA				
Source of variation	*df*	*MS*	F -statistic	*p*-value
Mortality				
Salinity x Cd conc.	4	3.704	3.226	0.037
Weight variation				
Salinity x Cd conc.	4	0.908	14.185	0.000
Dissolved Cd				
Cd conc.	2	140653.590	10.108	0.001

Post-hoc tests			
Dependent variable and factors tested	Test	Condition	*p*-value
Mortality Interaction: salinity x Cd conc.	Bonferroni	Salinity 0 Comparison: 0µg $Cd.l^{-1}$and 50µg $Cd.l^{-1}$	0.047
Weight variation Interaction: salinity x Cd conc.	Bonferroni	Salinity 10 Comparison: 0µg $Cd.l^{-1}$and 50µg $Cd.l^{-1}$ Comparison: 0µg $Cd.l^{-1}$and 100µg $Cd.l^{-1}$	0.000 0.000
Dissolved Cd Cd concentration	Tukey HSD	Comparison: 0µg $Cd.l^{-1}$and 50µg $Cd.l^{-1}$ Comparison: 0µg $Cd.l^{-1}$and 100µg $Cd.l^{-1}$	0.001 0.034
	LSD	Comparison: 0µg $Cd.l^{-1}$and 50µg $Cd.l^{-1}$ Comparison: 0µg $Cd.l^{-1}$and 100µg $Cd.l^{-1}$	0.000 0.013

Table 3

Mortality[a], larvae occurrence[b] and concentration[c] of Cd observed in the tissues of the surviving plants of *S. ramosissima*, after one month of treatment with different salinities and Cd concentrations.

Treatment	Mortality	Larvae occurrence (%)	Roots ($mg.kg^{-1}$ dry weight)	Aerial portions ($mg.kg^{-1}$ dry weight)
S0Cd0	**8**	**67**	*****	*****
S0Cd50	**0[d]**	**0**	**21.31 (18.19)**	**4.61 (3.90)**
S0Cd100	**5**	**78**	**85.95 (62.69)**	*****
S5Cd0	**4[d]**	**33**	**4.19 (3.24)**	*****
S5Cd50	**7**	**78**	**38.45 (27.19)**	*****
S5Cd100	**3[d]**	**55**	**64.86 (59.21)**	**1.40 (1.28)**
S10Cd0	**3[d]**	**0**	*****	*****
S10Cd50	**8**	**78**	**22.18 (0)**	*****
S10Cd100	**4**	**67**	**18.89 (16.89)**	**0.30 (0.26)**

* Value below the detection limit (0.03 µg/kg).

[a] Total dead plants in the end of each treatment, n=9. [b] Calculated for n=9 plants in each treatment. [c] Mean values (standard error). [d] Treatments where the larvae had no effect on the mortality of the plants.

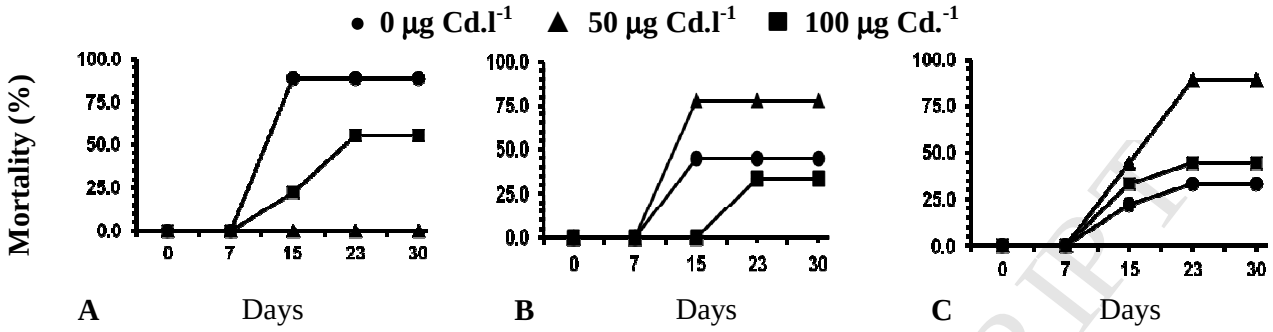

Figure 1

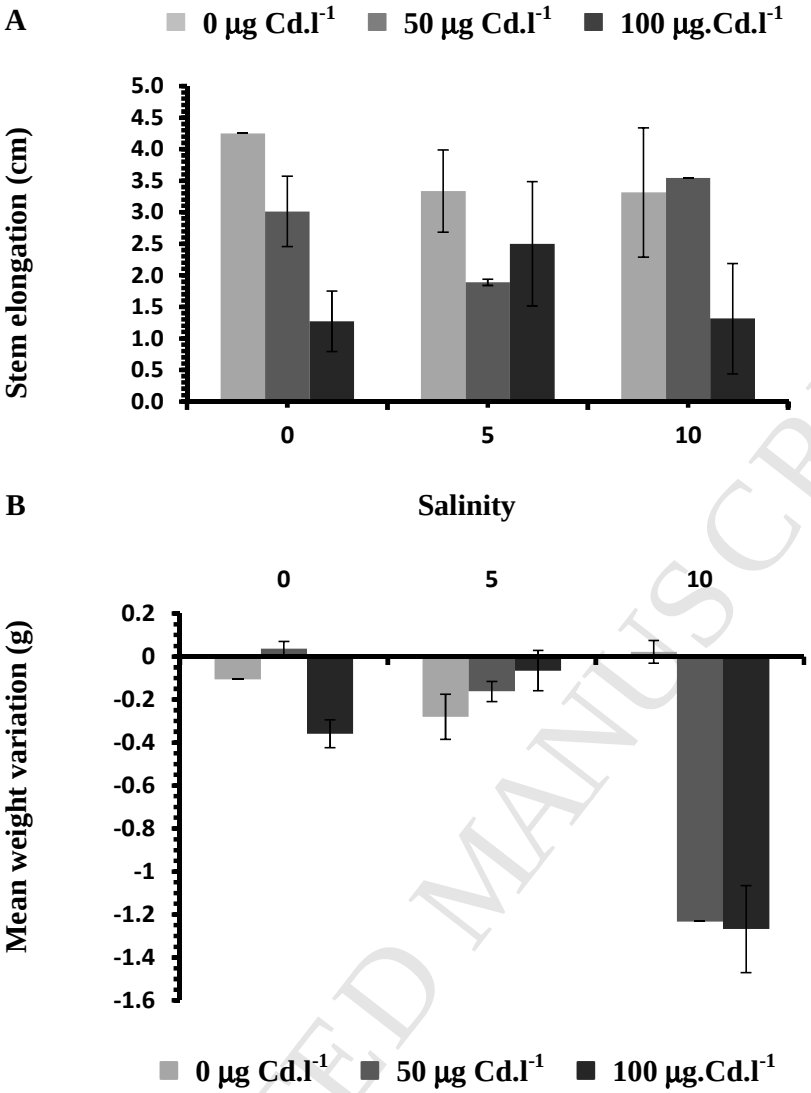

Figure 2

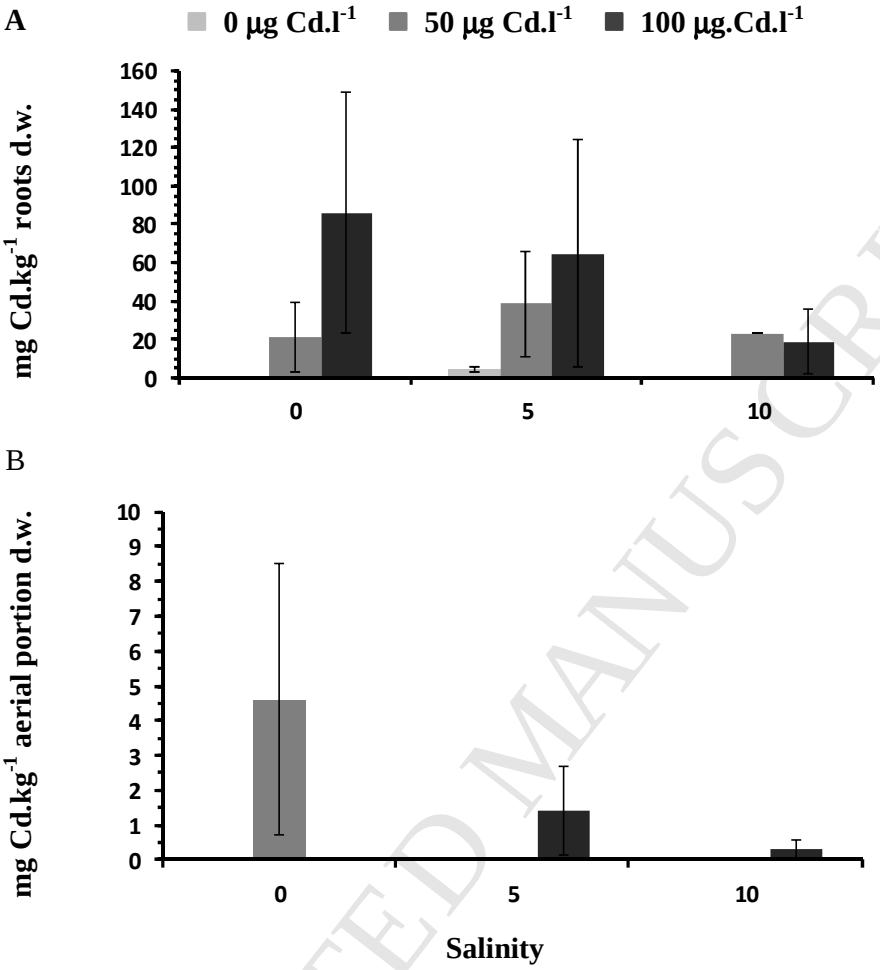

Figure 3

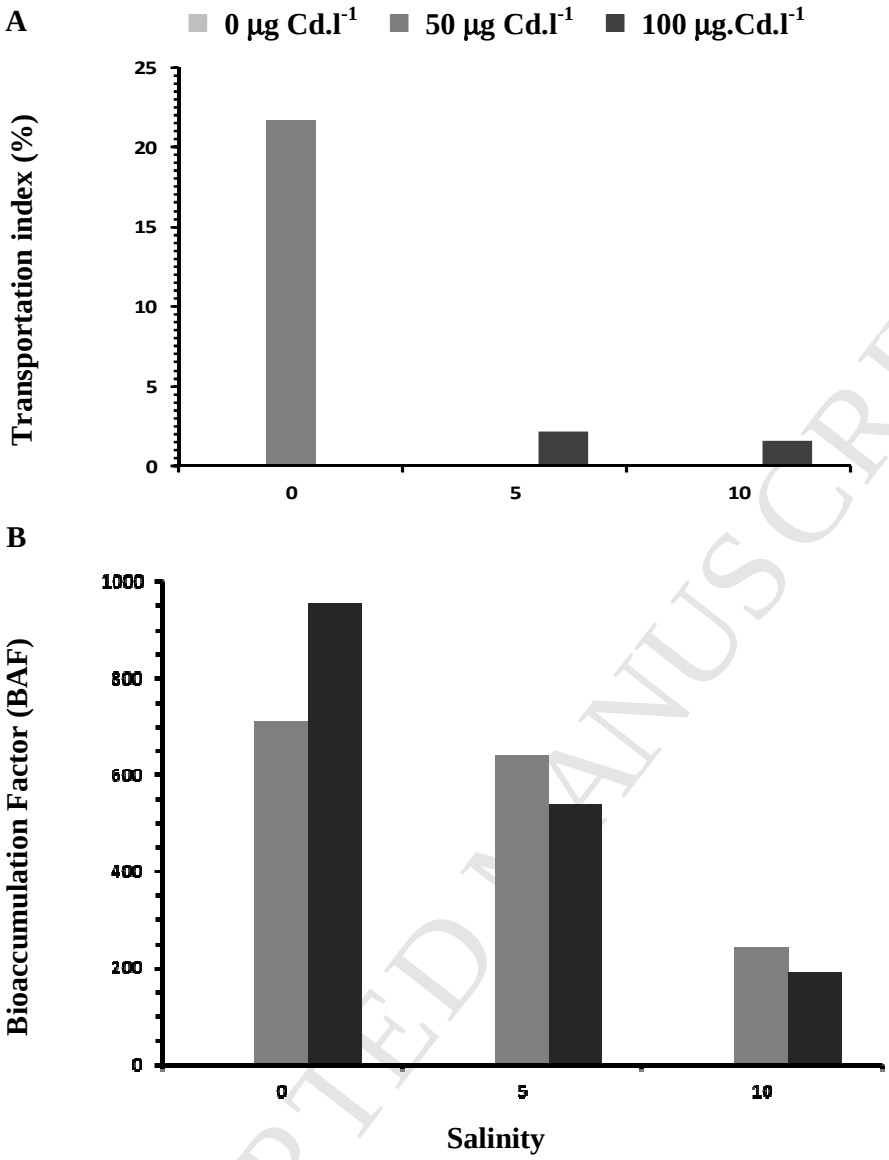

Figure 4

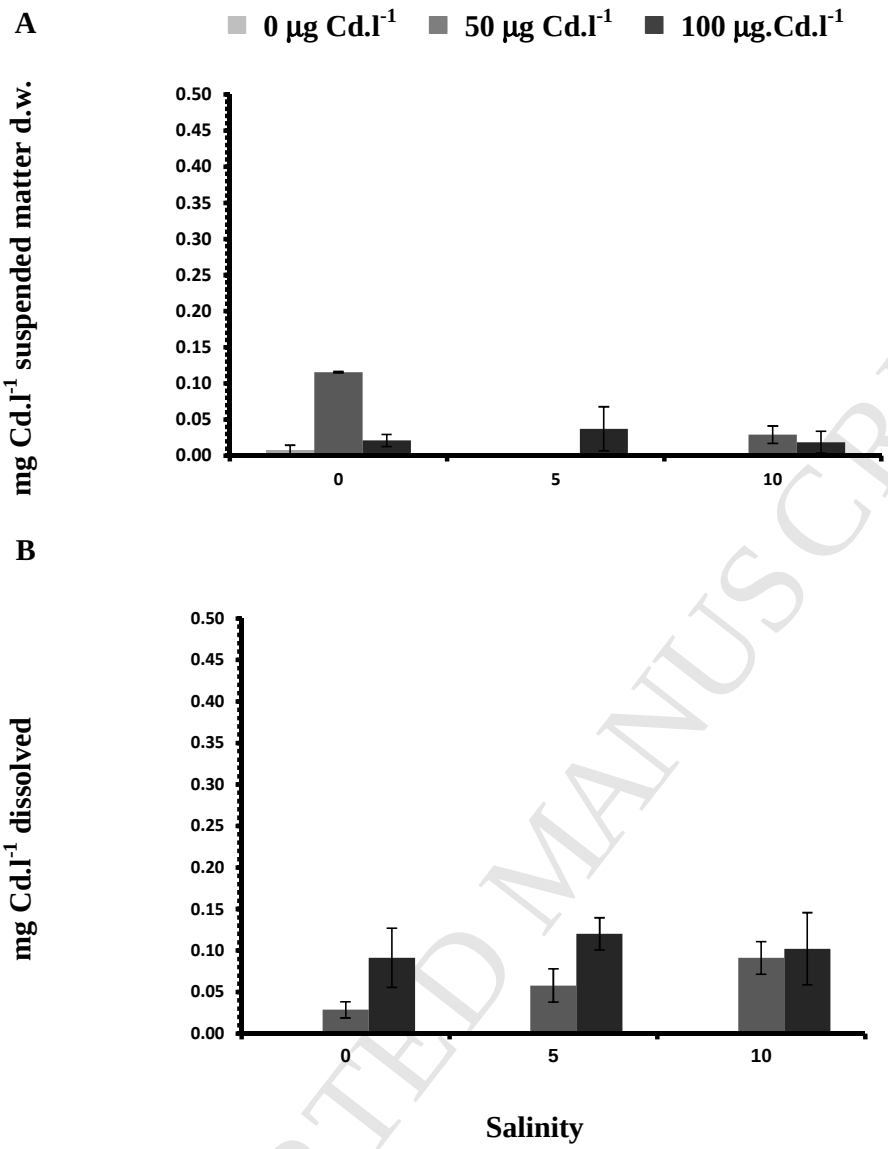

Figure 5

Highlights

- An experiment, using 2 Cd concentrations at different salinities, was performed.
- The capacity of *Salicornia ramosissima* on Cd phytoremediation was evaluated.
- Salinity and Cd affected the plants survival, growth and bioaccumulation capacity.
- Cd bioaccumulation decreased with the increase of salinity and Cd concentration.
- *Salicornia ramosissima* accumulated Cd mainly on the roots.