

***How do salinity and heavy metal contamination affect
Salicornia ramosissima and the Cadmium accumulation
capacity?***

Carmen Alexandra Costa Pedro

[2011]



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Dissertation submitted to obtain the degree of Master in Biotechnology of Marine Resources.

Dissertation of Master performed under the orientation of Doctor Sílvia Gonçalves and co-orientation of Doctor Susana Ferreira.

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School of Tourism and Maritime Technology

Polytechnic Institute of Leiria

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This thesis is dedicated to my parents, my brother
and friends

Resumo

Este estudo teve como principal objectivo analisar a capacidade de fitoremediação de cádmio pela halófito *Salicornia ramosissima*, presente na Lagoa de Óbidos (Portugal), quando sujeita a diferentes salinidades e concentrações de Cádmio (Cd), durante um mês. Duas concentrações de Cd foram testadas (50 e 100 $\mu\text{g l}^{-1}$) para avaliar a capacidade da planta para acumular cádmio, além dos efeitos tóxicos que este metal pesado possa ter no desenvolvimento da própria planta. Os níveis de contaminação foram testados em diferentes condições de salinidade da água (valores iguais a 0.0, 5.0 e 10.0), considerando as condições a que esta planta normalmente está sujeita no seu ambiente natural.

A mortalidade de *S. ramosissima*, assim como os parâmetros de crescimento analisados (comprimento e peso) não apresentaram diferenças significativas entre os tratamentos, o quer dizer que não foram afetados por qualquer um desses fatores, nem pela interação de ambos. No entanto, tanto o incremento do comprimento da planta como a redução de biomassa, verificados no final da experiência, poderão ter sido induzidos pelo aumento da salinidade e concentração de Cd da água. As plantas apresentaram uma maior concentração de Cd nas raízes, do que na parte aérea. A acumulação de cádmio nas raízes diminuiu com o aumento da salinidade e concentração de Cd, ainda que estatisticamente não se tenham detectado diferenças entre os tratamentos.

Houve desenvolvimento de biofilme nos diversos tratamentos, salientando-se o de salinidade 0 x 50 $\mu\text{g Cd.l}^{-1}$, onde os microrganismos e eventual matéria orgânica (da própria planta) em suspensão revelaram uma maior quantidade de Cd relativamente aos valores iniciais presentes na água. A concentração de Cd dissolvido na água, determinada no final da experiência, esteve em acordo com os valores de contaminação iniciais escolhidos para este estudo. No entanto verificou-se algum aumento de Cd, possivelmente em resultado da degradação de tecidos das plantas mortas com consequente libertação de Cd para a água.

A planta *S. ramosissima* é uma potencial candidata para a fitoremediação de Cd, em habitats de salinidades próximas de 0, onde o seu desenvolvimento é máximo, bem como a sua capacidade de bioacumular Cd. A optimização de processos de fitoremediação com *S. ramosissima* pode auxiliar na bioremediação de ecossistemas de água doce.

Palavras-chave: fitoremediação, halófitas, metais pesados, cádmio, sapal

Abstract

The major aim of this study was to evaluate the capacity of *Salicornia ramosissima*, a halophyte present in Óbidos lagoon (Portugal), on Cadmium (Cd) phytoremediation, when submitted to different salinities and Cd concentrations, for a month. Two Cd concentrations were used to evaluate this capacity and, consequently, the toxic effect of Cd on the plant development. The contamination levels were tested in different salinities (0, 5 and 10), taking into consideration the natural conditions that *S. ramosissima* is subject in its natural environment.

The mortality registered in the end of the experiment, as the growth parameters measured (length and weight), did not showed significantly differences between the treatments, which means were not affected for the salinity or Cd concentrations, neither for the interaction of those two stress factors. However, length elongation and the variation of weight, observed in the end of the experiment, may have been induced by the increase of salinity and Cd concentration. A highest Cd accumulation was detected in the roots of *S. ramosissima*, compared with the aerial portion. The Cd accumulation in roots decreased with the increase of salinity and Cd concentration, even though statistically there were no significant differences between treatments.

In all treatments, a biofilm was form, highlighting treatment salinity 0 x 50 $\mu\text{g Cd.l}^{-1}$, where microorganisms and, eventually, organic matter (from the plant), suspended revealed a higher Cd amount relatively to the water initial Cd concentrations. The dissolved Cd concentration, detected in the final of the experiment, was in accordance with the initial contamination levels used in this study; however an increase was observed for some treatments, possible as a result of decomposition of organic matter and consequent release of Cd to the water.

Salicornia ramosissima is a potential candidate for Cd phytoremediation at salinities close to 0, where development is maximum and, consequently, is Cd accumulation capacity. The optimization of phytoremediation processes by *S. ramosissima* could turn possible the use of this plant in freshwater ecosystems.

Key words: phytoremediation, halophytes, heavy metals, Cadmium, salt marsh

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1. Introduction

1.1. Biotechnology and environmental preservation

Environmental impacts in marine environments

As the human population has grown in size, human activities and technological capabilities have intruded in one way or another into the dynamics of most ecosystems. Even where we have not completely destroyed a natural system, our actions have disrupted the trophic structure, energy flow, and chemical cycling of ecosystems in most areas of the world (Campbell and Reece, 2002). The sustainability of the planet Earth can only be guaranteed by ensuring the sustainable management of natural resources and minimum environmental impact.

The fast increase of human population and activities during the last century led to an increment of environmental impact, with the pollution assuming the primary role. Industrialization in Europe and elsewhere caused the increase of production, use and release of contaminants into the environment. A large number of contaminants, particularly those structurally related to natural compounds, are readily degraded or removed by microorganisms found in soil and water. However, superimposed on the wide variety of pollutants present in the environment is an increase on the number of compounds rarely found in nature or in small amounts, namely heavy metals. These compounds are usually removed slowly and tend to accumulate in the environment. Due to the high degree of toxicity, their accumulation can cause severe environmental problems (Benavides *et al.*, 2005). Pollution, which may affect both the biotic and abiotic components of the ecosystem, is therefore one of the main threats to the marine environments.

Most marine pollution occurs in coastal regions but often originates from terrestrial sources. Industrialization, urbanization, effluent disposal, intensive agriculture, aquaculture and coastal engineering place increased demands in marine ecosystems, namely in salt marshes (Malhadas *et al.*, 2009). The ocean burden of heavy metals and different kinds of pollution is now a serious environmental concern and public interest regarding this issue has been increasing. Research efforts have focused primarily on salt marshes, estuaries and coastal environments since these highly productive and sensitive areas are often directly and most seriously affected and exposed to this problem because of their proximity to sources of pollution (Cohen *et al.*, 2001) and are often considered sinks for pollutants, especially for metal pollutants (Doyle and Otte, 1997).

Rivers and rain carry many agricultural products, such as fertilizers, pesticides, herbicides, and other industrial wastes – both inorganic (heavy metals) and organic (xenobiotics) – to coastal regions. Periodical tidal flooding of salt marshes provides large quantities of these pollutants to the marsh ecosystem. This will cause physical and chemical changes to the quality of the receiving waters. These changes may include increased dissolved nutrients which may result in eutrophication, changes in stream temperatures and bottom characteristics which lead to habitat destruction and alteration of species diversity and the addition of toxic substances which can have either acute or chronic effects on aquatic organisms (Sanders, 1997).

Although wetlands contribute to ameliorate the quality of surface waters, the increase of the pollutants supply can cause deterioration of water quality and eutrophication problems. Coastal lagoons are particularly vulnerable to these problems, since they are subjected to little turbulent mixing and restricted exchange with the adjacent ocean and, thus may accumulate pollutants supplied by the surrounding watershed. The European Environmental Agency calls such zones physically sensitive areas (Newton *et al.*, 2003).

Salt marsh as habitats

Coastal lagoons, that occupy about 13% of the world coastline, are semienclosed water bodies, characterized by small river flows and by the influence of tides. These systems are typically shallow, with high salinities, and are separated from the ocean by sandbars or barrier islands. They are connected to the ocean by constricted inlets, which may be seasonally closed (Fortunato and Oliveira, 2007). These environments are subject to rapid morphological evolution and constant change of hydrodynamic and sedimentary conditions, and are influenced by a number of factors. For example, salinity which in turn depend on a great variety of conditions, like the extent and frequency of tidal flooding, evapotranspiration, and vegetation (Vernberg, 1993 *in* Silva *et al.*, 2007), may change from freshwater conditions after heavy rains to hypersaline after prolonged evaporation periods (Liebezeit *et al.*, 1999). These productive ecosystems, are among the most productive and valuable aquatic ecosystems on earth, however their productivity is affected by several inter-related parameters, such as the tidal pattern (flooding frequency

and duration), salinity, temperature, nutrient availability, oxygen levels and sediment type (Ibañez *et al.*, 2000).

As transitional ecosystems between marine and continental waters, lagoons present a mosaic of different features, which correspond to the different and extremely complex ecological niches, what is reflected in a clear zonation in the distribution of the organisms that live here (Sousa, 2010).

Coastal lagoons, such as Óbidos Lagoon, the most extensive of the Portuguese coast, bear an undeniable economic, social and ecological value that must be preserved. Therefore it is necessary to recognize their potential and sensitivities, and so monitoring these ecosystems is of crucial importance.

Salt marshes, habitats present in estuarine ecosystems, such as lagoons, occur in low energy environments usually restricted to relatively sheltered areas (Best *et al.*, 2007). Those habitats present a high biodiversity, and thus, they are considered of great ecological and economic value. Besides providing habitats for many plants and animals (birds, mammals, reptiles, amphibians, fishes and invertebrates), they represent the main nursery areas, for fish, and breeding sites, for birds, since there is an abundance of benthic invertebrates, the main prey (França *et al.*, 2005; Sousa *et al.*, 2008). They also provide erosion mitigation and stabilization for adjacent coastal landforms (MacFarlane *et al.*, 2007). Salt marshes also playing a crucial role in nutrient cycling, either in transformation and sequestration of nutrients, as well as phytoremediation, namely of heavy metals (Sousa *et al.*, 2008).

The development of salt marsh vegetation is dependent on the presence of intertidal mudflats and other supplies of sediment, in which the halophytes (salt-tolerant vegetation, capable of completing their life cycle under highly saline conditions) play an important role in sequestering large quantities of nutrients and metals from the environment by storing them in the roots and/or shoots (Weis and Weis, 2004; Reboreda and Caçador, 2007).

Plant roots can alter physical and chemical properties of the sediment, influencing geochemical fractionation of metals and thus availability to the plant (Doyle and Otte, 1997; Caetano *et al.*, 2008; Mucha *et al.*, 2008). The extraction of metals is induced by the release of oxygen and exudates to the root surrounding sediments. The excess of oxygen

that is not consumed during respiration in belowground biomass diffuses to the surrounding sediment oxidizing reduced species in solid sediment and pore water components. As roots with a large surface area take up considerable quantities of water and nutrients, other solutes like metals are transported towards the roots (Weis and Weis, 2004). Metals that are up taken by the belowground plant tissues may be sequestered in their chemical structures or translocated to aboveground plant parts (Caetano *et al.*, 2008).

The high remediation potential is due to general fast growth and high biomass production of the salt marshes vegetation, although, salt marshes are commonly characterized by a relatively small number of highly productive marsh species (Lefeuvre *et al.*, 2003). Therefore, knowing their sensitivities to toxics that accumulate in their habitat is a foundation for safeguarding salt marsh ecosystems (Lytle and Lytle, 2001).

In warm temperate climates salt marshes are often colonized by halophyte plants belonging to a small number of cosmopolitan genera, including *Salicornia*, *Spartina*, *Juncus*, *Plantago*, *Scirpus*, *Sarcocornia* and *Limonium*. Pioneering salt marsh plants belonging to *Salicornia* and *Spartina* genera are often characteristic of the first stage in the successional development of salt marsh vegetation. By colonizing open mud they promote sedimentation by slowing local water movement, and reduce erosion by binding the sediment with their roots. Therefore factors which affect the abundance and distribution of such plants may have important consequences for sediment stability and salt marsh development (Gerdol and Hughes, 1993).

Salt marshes are recognized by intergovernmental agreements (e.g. Ramsar Convention on Wetlands, <http://www.ramsar.org>) and/or directives (e.g. EU Habitats Directive) that provide the framework for national action and international cooperation for the conservation and wise use of wetlands, including salt marshes, and their resources. In addition, salt marshes are classified as sensitive habitat under the European Habitats Directive (Sousa, 2010).

Presence of heavy metals in the environment and its consequences

Heavy metals are metals having a density of 5 g/cc, and include elements such as Copper (Cu), Zinc (Zn), Cadmium (Cd), Chromium (Cr), Arsenium (As), Manganese (Mn),

Iron (Fe), Lead (Pb) and Nickel (Ni) (Vardanyan and Ingole, 2006). Ecosystems contamination by heavy metals as a result of human activities is a serious environmental issue all over the world, particularly as they cannot be biodegraded.

Pollution in the biosphere with heavy metals has accelerated dramatically over the years, both as a result of natural processes and of human activities (España *et al.*, 1998). Many industries (such as automotive, metal finishing, electroplating, battery manufacturing, mining, electric cable manufacturing, tannery, steel and textile), and the application of fertilizer and pesticides, sludge dumping and generation of municipal waste have been identified as the principal sources of contamination by heavy metals (Demirezen Yilmaz, 2006; Bhattacharya *et al.*, 2006; Ogundiran *et al.*, 2008; Hu *et al.*, 2010).

Although many metals are essential in small amounts for the correct progress of biological cycles and required by living organisms for their normal function, most of them exert toxic effects at concentrations encountered in polluted environments (Malandrino *et al.*, 2006).

In aquatic systems, heavy metals tend to be adsorbed onto suspended particles and removed from the water column into sediments (Monterroso, 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, thus affecting the marine food chain (Warwick *et al.*, 1998) or be remobilized when sediments are dredged and disposed into water bodies (Monterroso, 2003).

Metals, present in sediments, pore water and water column, can occur in different forms (or speciation), depending on many factors, such as cation exchange, pH, organic matter and organic carbon content and plant species (Soon and Bates, 1982; Davies, 1992; He and Singh, 1993; *in* Ololade, 2007), which may control their bioavailability and toxicity (Chaney and Hornick, 1978 *in* Ololade, 2007). Potentially toxic metals and metalloids may be mobilized in solution as free cations (e.g., Cu^{2+} , Zn^{2+}), or ionic complexes (e.g. H_2AsO_3 , H_2AsO_4) (Mucha *et al.*, 2008; Madejón *et al.*, 2006). In the case of Cadmium, a relatively rare element (0.2 mg/kg in the earth crust) (Ololade, 2007), the divalent form is soluble and therefore bioavailable, but it can complex with oxides and organic compounds and is not soluble above pH 7.5. In seawater, chloride commonly forms complexes with Zn, Cd, and, to a lesser extent, Cu, but not with Pb, and thus the

free ion concentration of the former metals will be reduced (Förstner, 1979; Williams *et al.*, 1994).

These heavy metals can be accumulated by organisms in tolerance levels. Despite the heavy metals are not biodegradable, they can be absorbed by some organisms, namely by plants, that should allow their concentration and disposal. However, at concentrations exceeding the physiological demands of plants, heavy metals not only could administer toxic effect in them, but also could enter food chains, get biomagnified and pose a potential threat to human health (Sugiyama, 1994 *in* Hu *et al.*, 2010).

The toxic effects of heavy metals on biological systems have been reported by several authors. Heavy metals, when present in excess, disturb plant metabolism, affecting respiration, photosynthesis, stomata opening and growth (Malandrino *et al.*, 2011), being one of the reasons for decreased agriculture yield worldwide (Sharma *et al.*, 2010). Roots and leaves retain higher metal concentration of heavy metal than stems and fruits. Therefore, there is need to know the concentration of heavy metals in crops particularly leafy vegetables which are consumed by humans. Also, some heavy metals are known to induce oxidative stress by generating oxyradicals causing significant physiological damage in animals and humans (Mittler, 2002).

Cadmium is non-essential and recognized as an extremely significant pollutant due to its large solubility in water and highly toxic to most organisms, with toxicities 2 to 20 times higher than many other heavy metals (Vassilev *et al.*, 1998), been 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). The ingestion of Cd and other heavy metals containing vegetables by humans and animals can lead to series of clinical manifestation, such as emphysema of the lungs, bronchitis and cancer, high blood pressure, destruction of testicular tissue and red blood cells (Bowen, 1966; Bryce Smith, 1977 *in* Ololade, 2007).

Therefore, the determination of these toxic metals in the environment is of substantial importance for environmental monitoring, ensuring that the levels of heavy metals are within the legal limits imposed (Decret Law 236/98 of the Portuguese Legislation).

Toxic metal contamination of soil, aqueous waste streams, wetlands and groundwater are still in need of an effective and affordable technological solution (Bhattacharya *et al*, 2006).

Environmental biotechnology: phytoremediation

The European Federation of Biotechnology describes, in 1988, biotechnology as "the integrated use of biochemistry, microbiology and engineering sciences in order to achieve applications of the capabilities of microorganisms, cultured animal cells or plant cells or parts thereof in industry, agriculture, health care and in environmental processes".

To preserve the natural environment, Environmental Biotechnology, emerges as an effective, affordable and reliable biological (safer and greener) solution to some problems associated with the pollution of the ecosystems. Usually, the treatment of contaminated sites uses physical and chemical methods, such as immobilization, removal (dig and dump), thermal, and solvent treatments. However, advances in biotechnology have seen the development of biological methods of contaminant degradation and removal, a process known as Bioremediation. One of the strategies for bioremediation in both soil and water is Phytoremediation.

As defined by Cunningham and Berti (1995) phytoremediation is the use of vascular plants to remove pollutants from the environment or to render them harmless. This strategy provides an aesthetically pleasing option, minimal disruption to the environment, efficiency with low levels of mixed contamination, possibility of recovery of metals, and is inexpensive, some 50-80% less than the alternatives (Pullford and Watson, 2003). Yet, since it makes use of plants, there are some limitations to be considered in this process. It can be slower, and involve a number of growing seasons, the contaminant may reduce growth, and the plant could be a threat to the biotic community, via the food chains.

There are different types of phytoremediation, depending on the process that occurs, which are: phytoaccumulation (or phytoextraction), phytodegradation, phytovolatilization and phytostabilization. In the case of heavy metals, since they are clearly not degraded, what may occur is phytoaccumulation, which means the uptake by

the roots (with very large area containing high-affinity chemical receptors) and their storage in roots, leaves, and stems; but also phytostabilization, by reduced heavy metal mobility and therefore reducing metals bioavailability (Reboreda and Caçador, 2007). Nevertheless, the role of a plant depends on multiple factors, including environmental characteristics and plant species. Plants used for phytoextraction should be fast growing, deep rooted, easily propagated and accumulate the target metal. Ideally the species should have a high bioconcentration factor (BCF), which is defined as the plant/soil metal concentration (Ghosh and Singh, 2005).

Some plant species have been reported to have the ability to accumulate heavy metals at very high concentrations without dramatically being physiologically impacted (Reeves and Brooks, 1983). These plant species have developed strategies for avoiding phytotoxic effects, such as storing heavy metals away from metabolically active compartments (Álvarez, 2008). Also, plants are known to be able to alter metal speciation in the sediment surrounding its roots (Almeida *et al.*, 2004; Reboreda and Caçador, 2007), therefore reducing metal mobility. In fact, plants have the ability of oxidizing the sediment through the movement of oxygen towards the roots (Weis and Weis, 2004) or acidifying its rhizosphere through the release of root exudates (Mucha *et al.*, 2008).

Nevertheless, the phytoremediation of contaminated sites requires a detailed understanding of how contaminants and plants behave in a particular ecosystem, before remedial activities are carried out. Many studies have been made with this purpose (Almeida *et al.*, 2006; Reboreda and Caçador, 2007; Almeida *et al.*, 2010).

1.2. The plant *Salicornia ramosissima*

The *Salicornia* species, of Chenopodiaceae family, are small, usually less than 40 cm tall, and might be annual, biannual or perenne. The stem is succulent, prostrate to erect, simple to many-branched, apparently jointed and fleshy when young, becoming not jointed and somewhat woody with age. The leaves are small, opposite and scale-like and as such the plant may appear leafless. The inflorescences are terminal on each stem. The flowering occurs between May and November (Castroviejo *et al.*, 1990).

Salicornia genus, commonly called glassworts, are halophyte plants growing in periodically wet saline coastal or inland habitats, and are often referred to as pioneer

species. There is basically three ways that halophytes manage their salt load: exclusion, excretion and/or accumulation. *Salicornia* spp. are accumulator plants, sequester salts in the cell vacuoles as an osmoregulation mechanism and presumably to avoid toxic effects (Yensen, 2008).

The genus is widely distributed in Eurasia, North America and South Africa (Teege et al., 2011). Their extreme phenotypic plasticity, related to salinity-moisture-nutrients gradients, inbreeding, reduced leaf and flower morphology, provides few taxonomical characters, making it difficult to develop a satisfactory taxonomy of the genus (Kaligarič et al., 2008).

The young fleshy tips of this green vegetable are in high demand in gourmet kitchens, not only for their salty taste, but also for their high nutritional value. *Salicornia* aerial parts are not only a good source of minerals, but they also contain proteins, various vitamins (such as vitamin C and β -carotene, antioxidant compounds) and high total lipid and omega-3 contents (Lu et al., 2010).

The interest in *Salicornia* genus for its versatile commercial products, such as seed oil, protein meal, and fresh salad greens, is growing and make it a promising candidate for the development of novel halophytes as crop species (Ventura et al., 2011).

The genus *Salicornia* have also aroused interest in phytoremediation, in removing nutrients (Brown et al, 1999) and accumulated heavy metals (Sharma et al, 2010). Halophyte crops, especially accumulator type, are being developed that can eliminate toxic compounds and elements (especially Se, Pb, Cr, Cd, Zn, As and others), petroleum products, asphalt, or radio nucleides via halophyte phytoremediation and bioremediation. The previous study on the salt tolerance of *S. europaea* showed that this halophyte has the great capacity to accumulate Na^+ , K^+ , Mg^{2+} , and Ca^{2+} in the shoot (Ozawa et al. 2007). These metals seem to be sequestered in the well-developed vacuoles, which could be a good reservoir for heavy metals (Ozawa et al., 2009).

Salicornia ramosissima J. Woods (1851) is an annual halophyte, 3-40 cm tall, fleshy, with a unique stem articulated right and branched. The leaves are scale-like, opposite, welded together with the stem. The fertile knuckles are barrel-shaped (with convex sides), usually with a maximum width at the top (figure 1). Stems are generally

quite branched, usually purple after anthesis, and with branches erect-patent or upright (Castroviejo *et al.*, 1990).

The inflorescence is spike-like with two opposite three-flowered cymules in each segment, and each cymule has one large central flower (responsible for the production of a large seed) and two smaller lateral flowers (responsible for the production of small seeds). The life cycle of this species is well defined, with discrete generations and seed dimorphism (Silva *et al.*, 2007).

This halophyte is widely distributed in the salt marsh of Óbidos Lagoon and also present in many salt marshes of the Iberian Peninsula (Castroviejo *et al.*, 1990). This plant occupies preferentially small places not invaded by other halophytes, such as *Halimione portulacoides* and *Sarcocornia perennis* subsp. *perennis*, both Chenopodiaceae. Usually occupies the higher reaches of the marsh environments, where the salinity is lower. In fact, *S. ramosissima* has an optimum growth at low salinity (Silva *et al.*, 2007).



Figure 1. *Salicornia ramosissima*



Figure 2. *Salicornia ramosissima* in Óbidos Lagoon (Portugal) at July 23, 2011.

As a salt marsh pioneer, *S. ramosissima* is frequently the first higher plant to colonize intertidal zones; it is therefore, a very important species to include in strategies for management and conservation of salt marshes. This capability coupled with the potential for bioremediation of the genus *Salicornia* (Rosso *et al.*, 2005; Sharma *et al.*, 2010), makes *Salicornia ramosissima* an excellent candidate for phytoremediation.

1.3. Aim of the study

The ability to phytoremediation of heavy metals, recognized in many plant genera, is conditioned by several factors, and therefore, is variable depending on environmental conditions of each habitat. In addition, the load of contaminants that plants are subjected, and to what extent the development of the plant itself is affected, should also be considered.

Considering the potencial of *Salicornia ramosissima* in phytoremediation, the main objective of the present study is to evaluate the capability of this halophyte in the bioaccumulation of Cd, when submitted to different salinities (simulating distinct natural

conditions) and Cd concentrations, under controlled conditions. For these purpose, the following specific objectives are proposed:

- a) To analyse the effects of different Cd concentrations (0, 50 and 100 $\mu\text{g.l}^{-1}$) and salinities (0, 5 and 10) on *Salicornia ramosissima* survival and growth parameters;
- b) To assess the effects of different Cd concentrations (0, 50 and 100 $\mu\text{g.l}^{-1}$) and salinities in the bioaccumulation capacity of Cd, by *Salicornia ramosissima*;
- c) To determine the Cd compartmentation within the plant.

2. Materials and Methods

2.1. Study area

The Óbidos Lagoon is one of the most extended lagoons in Continental Portugal, located on the western central coast (39°24'N, 9°17'W) (Figure 3). With a surrounding area of 7 km², this lagoon has a medium depth of 3 m and is permanently connected to the Atlantic Ocean by a narrow inlet (Carvalho *et al.*, 2006). Tides are semidiurnal with a tidal range varying between 0.5 to 4.0 m depending upon location and tidal phase (Malhadas *et al.*, 2009). The influence of the tide extends to the entire lagoon, with pronounced longitudinal variation of salinity or stratification (Carvalho *et al.*, 2006).

The lagoon is characterized by two distinct regions, with different hydromorphological and sedimentary characteristics: the lower lagoon, with several sand banks and channels is characterized by strong velocities and low residence time; and the upper lagoon, characterized by low velocities, muddy sediments and high residence time (Malhadas *et al.*, 2009). The upper lagoon is divided into two main branches: Barrosa branch and Bom Sucesso branch. Barrosa branch (SE), is the less deep arm of the lagoon, with a mean depth of 0.5 – 1 m, and the water circulation is mostly driven by tides and by a small tributary (Cal River). The freshwater discharges are negligible in summer (<0.05 m³. s⁻¹) (Neves *et al.*, 2008) and annually amount to an average of 3 m³.s⁻¹ (Oliveira *et al.*, 2006). However, this input is responsible for the entrance of several pollutants, mostly from agricultural fields, from the cities nearby, like Caldas da Rainha town (Kowalski, 2009). Some of those pollutants are heavy metals, present in the water of the lagoon, such like cadmium, among others.

2.2. Sampling procedure (plants)

Green plants of *Salicornia ramosissima* without a senescent appearance and with similar size were collected in June 2011, at low tide, from the sampling site S1 (Figure 3), located in the Barrosa branch. Plants were carefully washed in lagoon water to remove sediments, using plastic gloves, placed in plastic buckets, and carried to the laboratory within 30 minutes. During collection of plants, a portable multiparameter probe (HANNA HI9828 analyser, Hanna Instruments, Vila do Conde, Portugal), was used to register values of salinity.

At the laboratory, plants were again carefully washed using tap water and then distilled water, to remove slurry, green algae's and other adherent particles. The washed samples were carefully dried of adherent water using absorbent paper. Fresh weights of

the plants were registered with a digital balance (Metler Toledo AB2045, Soquímica, Portugal), and their lengths (total, roots and aerial part) were measured. The plants looking healthy and of similar age/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

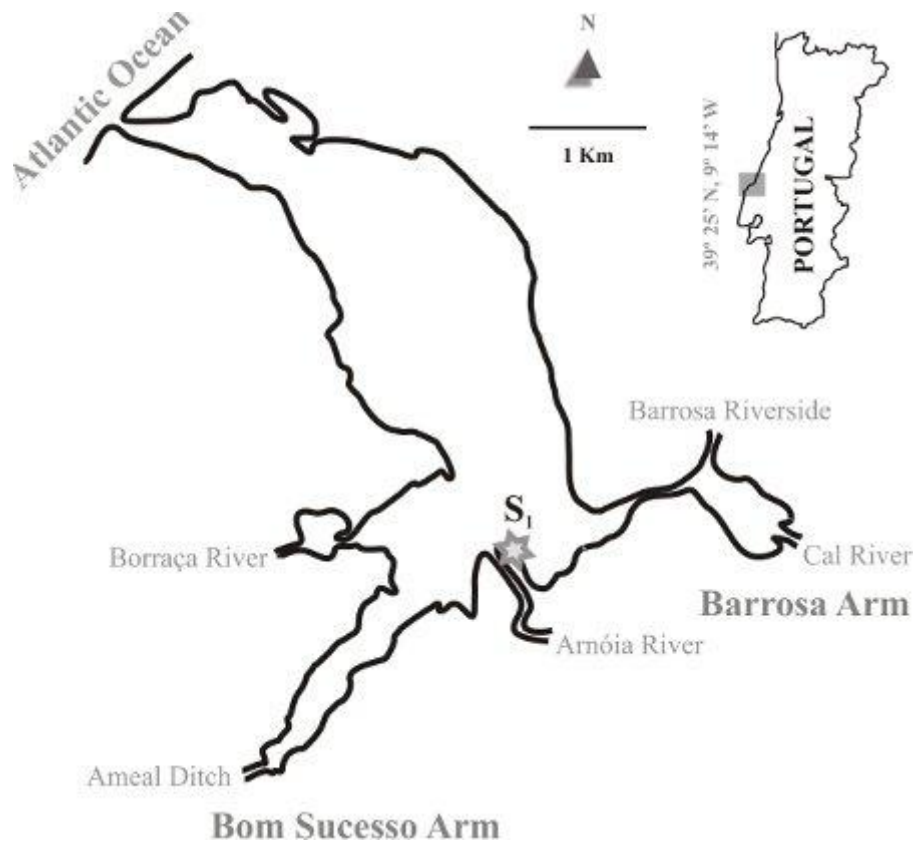


Figure 3. Location of the sampling site (S1) at the Óbidos lagoon.

2.3. Experiment design

All the glass and plastic materials were washed by immersion in 3% Derquim LM 02 (Neutral, phosphates free liquid, Panreac Química S.A.U., Spain) for 24 h, then in 25% HNO₃ (HNO₃ 69%, PA-ACS-ISO, Panreac Química S.A.U., Spain) for 24 h and finally rinsed with distilled water and dried. All the standard solutions were daily prepared with ultra-pure water (Water Purification System, Human Power I scholar UV) for metal analysis, from stock solutions. All the procedures of the experiment were conducted in a climate-controlled room in Escola Superior de Turismo e Tecnologia do Mar, Instituto Politécnico de Leiria (ESTM - IPL), in Peniche.

The plants collected were transplanted into plastic containers (3 in each container), containing 320 g (dry weight) of gravel, previously washed with 10% hydrochloric acid solution (HCl 37%, PA-ACS-ISO, Panreac Quimica, S.A.U., Spain) for 12 h and burned at 500 °C, 3 h in a muffle (Nabertheern Controler B170, VWR International – Material de Laboratório Lda, Portugal). Each of those containers (about 8 cmØ) was placed within a bigger plastic container (about 14 cmØ) (Figure4), which has 500 ml of artificial seawater, in order to ensure that water level was about 1 cm above substrate level. The artificial seawater was prepared to get a salinity of 2 (the same registered on the collection day), by dissolving sea salt Tropic Marine[®] (Sea Salt Tropic Marine, Germany) in distilled water, according to the manufacturer's instructions.



Figure 4. Experiment design.

For the acclimation period, all containers, a total of 54, were placed in a climate – controlled room, with an air temperature of 20 ± 1 °C and artificial lights (Lustek, TLD 10 W/765, Lustek Services, Australia) were used to create light intensity of 11.5 ± 12.5 $\mu\text{mol photons.cm}^{-2} \text{s}^{-1}$ (PAR Sensor, Apogee Logan, UT) 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 plants. To prepare the nutrient solution a source of N (620 mg N/l) (calcium nitrate 4-hydrate PRS, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, Panreac Química S.A.U., Spain) and a source of P (94 mg P/l) (Potassium di-hydrogen phosphate PA, KH_2PO_4 , Panreac Química S.A.U., Spain) were used.

About 15 days was given for acclimation and plants showing visible stress symptoms (e.g. wilting, chlorosis) were eliminated from the experiment.

To study the effects of salinity on the heavy metal uptake of *Salicornia ramosissima*, after the acclimation period, the water solutions were replaced for treatment solutions. Three solutions with different salinities were prepared (0, 5 and 10) by adding the synthetic Sea Salt to aerated ultra-pure water. Those salinities were chosen considering the most frequently observed in the Óbidos Lagoon, at the sampling site (S1) throughout the year

To prepare the heavy metal treatment, cadmium (cadmium nitrate in nitric acid, $\text{Cd}(\text{NO}_3)_2$ in HNO_3 0.5 mol/L, Scharlau Chemie S.A., Spain) were added to each salinized solutions, from a 1000 $\mu\text{g}\cdot\text{l}^{-1}$ stock solution, getting the final concentrations of 0, 50 and 100 $\mu\text{g}\cdot\text{l}^{-1}$. Those concentrations were selected considering the Portuguese legislation (Decree Law 236/98), which establish the maximum admissible concentration of Cd in “irrigation water” (surface or underground water of wastewater, which aims to meet or supplemented the water needs of crops or forestry) is 50 $\mu\text{g}\cdot\text{l}^{-1}$ and for water discharges Cd presented an emission limit value of 200 $\mu\text{g}\cdot\text{l}^{-1}$. Resulting in 9 different treatments: 3 salinities (0, 5 and 10) x 3 cadmium concentrations (0, 50 and 100 $\mu\text{g}\cdot\text{l}^{-1}$), with S 0 Cd 0 $\mu\text{g}\cdot\text{l}^{-1}$ as the control.

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 respected solution, in a total of 27 containers, and placed in the same climate – the controlled room.

The volume of the solution in each container was carefully monitored and kept at a constant level for the duration of the experiment to avoid changes in concentration due to water loss from evapotranspiration. Therefore the containers were watered following the same process used in the acclimation period.

During the acclimation and experiment periods, salinity, temperature (Seawater Refractometer HI96822, HANNA Instruments, Portugal) and pH (Symphony SP 70P, VWR, International – Material de Laboratório, Lda, Portugal) were registered, as in Lillebø *et al.* (2003).

Throughout the treatment, which ran for one month, the plants were monitored. During the experiment, it was been observed that some plants were infected with a

phytoparasite (whose presence was not detected during the acclimation. The phytoparasite (and their droppings) were removed, whenever possible, without disturbing the plant. Moreover plants showing signs of advanced senescence, or extensive damage, were eliminated (Rosso *et al.*, 2005). After one month of treatment, the plants were washed with distilled water and growth parameters such as total fresh length and weight, root (length and weight) and aerial part (length and weight) were measured.

2.4. Samples treatment (plant, water and sediment)

Plants were carefully washed using distilled water, measured and weighted, and the dry weight was measured after 48 h of desiccation in an oven at 80°C (Memmert Drying UFB 500, Germany) (Ghnaya *et al.*, 2005). Dried roots and aerial parts were weighted, after being separated, ground to a fine powder using a mortar and were acid digested by the following procedure. Approximately 0.1 g of dried material were digested with two times 3 ml of 69% nitric acid. The digested samples were dried on a hot plate (VHP seriesC-10, ceramic, VWR International-Material de Laboratório, Lda, Portugal) at 150 °C, until 1 ml solution remained (Sharma *et al.*, 2010). After allowing cooling, 3 ml of 1% HNO₃ was added and filtered (Whatman 41, filter papers, Ashless Circles, 55 mm Ø, GE HealthCare Company, UK). The filtered samples were diluted with ultra-pure water to make up the final volume of 50 mL, transferred to 50 mL plastic bottles and frozen (- 18 °C) until analysis.

The treatment solutions were filtered (Whatman OE 67, 0, 45 µm, Ø 47 mm, membrane filters, cellulose acetate, Whatman GmbH, Germany), under vacuum conditions (Rotovac Valve Control, Heidolph Instruments, Germany), for the analysis of dissolved and suspended cadmium (Pereira *et al.*, 2009).

For the analysis of dissolved cadmium, water filtered samples were acidified (69% HNO₃), to a pH<2, and then transferred to 50 ml plastic bottles and frozen (-18 °C). In case of suspended cadmium, the membrane filter of each sample was digested in 6 mL of 69% HNO₃, using a hot plate at 200 °C. Once more, after the digestion, the samples were filtered, diluted with ultra-pure water to final volume of 50 ml, and frozen at -18 °C.

In order to determine the organic matter, the sediment of all containers was dried in a oven at 60 °C, during 48 h, and then burned at 500 °C, during 3 h in a muffle (Kowalski, 2009).

2.5. Cadmium determinations

Determination of Cadmium was performed using a Atomic Absorption Spectrometry (AAS) (Thermo Scientific ICE 3500 Atomic Absorption Spectrometer, Thermo Unicam, Portugal), with graphite furnace (SOLAAR FS95 Furnace autosampler), using a cadmium Hollow cathode lamp (10 mA, Thermo Electron Corporation) and Argon (Air, Praxair Portugal Gases S.A., Portugal) (Pereira *et al.*, 2009). The cadmium concentrations were prepared using a standard solution (Cadmium standard solution, traceable to SRM from NIST Cd (NO₃)₂ in HNO 0.5 mol/l 1000 mg/l Cd CertiPUR®, © Merck KGaA, Darmstadt, Germany). Cadmium concentrations were also determined in plants collected in S1, in June 2011, which were immediately digested, to obtain the reference values on naturally conditions.

Metal concentrations were determined using the standard addition method and samples were re-analyzed when either the correlation coefficient for the calibration of six standards was <0.99. Procedure blanks were also carried out for quality assurance purposes.

2.6. Data analysis

After experiment, the following parameters were calculated for each treatment: mortality, 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.

Once during the experiment larvae were detected in plants, 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 translocate the metal species from roots to leaves at different concentrations. This index was calculated by applying the same equation used by Ghosh and Singh (2005), to each treatment plants:

$$Ti = \frac{\text{Cadmium in leaves (mg kg}^{-1}\text{)}}{\text{Cadmium in roots (mg kg}^{-1}\text{)}} \times 100$$

To determine the organic matter present in the substratum, in which the Cd could bind, the following equation was applied (Eleftheriou and McIntyre, 2005):

$$\text{O.M.(\%)} = \frac{\text{Dry weight} - \text{Ash weight}}{\text{Dry weight}} \times 100$$

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 the Cd 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 weigh 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.

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

Mortality

All the plants survived to the first two weeks of the experience (Figure 5). At the 15th day mortalities were registered at all the treatments, except for 50 $\mu\text{g Cd.l}^{-1}$, at salinity 0, where no deaths occurred during the entire experiment, and for 100 $\mu\text{g Cd.l}^{-1}$, at salinity 5, where dead plants were only observed on third week (23 days) of treatment.

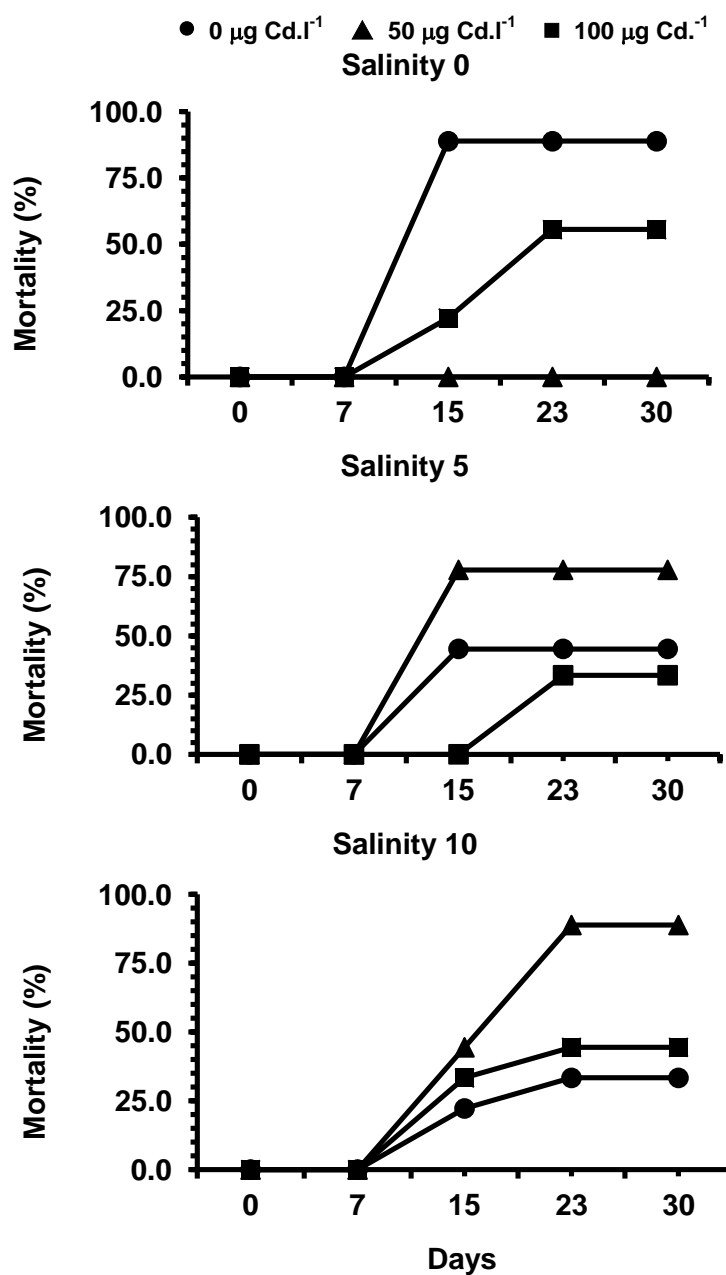


Figure 5. Percentage mortality of *S. ramosissima* exposed to different salinities and Cd concentrations over one month.

Although there was no uniform pattern in the percentage of mortality for the different treatments, the mortality rate was highest for 0 $\mu\text{g Cd.l}^{-1}$, at salinity 0, with a mortality rate of 88.9% and for 50 $\mu\text{g Cd.l}^{-1}$, at salinity 5 and 10, with a value of 77.8% and 88.9%, respectively.

Considering the concentration 0 $\mu\text{g Cd.l}^{-1}$, mortality decreased with the increase of salinity, while the opposite was observed for the concentration 50 $\mu\text{g Cd.l}^{-1}$ (figure 6). For the concentration 100 $\mu\text{g Cd.l}^{-1}$, the lowest mortality is observed for salinity 5 with a value of 33.3% (Figure 6).

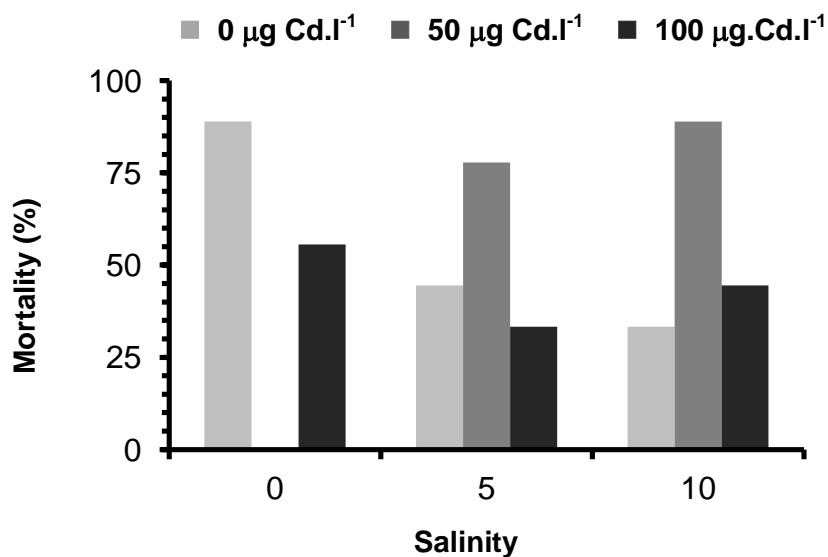


Figure 6. Percentage mortality of *Salicornia ramosissima* after one month of treatment with different salinities and Cd concentrations.

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 treatments 0 and 50 $\mu\text{g Cd.l}^{-1}$, at salinity 0 (Table I).

The presence of the larvae's, detected during the experience, had no effect on the mortality of the plants subjected to two of the three treatments with salinity 5 ($\chi_1^2 = 1.102$, $p = 0.294$, to 0 $\mu\text{g Cd.l}^{-1}$; $\chi_1^2 = 0.225$, $p = 0,635$, to 100 $\mu\text{g Cd.l}^{-1}$). Also for the treatments salinity 0 x 50 $\mu\text{g Cd.l}^{-1}$, and salinity 10 x 0 $\mu\text{g Cd.l}^{-1}$, the test was not applied, since all the plants survived the experiment in the case of the first treatment and dead plants with

larvae were not observed in the second treatment. As for the others treatments and according to the software, the chi-square approximation was most probably invalid or did not apply.

Table I. 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 $\mu\text{g.l}^{-1}$) 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: 0 $\mu\text{g Cd.l}^{-1}$ and 50 $\mu\text{g Cd.l}^{-1}$	0.047	
Weight variation				
Interaction: salinity x Cd conc.	Bonferroni	Salinity 10 Comparison: 0 $\mu\text{g Cd.l}^{-1}$ and 50 $\mu\text{g Cd.l}^{-1}$ Comparison: 0 $\mu\text{g Cd.l}^{-1}$ and 100 $\mu\text{g Cd.l}^{-1}$	0.000 0.000	
Dissolved Cd				
Cd concentration	Tukey HSD	Comparison: 0 $\mu\text{g Cd.l}^{-1}$ and 50 $\mu\text{g Cd.l}^{-1}$ Comparison: 0 $\mu\text{g Cd.l}^{-1}$ and 100 $\mu\text{g Cd.l}^{-1}$	0.001 0.034	
	LSD	Comparison: 0 $\mu\text{g Cd.l}^{-1}$ and 50 $\mu\text{g Cd.l}^{-1}$ Comparison: 0 $\mu\text{g Cd.l}^{-1}$ and 100 $\mu\text{g Cd.l}^{-1}$	0.000 0.013	

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 (Figure7). For the salinities 0 and 10, the growth decreased at the highest Cd concentration (100 $\mu\text{g.l}^{-1}$).

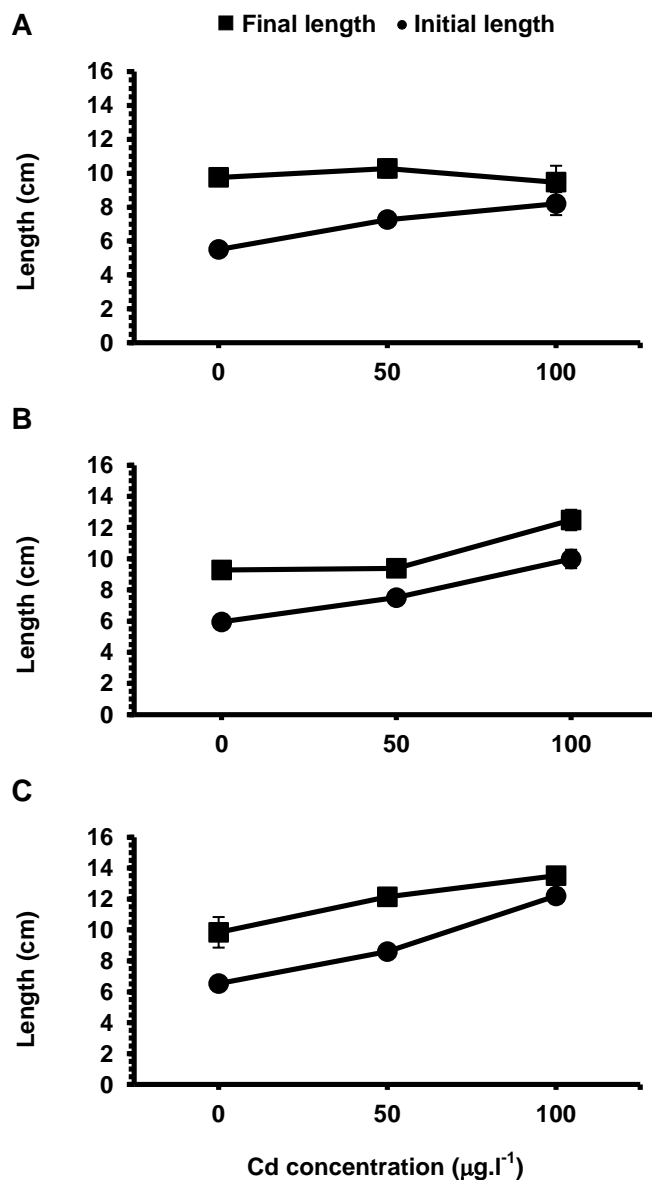


Figure 7. Comparison between initial and final length (average \pm standard error) of *S. ramosissima* aerial portion, exposed to different salinities and Cd concentrations. (A) Salinity 0; (B) Salinity 5; (C) Salinity 10.

Generally, *S. ramosissima* grew more when treated with the lowest Cd concentrations (0 and 50 $\mu\text{g Cd.l}^{-1}$) at salinity 0 and 10, with stem elongation reaching to 4.25 and 3.55 cm, respectively (Figure 8); mean stem elongation was only 1.27 and 1.31 cm, in treatment with 100 $\mu\text{g Cd.l}^{-1}$, at salinity 0 and 10, 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 $p = 0.771$).

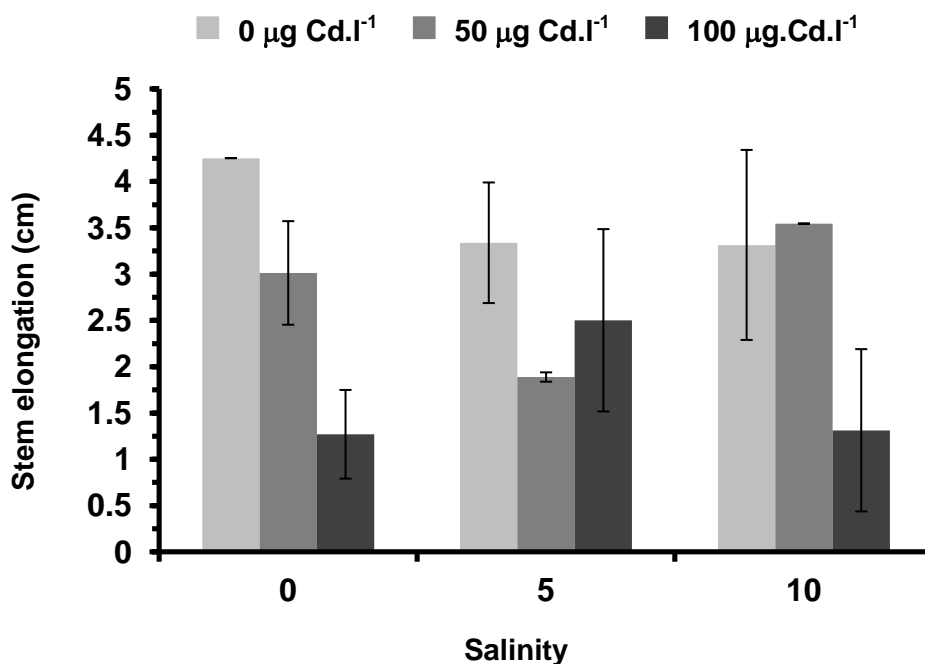


Figure 8. Mean stem elongation (\pm standard error) for *S. ramosissima* exposed to different salinities and Cd concentrations, after one month of treatment.

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 maximum 5 surviving plants were tested for this correlation (salinity 5 x 0 µg Cd.l⁻¹, salinity 5 x 100 µg Cd.l⁻¹, salinity 10 x 100 µg Cd.l⁻¹). 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 (Figure 9). This decrease is more evident for the higher salinity tested and increases with increasing concentration of Cd.

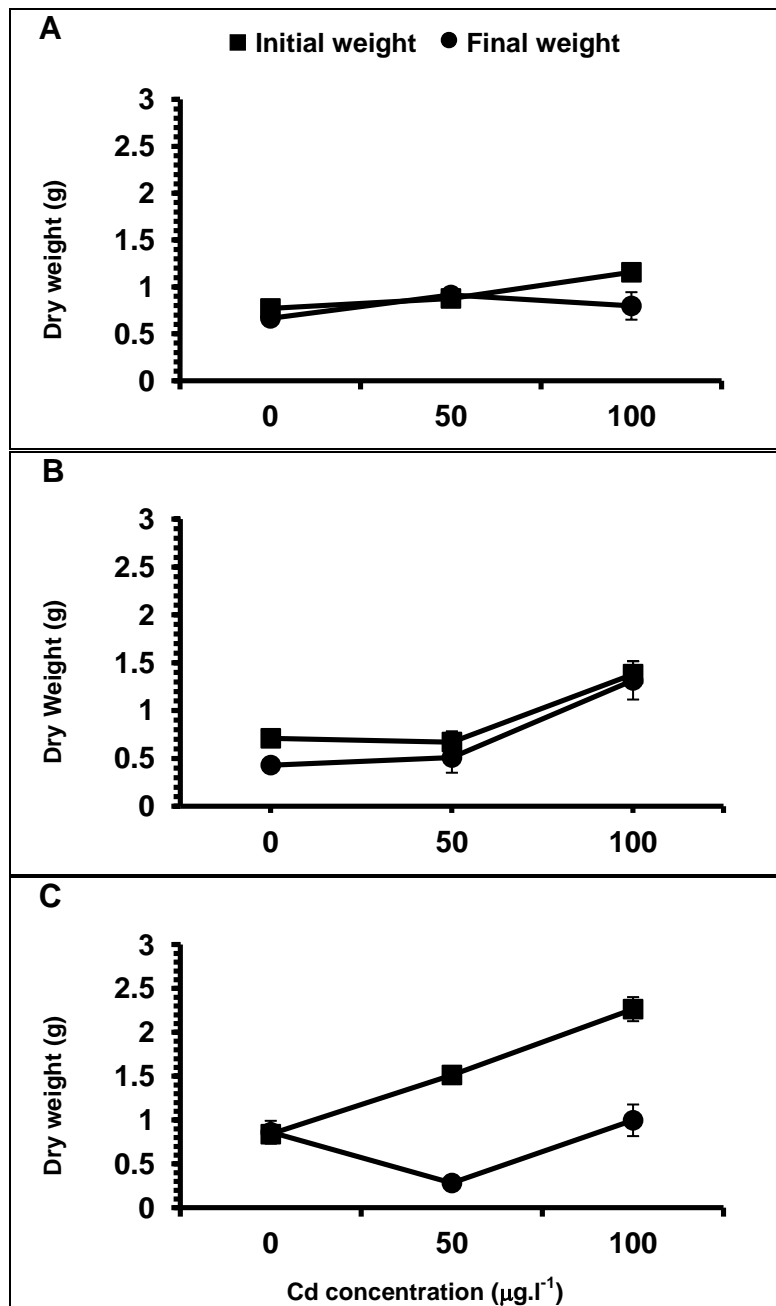


Figure 9. Comparison between initial and final weight (average \pm standard error) of *S. ramosissima*, exposed to different salinities and Cd concentrations. (A) Salinity 0; (B) Salinity 5; (C) Salinity 10.

Plants lost weight in almost all treatments, except in salinity 0 x 50 µg Cd.l⁻¹ and salinity 10 x 0 µg Cd.l⁻¹. However the great lost occur in the highest salinity with the highest Cd concentrations, with a value of 1.27 g (Figure 10). Salinity and Cd concentration influence the weight variations, exhibiting statistically significance when the

Two-Way ANOVA was performed ($p = 0.00$) (table 1). The application of Bonferroni test showed differences between treatments 50 and 100 $\mu\text{g Cd.l}^{-1}$ ($p = 0.049$) on salinity 0, and in salinity 10, between treatments 0 and 50 $\mu\text{g Cd.l}^{-1}$ ($p = 0.000$) and 0 and 100 $\mu\text{g Cd.l}^{-1}$ ($p = 0.000$).

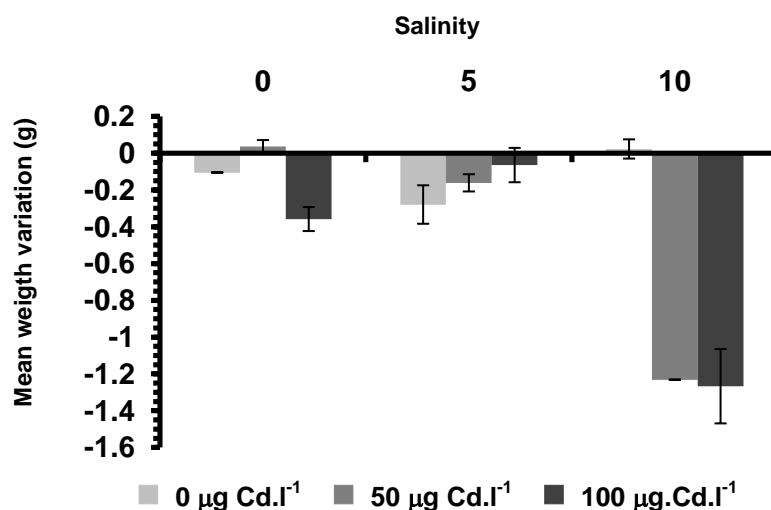


Figure 10. Mean weight variation (\pm standard error) of *S. ramosissima* exposed to different salinities and Cd concentrations, after one month of treatment.

The organic matter present in the sediment where plants were developing during the experiment it was negligible, with the highest value, 0.49%, in salinity 10 with 0 $\mu\text{g Cd.l}^{-1}$ and the lowest value, 0.28%, in salinity 5 with 50 $\mu\text{g Cd.l}^{-1}$ (Fig 11)

Cadmium accumulation

At the end of the experiment the Cd accumulated both in roots and aerial portions decreased, with the increase of salinity (Figure12). However in roots the values continued to be highest when compared with the results obtained for the roots of reference plants (with a mean value of $7.50 \pm 1.59 \text{ mg Cd.kg}^{-1}$). In the case of the aerial portions, the Cd accumulation recorded for the reference plants ($1.453 \pm 1.18 \text{ mg Cd.kg}^{-1}$) was similar. Roots accumulated more Cd when submitted to salinity 0, with 50 $\mu\text{g Cd.l}^{-1}$, with 215.78 mg Cd.kg^{-1} , as aerial portion accumulated more when submitted to salinity 5, with 0 $\mu\text{g Cd.l}^{-1}$, ascertaining a concentration of 9.99 mg Cd.kg^{-1} .

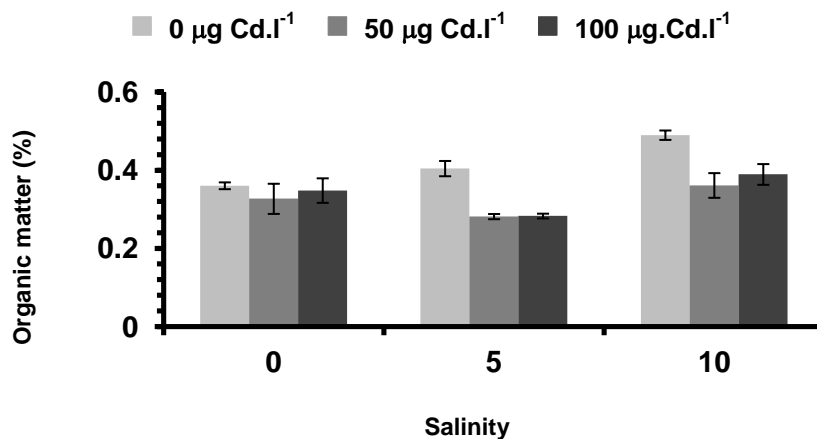


Figure 11. Percentage of organic matter present in the sediment of each treatment.

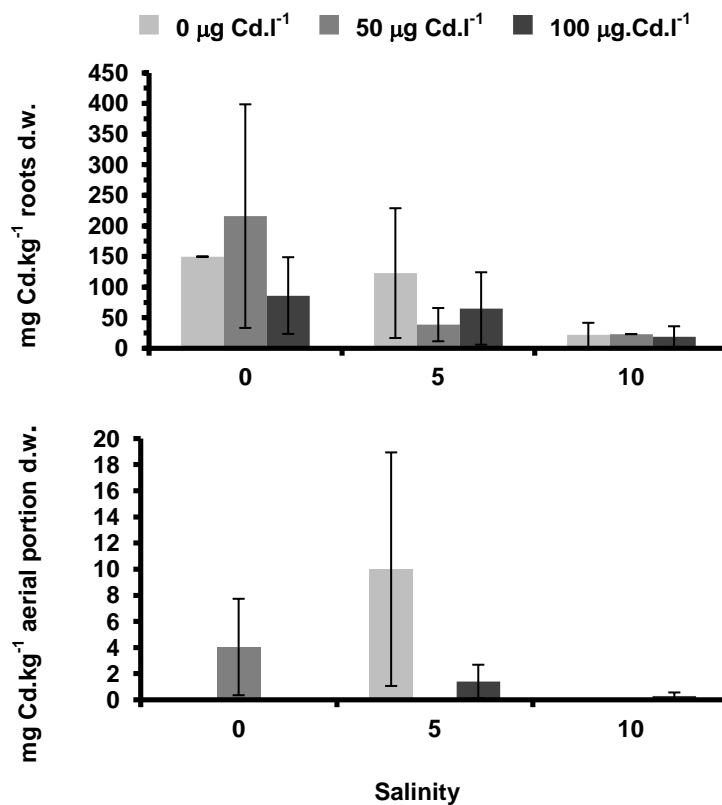


Figure 12. Mean accumulation (± standard errors) of Cd in *S. ramosissima* portions (roots and aerial portion) treated for one month with Cd concentrations of 0, 50 and 100 µg.l⁻¹ and salinity of 0, 5 and 10.

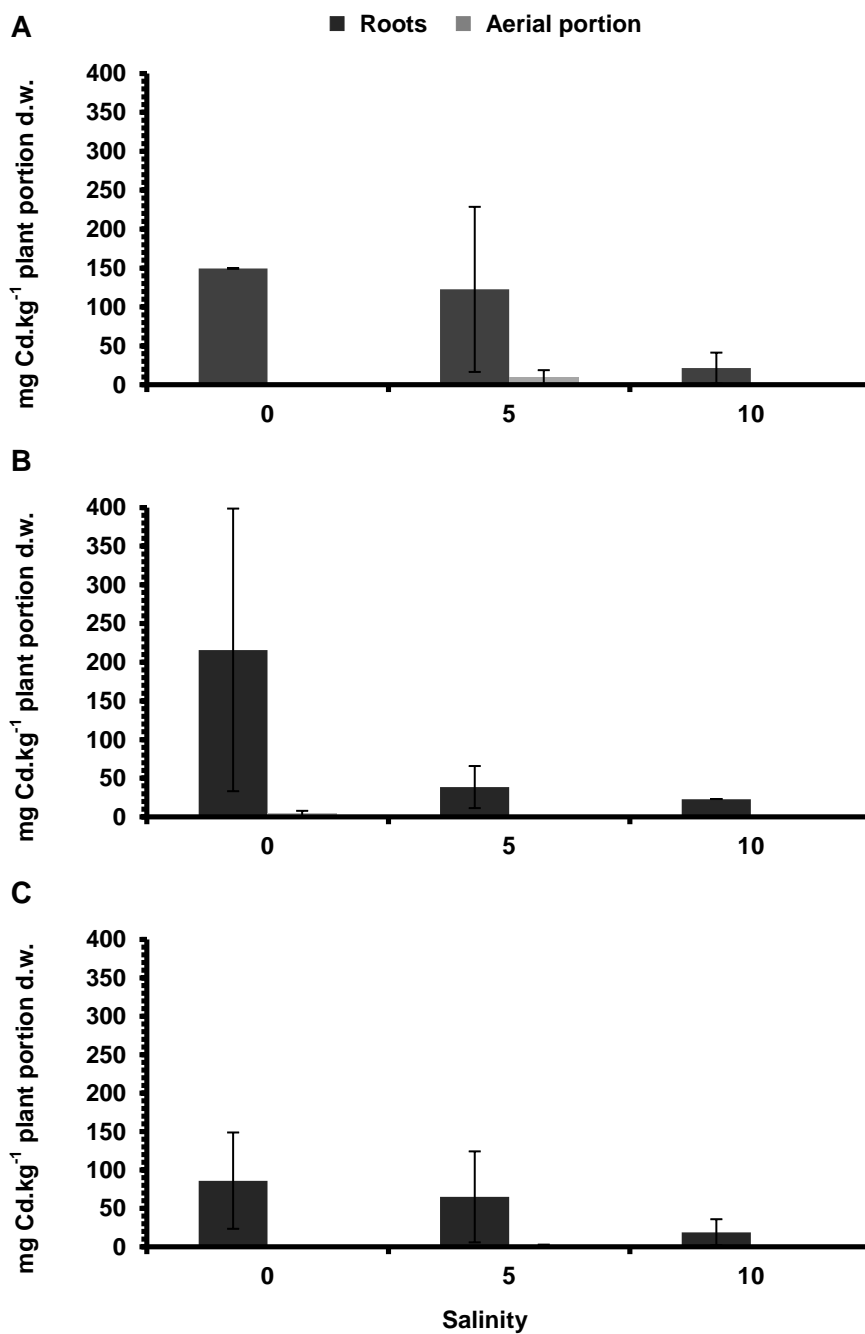


Figure 13. Mean cadmium accumulation (mg kg⁻¹) (\pm standard errors) by roots and aerial portions of *S. ramosissima* submitted at different salinities and Cd concentration, after one month of treatment. (A) 0 µg Cd.l⁻¹; (B) 50 µg Cd.l⁻¹; (C) 100 µg Cd.l⁻¹.

The Cd accumulation in the roots exceeded that of the aerial portions, for all treatments (Figure 13). In fact, in most treatments Cd concentration in aerial portions was not detectable. Maximum transport was observed at salinity 5, 0 µg Cd.l⁻¹, with a Ti

maximum not exceeding 9%. For the other treatments it was less than 2% (Figure 14). Salinity and Cd concentration have not influence the Cd accumulation on plants, there was not statistically significance when the Two-Way ANOVA was performed ($p > 0.05$).

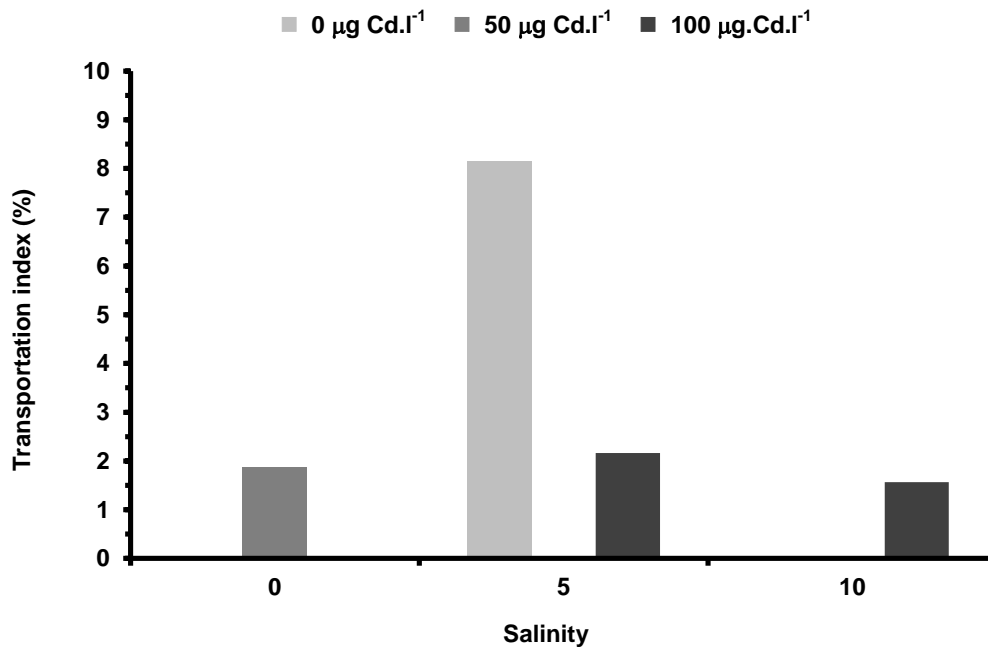


Figure 14. The leaf/root cadmium concentration index (Ti), calculated for each treatment, after one month of experiment.

At the end of the experiment the amount of Cd dissolved in the solution treatment is higher compared to Cd associated with suspended matter in these solutions (Figures 15 and 16). Salinity 0, with 50 µg Cd.l⁻¹ was the treatment with the highest Cd suspended concentration, 0.115 mg Cd.l⁻¹, the other treatments presented values below 0.056 mgCd.l⁻¹, pointing out the treatments with 0 µg Cd.l⁻¹, where the values were close to 0 mgCd.l⁻¹ (0.008 mg Cd.l⁻¹ in salinity 0) or even not detected (salinity 5 and 10). In relation to Cd dissolved, the maximum value, 0.300 mg Cd.l⁻¹, was detected at salinity 5, with 50 µg Cd.l⁻¹, while at salinities 0 and 5, with 0 µg Cd.l⁻¹, there was no detected Cd. Although there were no statistically significant differences on the influence of salinity and Cd concentrations in suspended Cd results, in respect to dissolved Cd, significant differences were observed between the treatments with different Cd concentrations used on the experience when the Two-Way ANOVA was performed ($p=0.001$), when applied the Bonferroni test, was manifested. differences between 0 and 50 µg Cd.l⁻¹ ($p = 0.001$) and between 0 and 100 µgCd.l⁻¹ ($p = 0.040$) (table I).

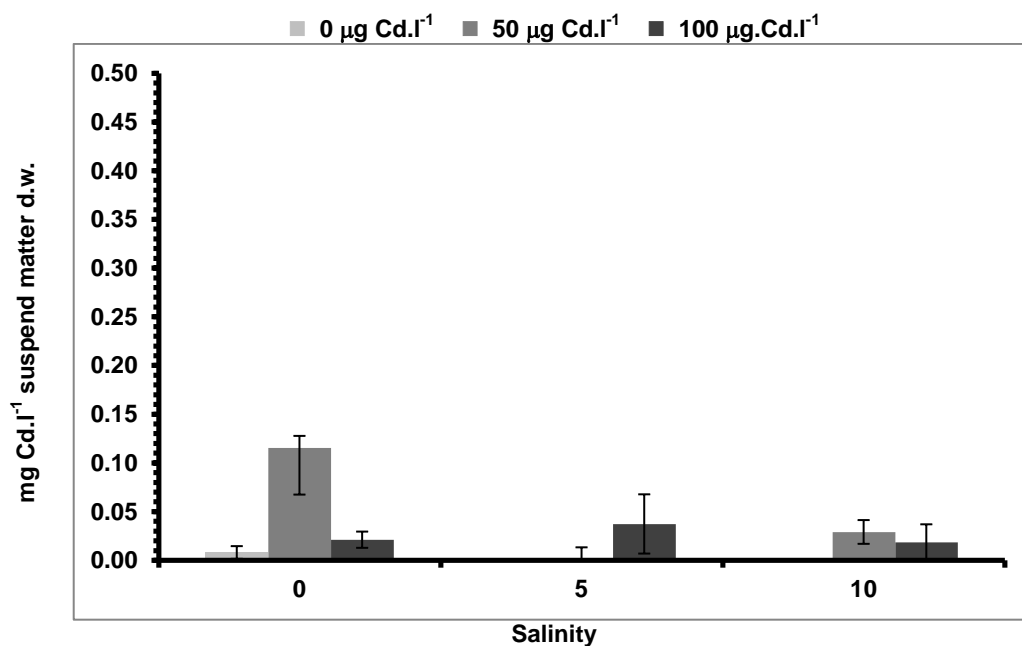


Figure 15. Mean suspended Cd (\pm standard errors) in solutions treatment (Cd concentrations of 0, 50 and 100 $\mu\text{g.l}^{-1}$ and salinity of 0, 5 and 10), after one month of treatment.

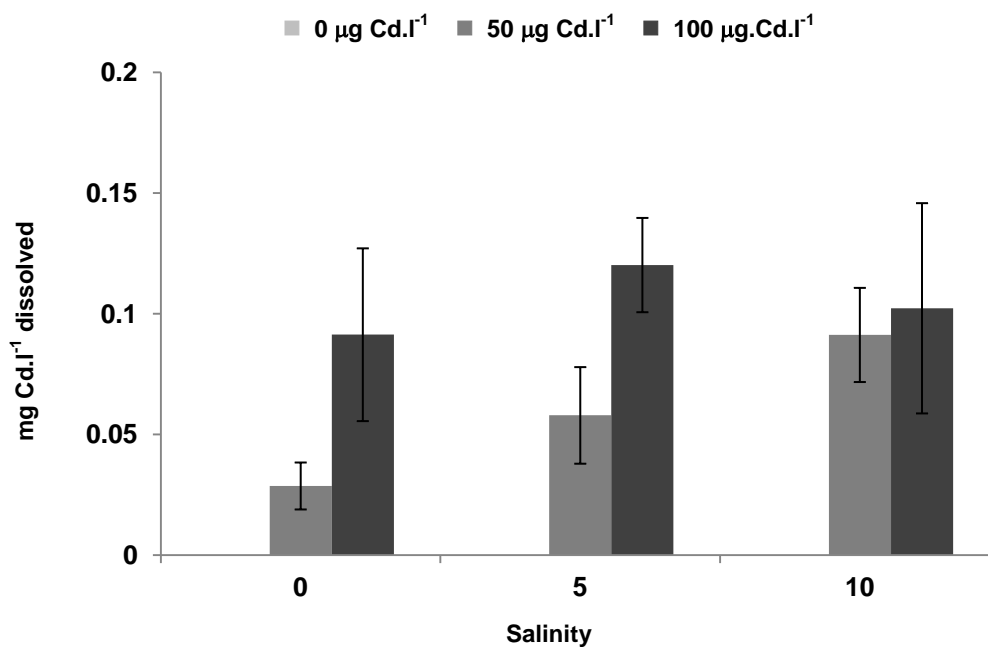


Figure 16. Mean dissolved Cd (\pm standard errors) in solutions treatment (Cd concentrations of 0, 50 and 100 $\mu\text{g.l}^{-1}$ and salinity of 0, 5 and 10), after one month of treatment.

4. Discussion

As a pioneer halophyte in salt marshes, *Salicornia* spp. has been intriguing to researchers and used in several studies (Brown *et al.*, 1999; Khan *et al.*, 2001; Rosso *et al.*, 2005; Silva *et al.*, 2006; Costa and Neves, 2006; Lu *et al.*, 2010; Sharma *et al.*, 2010) to confirm the several biotechnological applications. In the present study the main objective was to check the capacity of *Salicornia ramosissima* for phytoremediation of Cadmium, after exposed to different salinities and Cd concentrations.

A comparison of the results obtained in the different treatments for each measured parameter did not permit to establish a common pattern of the observed variations, which were not even significant in most cases. However, it was possible to make some observations.

Unlike the expected, the exposure of *S. ramosissima* to different salinities and Cd concentrations for one month just revealed significantly differences on mortality between the treatments with 0 and 50 $\mu\text{g Cd.l}^{-1}$, on salinity 0.

Regarding the presence of the larvae, chi-square tests proved that the parasites had no effect on the mortality of the plants subjected to two of the three treatments with salinity 5 (0 and 100 $\mu\text{g Cd.l}^{-1}$). Also for the treatments salinity 0 x 50 $\mu\text{g Cd.l}^{-1}$ (no presence of larvae), and salinity 10 x 0 $\mu\text{g Cd.l}^{-1}$ (about 78% of plants with larvae, but all of these plants survived to the experience) the effect of the larvae on plants mortality is completely excluded. However, as for the remaining treatments, the chi-square test was not conclusive. In fact, the treatments mentioned above match to the lowest mortalities found during the experiment, since all of them present mortalities inferior to 50 %, with emphasis on the treatment salinity 0 x 50 $\mu\text{g Cd.l}^{-1}$ where the survival of the plants was 100 %. For the remaining treatments, and even though the statistical test was not conclusive, the mortality observed was, almost entirely, superior to 50% (salinity 0 x 0 and 100 $\mu\text{g Cd.l}^{-1}$, salinity 5 x 50 $\mu\text{g Cd.l}^{-1}$, salinity 10 x 50 and 100 $\mu\text{g Cd.l}^{-1}$, with a mortality of 88.9%, 55.6%, 77.8%, 88.9% and 45%, respectively). Therefore it is not possible to exclude some influence of the larvae on the mortality of the plants, specially on the treatments: (i) salinity 0 x 0 $\mu\text{g Cd.l}^{-1}$; (ii) salinity 5 x 5 $\mu\text{g Cd.l}^{-1}$ and (iii) salinity 10 x 50 $\mu\text{g Cd.l}^{-1}$, since the incidence of the larvae was higher than 75% in these treatments.

The treatment salinity 10 x 100 $\mu\text{g Cd.l}^{-1}$ corresponds to the highest salinity and Cd concentration, so it must be considered the stress level to which plants are subjected, which will be higher relative to the other treatments in study. Indeed, according to Rand

(2002), herbivore impact might increase on host plants under more stressful conditions (Rand, 2002). However, a possible explanation for the lower mortality found in the present study may be related to the fact of the plants used in this treatment were slightly bigger, and therefore could be more resistant to damage caused by the larvae, which develop in mines within the plant. Cadmium has been found to cause reduction in photosynthesis and cell membrane damage (Rosso *et al.*, 2005), which could manifest in the growth parameters measured in this study.

In this study, stem elongation seems to be a parameter more tolerant to Cd and salinity, when compared with weight, 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 concentration. Despite of the careful taken when mounting the experience, to select plants with identical lengths, it was noted that for the treatment of higher salinity and higher Cd concentrations, i.e., salinity 10 and 50 and 100 $\mu\text{g.l}^{-1}$, the plants had a greater length and consequently, a higher biomass. The negative relation observed between initial length and growth increment, could be explained with the fact that the rate of plant growth is greater when the plant is younger, since their own metabolism and therefore the ability to absorb nutrients is more intense in the growth phase.

Considering only the effect of increase of salinity, i.e. in absence of Cd, stem elongation in general decreases. This is in agreement with other study on the effect of salinity on the growth of *S. ramosissima* (Silva *et al.*, 2007), that states that some halophytes develop better under non-saline conditions, despite their salinity tolerance.

According to Vassilev and Yordanov (1997) Cd conduct to a decrease in turgor potential and cell wall elasticity, resulting in smaller size of leaf cells formed with smaller intercellular space area (Ghnaya *et al.*, 2004). 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 $\mu\text{g Cd.l}^{-1}$. 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, 1997). Although *S. ramosissima* is a halophyte that develops well in low or moderate salinity

(Silva, 2007) and despite the salinities used in this study, the synergistic effect, once more, was involved.

Interestingly, Cd concentrations accumulated by plants, in each portion considered in this study (roots and aerial portion) do not always reflect the contamination levels administered at each treatment and no significant differences between treatments were observed.

This study showed that Cd accumulation in roots by *S. ramosissima* generally decreased with increasing salinity (Figure 13). The likely reason is the decreased availability of the Cd in the growth medium because of the complexes formed between chloride and metals (Förstner, 1979). Plants uptake Cd mainly in the form of Cd^{+2} into the cells. 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* and *Elodea canadensis* and *Potamogeton natans* (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). As previously mentioned, plants had a greater length and consequently, a higher biomass, on treatments at salinity 10 x 50 and 100 $\mu g Cd.l^{-1}$, and this fact might be related with the lowest Cd accumulation observed in the plants of those treatments. Apparently, plants with lower biomasses had higher metal accumulations than did plants with higher biomasses. This may be because a proportionally larger absorption area in relation to the external metal concentration may result in a dilution effect and thus a low internal metal concentration. Ekvall and Greger (2003) showed that this was the case for Cd in *Pinus sylvestris*, and Fritioff (2005) showed the same for *Elodea canadensis* and *Potamogeton natans*.

However, neither growth nor Cd accumulations by plants were significantly affected by salinity.

The uptake of essential elements may increase during the growth of the plant and their concentrations 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) channels has been pointed out as a way of Cd absorption by roots (Raskin and Ensley, 2000).

In the present study, a general decrease of Cd accumulation also occurred with the increase of Cd concentration, which is in disagreement with Ozawa (2009), that states that the amount of Cd in *S. europaea* increases with the concentration of Cd in the medium. However, the decreased tendency observed in this study could be explaining by the synergistic effect of salinity and Cd on plant metabolism. Nevertheless, once more, there was no significant effect detected on the accumulation of this heavy metal by the plants in this study.

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). Most metals tend to accumulate in the roots rather than in shoots (Fitzgerald et al., 2003). This could explain the larger bioaccumulation of Cd on roots of *S. ramosissima* (Figure 14) for all treatments, with emphasis on the value of 215.78 mg Cd.kg⁻¹ root dry weight on plants submitted to salinity 0 x 50 µg Cd.l⁻¹. Unexpectedly, the highest Cd accumulation on aerial portion, corresponding to a value of 9.99 mg Cd.kg⁻¹, was observed in salinity 5 x 0 µg Cd.l⁻¹, 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; it could be related with the accumulation of Cd in the Óbidos lagoon by plants, however, the Cd analyses on the references 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. However, and since for most of the treatments it was not possible to determine the accumulation of Cd in the aerial portions (since the concentration is below the detection limit of the recording equipment used), it is impossible to make any explanations related with the Transportation index; an interference occurred during the procedure applied for the

determination of Cd, namely on the acid digestion or in Atomic Absorption, is also a possibility that cannot be excluded.

The results of the dissolved and suspended Cd of the treatments solutions analyses at the end of the experiment and the Cd present in the reference plants (collected in the same sampling site and submitted to the same procedures for Cd determinations) did not contributed to clarify the observed absence of consistence in the results.

In the case of the presence of Cd in the suspended matter, the highest value was observed in the treatment salinity 0 x 50 $\mu\text{g Cd.l}^{-1}$, with 0.12 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 the Cd. For the other treatments (salinity 5 x 100 $\mu\text{g Cd.l}^{-1}$ and salinity 10 x 50 $\mu\text{g Cd.l}^{-1}$) 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 salinity 5 x 50 and 100 $\mu\text{g Cd.l}^{-1}$ and salinity 10 x 50 $\mu\text{g Cd.l}^{-1}$. Without excluding the possibility that contamination have occurred (which 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.

5. Conclusion

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.

If plant development is affected, so it is the Cd accumulation capacity of *S. ramosissima*, which appears to decrease with the increase of Cd concentrations.

Regarding the Cd compartmentation within the plant, the Cd accumulation occurs especially in the roots, where the concentration largely exceeds that detected in the aerial portions.

Based on these results, It can be concluded that this particular wetland specie can be successfully used for phytoremediation, namely on phytoaccumulation and phytostabilization, since plants were able to bioaccumulate Cd in plant roots, acting like a sink for this metal and preventing it from becoming available to other organisms. However, 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.

6. Future Perspectives

The halophyte plant *S. ramosissima* was not efficiency for bioremediation of cadmium in the highest conditions of salinity tested. As such, further studies could contribute for a much better understanding of their capabilities as a possible phytoaccumulator in coastal lagoons.

1) *Salicornia. ramosissima* could be studied for phytoaccumulation of other heavy metals, like Al, Cr, Cu, Fe, Mn, Ni, Pb and Zn. These other metals in the presence of the salinities (inferior or superior to salinity 10.0) could present a different result in terms of accumulation in a similar greenhouse experiment.

2) The presence of cadmium in sediments of the Óbidos lagoon has also been studied (authors, unpublished data). The amount of salt in the sediments could also be interesting to analyse, in order to verify the accumulation of cadmium or other heavy metals in accordance with salinity variations.

3) Other values of cadmium concentrations could be an option for the optimization of the phytoremediation technique with *S ramosissima*, more close to the MAC established for surface waters (but impossible to apply in brackish or marine water systems).

4) The analysis of the roots of *S ramosissima* and the identification of symbiotic microorganisms could be important to better understand the bioremediation possibilities that the halophyte plant presents.

5) Studies in greenhouse experiments, with *S. ramosissima* associated with other hyperaccumulator halophyte plants (such as *Scirpus maritimus*, also found in Óbidos Lagoon), could be interesting to analyze if the potential of bioremediation is more efficient by combining the effects of different species.

6) The study of the release rate of Cd from contaminated organic matter in decomposition, to assess the significance of this phenomenon in a subsequent contamination, considering the life cycle of *S. ramosissima*.

7) The monitoring for accumulated metal levels and different biochemical responses of *S. ramosissima* induced by the stress due to heavy metals accumulation, namely peroxidative damage and antioxidant (enzymatic and non-enzymatic).

8) To identify the common *S. ramosissima* herbivores and to understand their impact in plant development and survival, and therefore in heavy metals bioaccumulation capacity.

Given the above, 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.

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