Phytoremediation of cadmium at different salinities by **Scirpus maritimus** *from the Óbidos Lagoon (Portugal)*

Márcia Sofia da Silva Santos

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Phytoremediation of cadmium at different salinities by **Scirpus maritimus** *from the Óbidos Lagoon (Portugal)*

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This thesis is dedicated to my parents,
my grandmother,
my family
and friends.

Resumo

Este estudo teve como objectivo analisar se a fitoremediação de cádmio pode ser feita usando a macrófita *Scirpus maritimus* da Lagoa de Óbidos (Portugal), para estudar a influencia da salinidade da água na sua eficácia como planta acumuladora de metais pesados. Duas concentrações de cádmio foram testadas (50 e 100 $\mu g\ l^{-1}$) para avaliar a capacidade de acumular cádmio pela planta, além de efeitos de toxicidade do cádmio. Os níveis de contaminação foram testadas em diferentes condições de salinidade da água (valores iguais a 0.0, 5.0, 10.0 e 20.0), em que as plantas estão normalmente submersas no seu ambiente natural.

A mortalidade de S. *maritimus* foi principalmente determinada pela salinidade da água e não pela contaminação de cádmio, em que as plantas morreram em águas com salinidades mais elevadas. A presença de novos rebentos, o comprimento das plantas e o incremento de biomassa não foram afectados por qualquer desses fatores, no entanto, o comprimento da planta, a redução e perda de biomassa podem ser induzidos pelo aumento da salinidade da água.

O desenvolvimento de um biofilme em todos os vasos deste estudo foi observado independentemente do tipo de tratamento envolvido. Esses microrganismos em suspensão e matéria orgânica senescentes apresentaram mais cádmio, de acordo com os valores iniciais presentes na água. A concentração de cádmio dissolvida na água foi positivamente relacionada com os valores de contaminação escolhidos para este estudo, mas o aumento de cádmio foi proporcional ao aumento da salinidade da água, possivelmente um resultado da degradação de tecidos e da ruptura de plantas mortas, potenciado também pela presença de organismos simbiontes, também encontrados no biofilme. Alguns destes podem promover a decomposição de tecidos e a biodisponibilidade de cádmio dissolvido na água.

As plantas apresentaram mais cádmio nas raízes, seguido dos caules e das folhas. A maior acumulação de cádmio nas raízes foi um resultado que variou de acordo com o aumento de salinidade em águas contaminadas. Os resultados indicaram que a salinidade pode influenciar o transporte de cádmio das raízes para as folhas em *S. maritimus*. Contudo, a fitoremediação da Lagoa de Óbidos com esta planta pode não ser possível, devido ao stress provocado nas plantas pela salinidade, que interfere com a acumulação de cádmio e com a normal fisiologia das mesmas. A optimização de processos de

fitoremediação com *S. maritimus* pode auxiliar na bioremediação de ecossistemas de água doce.

Palavras-chave: biorremediação, plantas acumuladoras, metais pesados, planta halófita, espectrometria de absorção atómica, sapal

Abstract

A greenhouse experiment was performed, to verify if phytoremediation of cadmium can be done by using the macrophyte *Scirpus maritimus* from the Óbidos Lagoon (Portugal), plus if water salinity can influence its phytoremediation effectiveness. Two concentrations of cadmium were tested (50 and 100 μg l^{-1}) to evaluate the plant capability to accumulate cadmium, plus possible toxicity effects of this pollutant. The contamination levels were tested at different water salinity conditions (values equal to 0.0, 5.0, 10.0 and 20.0), in which the plants are usually submerged in, at their natural environment.

S. maritimus mortality was mostly determined by the water salinity and not for the cadmium contamination, in which more plants died at higher water salinities. The surge of new shoots, plant length and biomass increments were not proved to be affected by either of those factors, however, plant length and reduction and biomass loss can be induced by increasing water salinities. There was a biofilm development in all trial vessels, independently of the type of treatment involved. These suspended microorganisms and senescent organic matter presented more cadmium, according to initial water level of cadmium. The amount of water dissolved cadmium was positively related to the initial contamination levels of the experiment, but it increased also at higher water salinities. This may have resulted from tissue degradation and disruption from dead plants, possibly potentiated as well by symbiotic organisms and those from the biofilm, some of which may have promoted tissue decomposition and bioavailability of dissolved cadmium. The plants presented more cadmium in the rhizomes, followed by the stems, and less in the leaves. More cadmium accumulated in the plant's rhizomes, according to higher water contamination levels and lower water salinities. According to the results, salinity could influence the transport of cadmium in the plant, between roots and leaves.

However, the phytoremediation of the Óbidos Lagoon by *S. maritimus*, or other similar systems, may be not be possible, due to the salt stress of the plants, the salt interference with the cadmium accumulation, and the normal functions of a plant. But the optimization of phytoremediation processes by *S. maritimus* could turn possible the use of this plant in freshwater ecosystems.

Key words: bioremediation, accumulator plant, heavy metal, halophyte plant, absorption atomic spectrometry, salt marsh

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Annex II - Post-hoc tests results from the relations between salinity, cadmium concentrations ([Cd_0], [Cd_{50}], [Cd_{100}]) and the cadmium concentrations *versus* the amount of suspended organic matter and cadmium concentrations evaluated dissolved in water, within suspended particles, plus *S. maritimus* leaves, stems and rhizomes. The following symbols stand for: [S × Cd] - salinity and cadmium concentration($\mu g\ l^{-1}$) variable; df - degrees of freedom; MS - Mean Square

Introduction

1. Environmental Biotechnology

1.1. Bioremediation in water environments

In the last years, Biotechnology has been considered one of the most promising scientific areas of development. It combines knowledge from several sciences, applying biological organisms, or a concrete property of theirs, to technological use for different purposes. In the case of recovering endangered ecosystems, it is classified as Environmental Biotechnology, usually referred to as "Green Technology" (Kjelleberg, 2002).

There is a narrow line between Ecology and Environmental Biotechnology. Pollutants such as heavy metals, persistent organic/synthetic pollutants, polycyclic aromatic hydrocarbons, volatile organic compounds, environmental xenobiotics, as well as nutrients (like nitrogen and phosphorous) destabilize the biotic and abiotic interactions between organisms and their environment, when they are present in high concentration in water, soil and air (McMahon *et al*, 2007). The purpose of Environmental Biotechnology is to apply biological techniques, such as bioremediation, to control the concentration of toxic compounds, by using several organisms, primarily microorganisms to degrade several environmental contaminants to their less toxic forms, to safeguard human health and the environment (Vidali, 2001; Grommen & Verstraete, 2002).

Bioremediation is also a procedure applied in environmental monitoring, using bioremediation techniques *in situ* (Scrag, 2005). With organisms used as bioindicators, it is possible to identify and quantify the effects of pollutants, like heavy metals. This organisms presenting a geographical wide distribution, exist in the local subject of the environmental study, capable of providing quantitative information of the presence, amount and intensity of the exposure to a pollutant (sensibility for a specific contaminant) (Scrag, 2005).

Bioindicators can be able to allow evaluating the bioaccumulation (the accumulation of a certain substance in organic tissues) and the biomagnification (the increase of a given pollutant within the tissues of successive organisms from a food chain) of certain pollutants (Scrag, 2005; Gomes 2007). These results are important to establish the impact of contaminants in different organisms and ecosystems. (Gomes, 2007).

1.2. Phytoremediation

Phytoremediation ("phyto" = plant + "remedium" = clean or restore) is part of bioremediation processes, developed in Ecology and Environmental Biotechnology studies, through the use of plants with certain abilities to control pollutants (such as metals, pesticides, oils, etc) (Prasad & Freitas, 2003). This ecological procedure is considered a cost-effective “green” technology and environmental friendly. The handling of pollutant-accumulating plants presents less costs and waste volume than those from other procedures, like incineration. This remediation technique can be used to remove or degrade both organic and inorganic pollutants present in different substrates (air, liquid - e.g. water, or solid – e.g. soil/sediment (Raskin *et al*, 1997; Sousa *et al*, 2010).

Phytoremediation consists in different plant-based technologies, each having a different mechanism of action, for the remediation of polluted soil, sediment, or water, such as those contaminated by heavy metals (Kavamura *et al*, 2010; Sousa *et al*, 2011). Among those mechanisms are: 1) phytoextraction, that consists in adsorption of soil contaminant by the roots, subsequently stored and accumulated in the harvestable tissues; 2) rhizofiltration, which refers to the approach of using hydroponically cultivated plant roots to remediate contaminated water through absorption, concentration, and precipitation of pollutants; 3) phytostabilization, it concerns the reduction of mobility and bioavailability of contaminants by plants roots, which will stabilise and reduce pollutant transfer to other ecosystem compartments; 4) phytovolatilization, it is used for the removal of pollutants by plants into volatile compounds released to the atmosphere by evaporation; 5) phytoaccumulation, which results in accumulation of contaminants in plants’ biomass; 6) phytodegradation, consists in the rhizosphere degradation of pollutants into insoluble or non-toxic compounds, through metabolic processes and through interaction with microorganisms (Kavamura *et al*, 2010; Sousa *et al*,2011).

Plants are good candidates for bioremediation (especially phytoextraction), since they present a rapid growth, have profound roots in soil/sediment and are able to accumulate the pollutants. Plants can be divided into categories, according to these purposes: 1) accumulator - which can concentrate pollutants in their above-ground tissues to levels far exceeding those present in the soil or in the non-accumulating species growing nearby; 2) indicators – which accumulate contaminants concentrations in different plant tissues above-ground, generally reflecting the environmental levels *in situ*; or 3) excluders – which effectively prevent pollutants from entering the aerial parts, over a broad range of pollutant concentrations in the surrounding environment, although they can still contain large amounts in their roots (Ghosh & Singh, 2005; Lal, 2010).

Other criteria are also important to chose bioremediating plants: 1) the concentration of pollutants in the plant cells must be 10 to 500 times higher than the values found in the same species from non-polluted environments; 2) the contaminant concentration ratio between shoots and roots must be invariably higher than one. This indicates an efficient ability to transport pollutants from roots to shoots and, probably, the existence of tolerance mechanisms to support high concentrations of those contaminants; 3) the shoot/soil concentration ratio must be also higher than one, that indicates the increasing degree of contaminant uptake (Cheraghi *et al*, 2011). For instance, heavy metals' concentration in shoots needs to be superior to 1 000 mg kg^{-1} of copper, cobalt, chromium, nickel and lead, or 10 000 mg kg^{-1} of iron, manganese and zinc, in order to consider a certain plant as a metal accumulator. In the case of cadmium, accumulator plants present 100 mg kg^{-1} dry weight of that metal (Peer *et al,* 2005; Sun *et al,* 2008) .

Despite the promising possibilities and applications of phytoremediation, there are still some downsides to be resolved for a better understand of pollutants accumulation. Phytoremediation is a slow procedure for several reasons: 1) contaminants' concentration present in the water and sediment can be toxic to the plants, inducing sometimes death, which plants are most effective at remediating a given pollutant (Prasad & Freitas, 2003); 2) preliminary *ex situ* experiments to try phytoremediation present the first adversity of how to maintain the plants alive; 3) secondly, low plant biomass in these experiments may under- or overestimate phytoremediation results (Ghosh &Singh, 2005; Cheraghi *et al,*

2011); and when the procedures are established; 4) it can take many growing seasons to clean up a site; 5) the plants that absorbed toxic materials may contaminant the food chain, turning them bioavailable again after being inaccessible in deeper sediment/soil layers; 6) the volatization of compounds can transform a groundwater pollution problem to an air pollution problem; and 7) hydrophobic contaminants, which bind tightly to soil, are not efficiently removed by plants (Belz, 2007).

2. Environmental impacts of heavy metals contamination in coastal Lagoons

2.1. Heavy metals contamination in aquatic environments

Water environments are rich in different nutrients and metals, introduced by natural inputs, but also effluents from industrial, municipal wastewaters, landfills, agriculture, etc. Compounds like heavy metals can be harmful for the environment, when present in large quantities (Carvalho, 2006). Heavy metals are chemical elements with a specific gravity that is at least 5 times the specific gravity of water. They are pervasive and persistent metallic elements that are present in their elemental and organic/inorganic form. Heavy metals are dissolved in water, as vapour, in rocks as a mineral and in particulate forms. They have a relatively high density and they are toxic even at low concentrations. (Duruibe *et al*, 2007). In high concentrations can form unspecific compounds with cytotoxic effects for organisms. Heavy metal are sulphides, such as iron, arsenic, lead, lead-zinc, cobalt, gold silver. They cannot be degradated biologically, they can only be transformed from one oxidation state or organic complex to another (Lone *et al*, 2008). In general, iron, manganese and molybdenum are important micro-nutrients, while zinc, nickel, copper, cobalt, and chromium are toxic elements for plants. The elements cadmium, lead, mercury, and others are not involved in nutrient function (Benavides *et al*, 2005; Carvalho, 2006; Sousa, 2010).

The transportation of heavy metals trough rivers and streams is made under the form of either accumulated in aquatic organisms or in suspended sediments. The fate of pollutants transported in shallow waters is regulated by resuspension and deposition, both physical processes that strongly depend on tidal currents and wind, which have low expression in some Lagoon system (Kowalski, 2009). The contamination of the soil and

water resources with heavy metals results in damages to the ecosystems, leading to the loss of agricultural productivity, food chain deterioration and to human and animal health problems (Cheraghi, 2011).

2.2. Cadmium

Cadmium has been one of the most studied heavy metals. This non-essential metal is naturally present in the soil, in low concentrations; however it is extremely pollutant due to its great solubility in water and high toxicity for biological organisms. Benavides *et al*. (2005) established that cadmium did not occur in isolated forms at natural environments, but that it was associated with lead and zinc mineralization instead. This metal can also interfere with plants' nutrient uptake, transport and use of water, plus of other elements. According to Ghosh & Singh (2005), total cadmium concentration over 8 mg kg^{-1} dry weight, or bioavailable in a soluble form that exceeds 0.001 mg kg^{-1}, is toxic for most plants. The bioaccumulation of cadmium will depend on the soils' pH, redox potential and on the chemistry of roots.

The Portuguese legislation, plus other international environmental quality regulations, contemplates rules to control pollution and establish maximum levels of concentration for several pollutants in water, soil and air, in order to protect public and environmental health. Cadmium is classified as a priority substance according to the European Union Water Frame Directive (EU-WFD; Directive 2000/60/EC), which means that it is a substance that represents significant risk to the aquatic environment or others by its intermediation.

The Portuguese Decrete Law 103/2010 (which transposes the Directive 2008/105/EC to Portuguese National Law) states that superficial waters should not present cadmium above 0.45 – 1.5 µg l^{-1}, the Maximum Allowed Concentrations (MACs) for the lowest and highest water hardness classes. Atomic absorption spectrometry is the most usual procedure to determinate metals and metalloids' concentration in water and sediment samples. The most used methods are Flame Absorption Spectrometry, with a sensitivity to analyse concentrations in the order of mg l^{-1} (ppm –parts per million), and Graphite

Furnace Absorption Spectrometry, which allows detecting concentrations in the order of $\mu g\ l^{-1}$ (ppb – parts per billion).

2.3. Heavy metals contamination in coastal Lagoons

Coastal Lagoons are semi enclosed water bodies, influenced by tides and small rivers flows. They are very important water ecosystems, which communicate with the ocean through a permanent or intermittent barrier (Kjerfve, 1994). These ecosystems are brackish water environments, presenting a wide range of salinity values, varying from fresh (up to 0.5 salinity) to salt water (≈ 35.0 salinity) (Carvalho, 2006). They detain a particular fauna and flora, which endure extreme fluctuations of environmental conditions associated with the mixture of fresh and salt waters. Coastal Lagoon systems are responsible for elevated production of organic matter introduced in the oceans. Nerveless, the sediments and suspended matter that are fixed in the bottom are retained by filter organism and plants, like halophyte plants, distributed in important areas of preservation. At the same time, coastal Lagoons are also important nursery areas of several species. Vegetation within coastal systems can be classified as: reeds, marshes, meadows and salines (Carvalho,2006).

Salt marshes are found in brackish water systems, especially coastal Lagoons. (Reboreda & Caçador, 2007). They consist in an interface between terrestrial coast and maritime environment, extended around the coast and to the inside of estuarine systems. Salt marshes provide several benefits and goods, such as protecting the coastline from erosion. These places of great ecological diversity are also responsible for elevated productivity (values could exceed 2000 $g\ m^{-2}\ year^{-1}$), but lower specifies diversity (Silva, 2000).Such ecosystems involve several physical, chemical and biological activities, that results in unstable salt marshes, where factors like temperature, salinity, dissolved oxygen and others are very variable (Silva, 2000). Salinity is an important parameter that can influence salt marshes vegetation. High concentration of salinity in aquatic environments can affect plant growth and suppress shoot growth. If the values of salinity are inferior to 5.0, plants found in these areas will be characteristic from freshwater systems (Silva, 2000; Reboreda & Caçador, 2007), otherwise, halophytes will be the most representative plants of the salt marshes.

Halophytes are plants that tolerate high water/soil/sediment salinities (near or higher than 35.0), contrarily to glycophytes, that are damaged when submitted to those conditions. Plants that avoid the effects of salt (even though they live in a saline environment) may be referred to as "facultative halophytes" rather than "true" or "obligatory halophytes". Plants can minimize sodium (Na^+) uptake and transport to the areal parts, in order to avoid sodium chloride (NaCl) toxicity. The plants present such an osmotic balance, which is responsible for the adaptation, survival, growing conditions and energy expenses. (Silva, 2000; Reboreda & Caçador, 2007). Na^+ can be mostly accumulated in the leaves of plants (80%), but halophytes have other mechanisms to reduce salt levels: 1) accumulation in vesicular trichomes; 2) salt gland secretion; 3) lost of old leaves or 4) salt transfer to other organs (Silva, 2000). Accordingly, halophytes are promising candidates for soil desalination. These plants occur according to the salt marsh's topography, physical and chemical properties of sediment and interspecific competition conditions (Ghnaya *et al*, 2005; Sousa *et al*, 2010).

Salt marshes are classified as sensitive habitats under the European Habitats and Birds directives (European Directive 92/43/EEC; Portuguese Decrete Law 149/2004; European Directive 2009/147/EEC;). Heavy metals in aquatic environments are distributed in the sediments, water and plants. Such is the case of halophyte plants (Reboreda & Caçador, 2007). Halophyte plants accumulate heavy metals mostly in roots, but some are able to translocate them into the leaves and stems. The translocation of metals within plants depends on the mobility and disposal of heavy metals in the sediments. The availability and plant uptake of heavy metals will dependent of the pH, salinity, redox potential, organic matter content and t grain size of the sediments (Reboreda & Caçador, 2007). For example, metals like manganese, cadmium and zinc accumulate more within the aerial parts of the plants, because they are more mobile in the cytoplasm. The concentration a given heavy metal present in the different parts of a plant varies from species to species, causing different degrees of toxic effects and perturbations of the plants' physiology.

Phytoremediation using halophytes could be a possible bioremediation technique to apply in heavy metal contaminated coastal environments. Salt marshes are a source of plants for phytoremediation of metals, because of their capability to accumulate heavy

metals in their tissues, especially in roots cells. Those plants can contribute to the reduction of metals' bioavailability in the sediments. Heavy metals are bound to sulphides in anoxic sediments and plants are able to oxidize them in the roots, moving oxygen downwards through the aerenchyma pathway. This oxidation can remobilize metal contaminants, otherwise entrapped in the sediments, and taken the proper actions, their concentration can be decreased in the affected ecosystem. (Weis & Weis, 2004; Reboreda & Caçador, 2007). Numerous plant species have been identified and tested for their traits in the uptake and accumulation of different heavy metals for the recovery of several ecosystems. Phytoremediation using halophytes is being applied to try recovering polluted coastal Lagoons (Madejón *et al*, 2006; Lone *et al*, 2008);

2.4. Phytoremediation of cadmium by *Scirpus maritimus*

The presence of cadmium in water and sediments from salt marshes Lagoon is important to evaluate and to monitor. The halophyte plants present in salt marshes has been studied for phytoremediation, in order to study heavy metal Lagoon contamination (Madejón *et al*, 2006; Vardanyan & Ingole, 2006; Momudu & Anyakora, 2010; Cheraghi,2011; Marques *et al*, 2011). However, the relationship between heavy metal accumulation and salt-stress in plants remains poorly understood (Ylmaz, 2007)**.**

The plant *Scirpus maritimus* (Figure 1) is a facultative halophyte plant. This plant can be found in Europe, North America and South Africa, at wet marsh flats, seasonal and permanent wetlands, pond margins and estuaries (Shupping, 2008; Marques *et al*, 2011). *S. maritimus* belongs to the Cyperaceae family. It is a possible option for phytoremediation studies, due to the general geographical distribution and ecosystem occurrence. This plant presents emergent rhizomes, is widely distributed in shallow brackish water bodies of temperate regions and can reach a height of 1.2 m (Shuping, 2008).

S. maritimus presents three phases of development: 1) a juvenile phase, followed by 2) a mature phase, when the density of shoots are higher/heavy weighted, and 3) a senescent phase, with a dominance of belowground plant parts (Marques *et al*, 2011). This plant is well adapted to low temperatures and high carbon dioxide levels. *S. maritimus* rhizomes have tubers (main reserves of carbohydrates for plant growth) that sprout in the

autumn (wet season) and emerge from the sediment at the at the beginning of winter. During spring, these monoic plants undergo sexual reproduction, forming flowers and fruits afterwards. They can also undergo vegetative propagation, which allows them to form dense populations. The marshes dry up in the summer; consequently tubers become dormant, buried in the sediment (Madejón *et al*, 2006).

This plant can survive under both saline and non-saline conditions, plus it can endure total submersion in the water. In the study of Lillebø *et al*. (2003), *S. maritimus* tolerated high salinities for short periods of time and senescence was induced after a long exposure to salinities above 15.0. They concluded that high salinities result in a decreased growth rate, emergency of new shoots, seed germination and survival rates of this facultative halophyte plant.

S. maritimus is a metal-accumulator plant, which can present elevated values of heavy metals in their rhizomes, followed by leaves and stems (Carranza-Álvarez *et al*, 2008). This distribution in the plant can be influenced by: 1) seasonality and translocation 2) demand for essential micronutrients; 3) roots limitation storage; or 4) salinity conditions, (Shupping, 2008). Weis & Weis (2004) used "phytostabilization" properties of *S. maritimus* to immobilize metals.

Figure 1- *Scirpus maritimus* in the salt marshes of the Óbidos Lagoon (**A**), and the respective stems (**B**) and flowers (**C**) characteristics .

3. The Óbidos Lagoon as a case study

The Óbidos Lagoon was chosen for this study, due to its ecological importance. The Óbidos Lagoon is located in the west coast of Portugal (Figure 2) and is one of the

largest coastal Lagoons in Europe (Carvalho, 2006). For several years, the Óbidos Lagoon was potentially exposed to different sources of contamination, being eutrophication its worst problem (Carvalho, 2006; Pereira *et al*, 2009a; Pereira *et al*, 2009b). Agriculture and livestock farming practices have been the largest sources for nutrients and pesticides, as well as several discharges from domestic and industrial sewages (sewage treatment plants and pig farms) from the towns nearby, like Caldas da Rainha, Cadaval and Bombarral (Carvalho, 2006).

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Given the above, the Óbidos Lagoon is a good system to perform bioremediation studies by phytoremediation. *Scirpus maritimus* is a plant that exists in the mouths of rivers that run into the Lagoon, being subjected to periodic submersions in brackish waters with high salinities, larger than 30.0 in the summer (authors, unpublished data). This plant is a known bioaccumulator of cadmium (Carranza-Álvarez *et al*, 2008) and, therefore, is a good candidate to test cadmium phytoremediation. Nevertheless, as a facultative

halophyte, how efficient can *S. maritimus* be in accumulating cadmium in different places of the Óbidos Lagoon, where salinities reach higher values?

4. Aim of the study

Bioaccumulation of cadmium by *Scirpus maritimus* plants from the Óbidos Lagoon was assessed in a greenhouse experiment. The essay developed in this work tried to understand ways to apply *S. maritimus* capacity to accumulate cadmium for *in situ* phytoremediation of this heavy metal in coastal systems. In the case of coastal Lagoons, salinity is one of the most relevant environmental parameters to influence organisms' distribution and physiology. Thus, the capacity of *S. maritimus* to accumulate cadmium was also evaluated at different salinities, according of the regimes observed in the Óbidos Lagoon.

An essay was conducted for 30 days, in which two concentrations of cadmium were tested (50 and 100 $\mu g\ l^{-1}$) according to the Maximum Allowed Concentrations referred in The Portuguese Decrete Law 236/98. These values were used to evaluate the capability of *S. maritimus* from the Óbidos Lagoon to accumulate cadmium, plus possible toxicity effects of this pollutant for this plant. The different cadmium contamination levels were tested at different water salinity conditions (values equal to 0.0, 5.0, 10.0 and 20.0), in which the plants are usually submerged in, at their natural environment. This was done to assess the cadmium accumulation efficiency of *S. maritimus* at different water salinities, in order to establish if it can be used as a phytoremediation plant in coastal Lagoons' salt marshes and, if so, in what extent.

To accomplish these goals, cadmium concentration in water, biofilm, rhizomes, stems and leaves was evaluated by Graphite Furnace Absorption Spectrometry, at the end of the experiment. Plus, plant mortality, elongation, biomass and formation of new shoots were also appraised. In the end, cadmium translocation index from rhizomes to leaves were estimated, as well as *S. maritimus* cadmium accumulation levels within its different plant parts (rhizomes, stems and leaves).

Materials and Methods

1. Study area

The Óbidos Lagoon (Figure 1) is located in the western central coast of Portugal. Is influenced by a transition-Atlantic Mediterranean, mesothermal and humid weather. It is a shallow coastal Lagoon, with an elongated form (4.5 km length, 1.8 km large), being the largest system of its kind in the Portuguese coast. It has a mean an area of 7 km^2 and a mean depth of 3 m. It is permanently connected to the Atlantic Ocean by a narrow inlet and presents a perpendicular position to the coastline (Carvalho, 2006).

The lower section of the Lagoon has several channels that cut through large sand banks. The bottom sediments are manly sand and the tides are not faster than 1 m s^{-1}. The sand barrier, in the middle section, is the result of mixed semidiurnal tide regime (two flood and two ebb tides, alternately), with a medium amplitude of 1 to 3 m, according to the annual season (Carvalho, 2006). There is a sediment deposition on the central Lagoon, due to a decrease in tidal water velocity, which leads to a progressive reduction of the average depth and the surface area (Fortunato & Oliveira, 2005).

The Lagoon has a surface area of 3.6 km^2 at mean sea level and 5.3 km^2 during a high spring tide. The bottom sediments are mud. The tidal ranges vary between 2 and 4 m, near the coastline, and between 1 and 2 m on the inner areas of the Lagoon. Ripple effects significantly the morphodynamics of the Lagoon, where the sea waves exceed 1 m high at the opening, during 88% of the time (Fortunato & Oliveira, 2005), preventing the water to run out of the Lagoon during low tides.

The upper Lagoon is divided into two arms: the Barrosa arm at southeast (maximum of 1.5 m depth) and the Bom Sucesso at southwest (4.5 - 5 m depth). In the Barrosa branch occurs an outflow of freshwater from the Barrosa Riverside and the Cal River (Pereira *et al*, 2009). In the Bom Sucesso arm, freshwater inputs result from the Ameal ditch. The Borraça River flows into a small puddle in the middle section of the Lagoon and the Arnóia River reaches the Lagoon in the area between the inner arms, This last river drains the main agricultural areas of the region, running an annual average flow

of 3 m^3 s^{-1}, being negligible in the summer (< 0.005 m^3 s^{-1}) (Pereira *et al*, 2009b), which represents 90% of the freshwater input into the Lagoon.

The Óbidos Lagoon presents different salinity values throughout the year, depending on the precipitation, plus income of fresh water and seawater into the Lagoon, during the different annual seasons. The salinity values registered near Barrosa and Bom Sucesso arms are usually lower than the remaining areas (Pereira *et al*, 2009b).

Figure 2 – Map of the Óbidos Lagoon, situated in the western coast of Portugal, along with the location of the sampling station where *Scirpus maritimus* plants were collected for the essay.

2. Sample collection and acclimation period

Scirpus maritimus plants were collected in the Óbidos Lagoon, in April 2011, near the mouth of the Arnóia River. Those plants were collected carefully. They were washed in the water from the collection site, to remove attached sediment, and then transported to laboratory, in plastic containers (Carranza-Álvarez *et al*, 2008). At the same time, a portable multiparameter probe (HANNA HI9828 analyser, Hanna Instruments, Vila do Conde, Portugal), was used to register the values of salinity, dissolved oxygen, temperature and pH.

At the laboratory, all plants were cleaned with distilled water, to remove slurry and epiphytic algae. Healthy non-damaged plants with similar age/size were chosen for the experiment (Almeida, 2006; Carranza-Álvarez, 2008). All plants were dried with paper towels, fresh weighted (Metler Toledo AB2045, Soquímica, Portugal) and measured (Almeida *et al*, 2004; Almeida *et al*, 2011).).

In order to avoid metal contamination, all the glass and plastic materials were washed for 24 hours with Derquim LM 02 (Neutral, phosphates free liquid, Panreac Química S.A.U., Spain), followed by a 24 hours bath in a nitric acid solution (25%) (HNO_3 69%, PA-ACS-ISO, Panreac Química S.A.U., Spain) and rinsed with ultrapure water. No metal utensils were used (Monterroso *et al*, 2003).

For this experiment, three plants of *S. maritimus* were put in perforated plastic cups (≈ 4 cm diameter), with 240 g of gravel in the bottom that covered the plants' rhizomes entirely (Figure 3). The gravel was previously burned at 500ºC for 6 h (Nabertheern Controler B170, VWR International), in order to eliminate possible organic (Lillebø *et al*, 2003), and washed with a 10% hydrochloric acid solution (HCL 37%, PA-ACS-ISO, Panreac Quimica) for 12 h, to avoid metal contamination. Each cup containing the plants were inserted in a single plastic vase (≈ 18 cm diameter), with 500 ml of artificial brackish water (ultra pure water plus sea salt; Tropic Marin, Germany) set for a salinity of 2.0, which was registered *in situ* at the time of the plants' collection. The plants were submerged until ≈ 1 cm high above the gravel (Lillebø *et al*, 2003). In total, 54 vases were prepared and placed in an acclimation chamber, with constant temperature (22.5 ± 1.0 ºC) and artificial light (Lustek, TLD 18W/765 daylight, Lustek Services, Australia), with a photoperiod of 16:8 hours of light:dark, at 11.5 ± 12.5 µmol photon cm^2 s^{-1} (PAR Sensor, Apogee Logan, UT). The acclimation endured one and a half months.

Throughout the entire acclimation and experimental periods, the water volume lost by evapotranspiration was compensated every 3 days, by adding a plant nutrient solution (Lillebø *et al*., 2003), with a source of nitrogen (N - 620 mg l^{-1}: calcium nitrate 4-hydrate PRS, Panreac Química S.A.U., Spain) and phosphorous (P - 94 mg l^{-1}: potassium di-hydrogen phosphate PA, Panreac Química S.A.U., Spain). At the same time, salinity, temperature (Seawater Refractometer HI96822, HANNA Instruments, Portugal) and pH

(Symphony SP 70P, VWR, International- Material de Laboratório, Lda, Portugal) were regularly monitored (Lillebø *et al*., 2003).

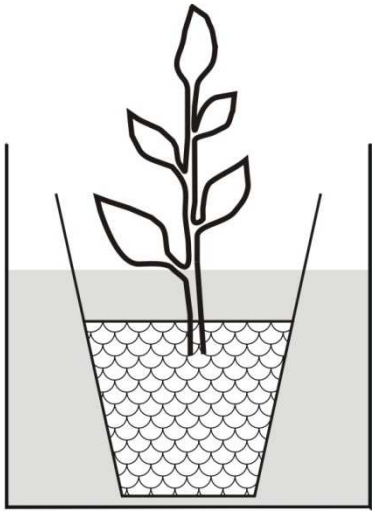

Figure 3 – Schematic representation of the container vessels used to acclimate *S. maritimus* to the experimental set-up.

3. Experimental design

To study the relation between salinity and cadmium absorption by plants, four salinities were chosen (0.0, 5.0, 10.0 and 20.0) and three cadmium concentrations (0, 50 and 100 $\mu g\ l^{-1}$). Brackish waters were prepared by adding the necessary quantity of sea salt (Tropic Marin, Germany) to ultrapure water, for obtaining the pretended salinities. Afterwards, those waters were contaminated, by adding the respective cadmium volume from a cadmium stock solution (cadmium nitrate, Scharlau Chemie S.A., Spain), for achieving the desired concentrations.

The [Cd] = 0 $\mu g\ l^{-1}$ was used for a reference situation of no cadmium contamination and [Cd] = 50 $\mu g\ l^{-1}$ was chosen for being the Maximum Allowed Concentration (MAC) for irrigation waters, according to the Portuguese Decrete Law 236/98. This document also states that residual water discharges should have an Emission Limit Value (ELV) = 200 $\mu g\ l^{-1}$, but it mentions that plants like beans, beets and turnips are affected by nutrient solutions with [Cd] = 100 $\mu g\ l^{-1}$. Therefore, this concentration was used to simulate a discharge with high concentration of cadmium, without risking extreme toxicity

for the plants. These treatments will be referred to as [Cd_0], [Cd_{50}] and [Cd_{100}], respectively.

The salinities were chosen according to the essay performed by Lillebø *et al* (2003), testing salinity as a major role in the annual dynamics of *S. maritimus*, which agrees with the range of salinities recorded in the field station where those plants were collected at the Óbidos Lagoon (authors, unpublished data). These treatments will be referred to as S_0, S_5, S_{10} and S_{20}, respectively.

During the acclimation period, all the aerial parts of the plants *S. maritimus* went senescent and new shoots were born. The vases were grouped according to similar number, weight and size of new shoots, for each experimental treatment (Table I) . The experiment began after the development of the new shoots, when these reached approximately 10 cm high (Lillebø *et al*, 2003). Three vase replicates were used for each combined treatment salinity x cadmium concentration. The aquaria were placed randomly and moved frequently to avoid temperature and light gradient effects.

Table I- Representation of the number of plants *S. maritimus*, with 3 replicates for each combined treatment.

Cadmium (µg l^{-1})					
		[Cd_0]	**[Cd_{50}]**	**[Cd_{100}]**	
	S_0	3 × 1	3 × 2	3 × 3	
Salinity	**S_5**	3 × 1	3 × 1	3 × 1	**Vessels × Plants**
	S_{10}	3 × 2	3 × 2	3 × 1	
	S_{20}	3 × 1	3 × 1	3 × 2	

4. Cadmium analyses

After the experiment, the water of each replicate was filtered with cellulose acetate membranes with 0.45 µm pore size and 47 mm ∅ (Whatman OE 67, Whatman GmbH, Dassel, Germany), under vacuum conditions (Rotavac Valve control, Heidolph Instruments, Germany), for the analysis of dissolved and suspended cadmium (Pereira *et al*, 2009b).

In order to determine the concentration of cadmium dissolved in the water samples, the filtered water was acidified with nitric acid 69% to a pH < 2. Then, the samples were analysed by Graphite Furnace Atomic Absorption Spectrometry, using a Thermo Scientific iCE 3500 Atomic Absorption Spectrometer (Thermo Unicam, Leça da Palmeira, Portugal), with a graphite furnace SOLAAR FS95 Furnace autosampler and a cadmium hollow cathode lamp (10 mA, Thermo Electron Corporation). Argon was the used gas (Praxair Portugal Gases S.A., Portugal). All the standard solutions were daily prepared for metal analysis, from a cadmium stock solution ([Cd] = 1000 mg l^{-1}, traceable to SRM from NIST Cd $(NO_3)_2$ in HNO 0.5 mol l^{-1}; CertiPUR®, © Merck KGaA, Darmstadt, Germany).

For the analysis of the suspended cadmium, the cellulose acetate membrane filters of the water samples were submitted to a nitric acid digestion. The filters were weighed, dried at 60ºC for 48 hours. Then they were digested in 3 ml of nitric acid (Hseu, 2004) and heaten at 200 ºC in a hotplate (VHP series C - 10, ceramic, VWR International-Material de Laboratório, Lda, Portugal). After digestion, the samples were filtered through ashless quantitative filter papers Whatman grade no. 41, (0.45 µm pore size, 55 mm ∅, Whatman™, GE HealthCare Company, UK) and diluted to a final volume of 50 ml, with ultra pure water. These samples were processed afterwards, in the same way as the water samples for dissolved cadmium.

At the end of the essay, all plants were removed from the sediment. They were fresh weighted (Mettler Toledo, AB 2045, Soquímica, Portugal), measured and then dried at 60 ºC, for 48 h (Memmert Drying UFB 500, Germany). The plants were divided into rhizomes, stems and leaves, with the help of a ceramic knife (MUSSLA, Inter IKEA Systems B.V, Sweden). All these parts were weighted and grinded to powder by using a glass mortar. Afterwards, they were submitted to an acid digestion (Bragato *et al*, 2006; Cambrollé *et al*, 2008). About 100 mg of plant sample was subjected to acid digestion with 3 ml of nitric acid 69 % added twice, at 150 ºC (HSeu, 2004). After the digestion, 3 ml of nitric acid 1 %, were added, to stabilize the samples. The volume obtained and was diluted with ultra pure water, until 50 ml. Later on, they were processed in the same way as the acetate membrane filters for the quantification of cadmium.

5. Statistical analyses

The transportation index (Ti) evaluates the proportion between leaf and rhizomes metal concentrations and depicts the plants' ability for translocating the metal ion form from rhizomes to leaves, in the presence of several heavy metal concentrations (Ghosh &Singh, 2005). I is calculated as $\mathrm{Ti} = \frac{[\mathrm{Cd}]_{\mathrm{leaves}}}{[\mathrm{Cd}]_{\mathrm{rhizomes}}} \times 100.$

The accumulation of cadmium within the rhizomes, stems and leaves' tissues was determined as being the difference between the cadmium concentrations of plants subjected to contaminated water ($[Cd_{50}]$ and $[Cd_{100}]$) and those from the [Cd] = 0 μg l^{-1}, used for a reference situation of no cadmium contamination. So, $\mathrm{Cadmium\ accumulation} = [\mathrm{Cd}_{>0}] - [\mathrm{Cd}_0]$.

Statistical analyses were performed using the IBMM SPSS 19 software package. The One-Way ANOVA was applied to the relation between cadmium concentrations (0, 50, 100 μg l^{-1}) and salinity established for the mortality results of *S. maritimus*. For this factors were realized Two-Way ANOVA, with post-hot testes: Tukey HSD, LSD and Bonferroni tests. These statistic analyses were also used to evaluate the relationship with other parameters: number of shoots and biomass of *S. maritimus*. The same tests were also applied for the cadmium concentrations measured dissolved in the brackish water samples (μg l^{-1}), in the water suspended matter and in the plants rhizomes, stems and leaves (mg kg^{-1})."A Kolmogorov-Smirnov Test was performed to test the normality distribution for cadmium found in roots, stems and leaves of *S. maritimus*, with several transformations (Log, Log_2, Square root and Ln). These transformations were made in agreement with Underwood (1997).

Results

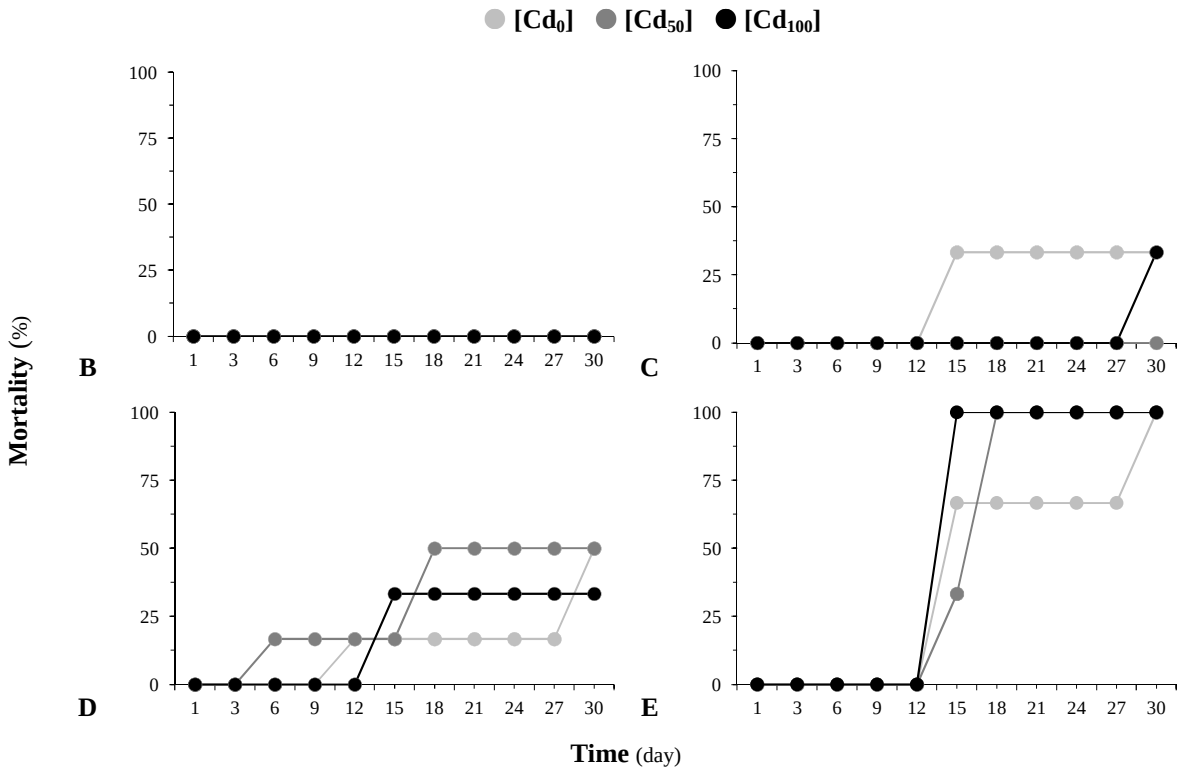

Figure 4 – Relative frequency of *S. maritimus*' cumulative mortality for the cadmium concentration treatments [Cd_0], [Cd_{50}] and [Cd_{100}], submitted to four salinity treatments S_0 (**A**), S_5 (**B**), S_{10} (**C**) and S_{20} (**D**), throughout the month in which the essay was conducted (mean; n = 3).

After one month, *S. maritimus* mortality showed a gradient dependent on the salinity values and not on cadmium concentrations, statistically supported by a two-way ANOVA (Table II). Higher plant mortality was observed at higher salinities. No plant mortality was observed in all cadmium treatments at salinity S_0 (Figure 4A). However, all plants died at S_{20}, after the 18th day, except for one plant of [Cd_0] that lasted until the 27th day (Figure 4E). Plants in vessels S_{10} and S_5 (Figure 4C and 4D, respectively) start to died at different days in the presence of different cadmium concentrations, reaching no more that 50% mortality.

After the beginning of the experiment, new shoots started to appear within the different treatments (Figure 5A). The treatments [Cd_{50}] and [Cd_{100}] from the two lower salinities, presented the highest number of new shoots. These shoots were not measured or

analysed, once that they went senescent almost immediately after their appearance. A two-way ANOVA test (Table II) did not reveal any influence of salinity and cadmium (individually and combined together) in the appearance of new shoots, although it seems to be a tendency for being most abundant at lower salinities, within treatments with cadmium.

Table II – Two-way ANOVA tests results for the greenhouse experiment with *S. maritimus* to test the relations between salinity, cadmium concentrations ([Cd_0], [Cd_{50}] and [Cd_{100}]) and plant parameters as factors. The following symbols stand for: S × [Cd] - salinity and cadmium concentration variable; df - degrees of freedom; MS - Mean Square.

Two-way ANOVA	**Source of variation**			
Experiment parameters	**df**	**MS**	**F-statistic**	***p-Value***
Mortality (%)				
Salinity × death plants	3	1.806	16.250	0.000
Cadmium × death plants	2	0.111	1.000	0.383
[S × Cd] × death plants	6	0.111	1.000	0.448
Shoots				
Salinity × shoots	3	0.296	0.593	0.626
Cadmium × shoots	2	0.861	1.722	0.200
[S × Cd] × shoots	6	0.380	0.759	0.609
Stem elongation (cm)				
Salinity × stem elongation	2	3.728	0.123	0.885
Cadmium × stem elongation	2	10.893	0.360	0.701
[S × Cd] × stem elongation	4	5.515	0.182	0.946
Plant Fresh weight (g FW)				
Salinity × plant weight	2	54.770	0.826	0.457
Cadmium × plant weight	2	40.304	0.608	0.557
[S × Cd] × plant weight	4	110.799	1.671	0.209

The *S. maritimus* length increment of all living plants (Figure 5B), at the end of the experiment, showed to be related with the size of the plants, analysed by Cubic Regression (length increment = -0.001 (initial length)^3 + 0,06 (initial length)^2 - 2,108 (initial length) + 24,152; R^2 = 0.679; N = 35) and a one-way ANOVA test (F = 21.856; MS = 196.029; df = 3; *p-value* < 0.05). Smaller plants showed a larger increment than the tallest ones. The plants' length increment showed no significant influence of salinity, cadmium, or any interaction between the two factors (Table II). The plants from S_0 treatments showed

increased length in all cadmium concentrations. Lower increases were observed in S_5 and S_{10} (although, plants from S_{10} × [Cd_{100}] verified noticeable increment and those from S_5 × [Cd_0], showed a tendency to shrink), all presenting a large value variation. No plant survived in S_{20} treatments, reason for which they were not measured, due to senescent processes that wizen and shrink the plants.

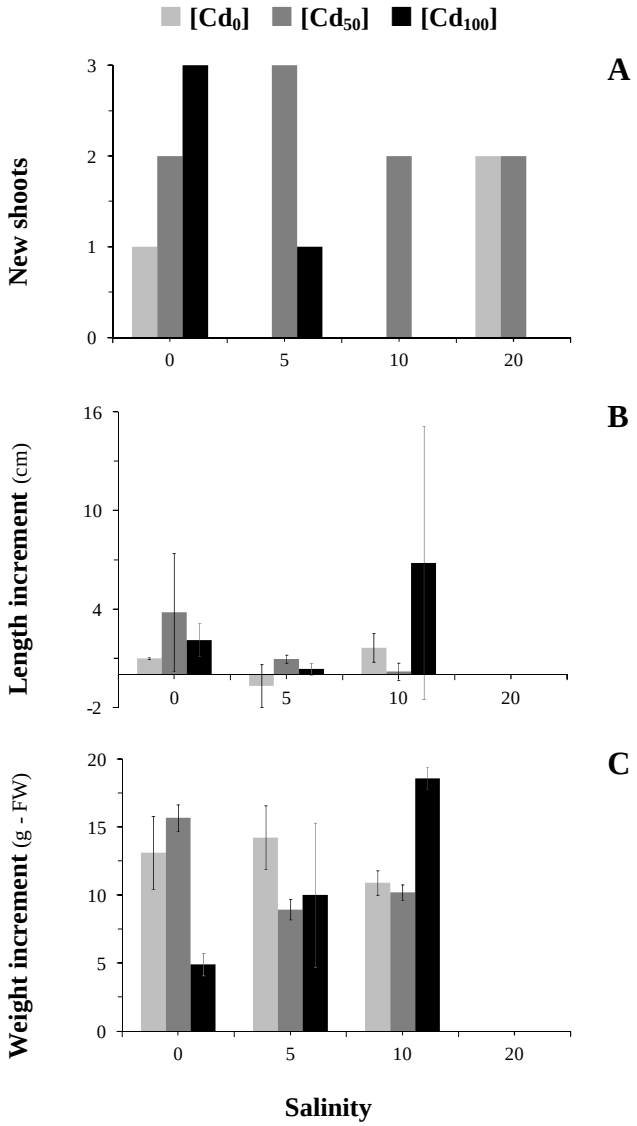

Figure 5 – (**A**) Number of new shoots *S. maritimus* observed in each treatment, since the beginning of the experiment and after the acclimation period (mean; n = 3). (**B**) Stem elongation increment of *S. maritimus* living plants in each treatment, since the beginning of the experiment (mean ± standard error; n = 3). (**C**) Biomass increment of *S. maritimus* (measured as FW – Fresh Weight) in each treatment, since the beginning of the experiment (mean ± standard error; n = 3).

All treatments presented a biomass increase (Figure 5C). The lowest increment of weight of *S. maritimus* occurred in the treatment S_0 × [Cd_{100}] and the largest in S_{10} ×

[Cd_{100}]. No tendency for the influence of salinity, cadmium or interaction between these two factors is perceptible. This is supported by the absence of statistically significant differences between the plants of the multiple treatments, tested by a two-way ANOVA (Table II).

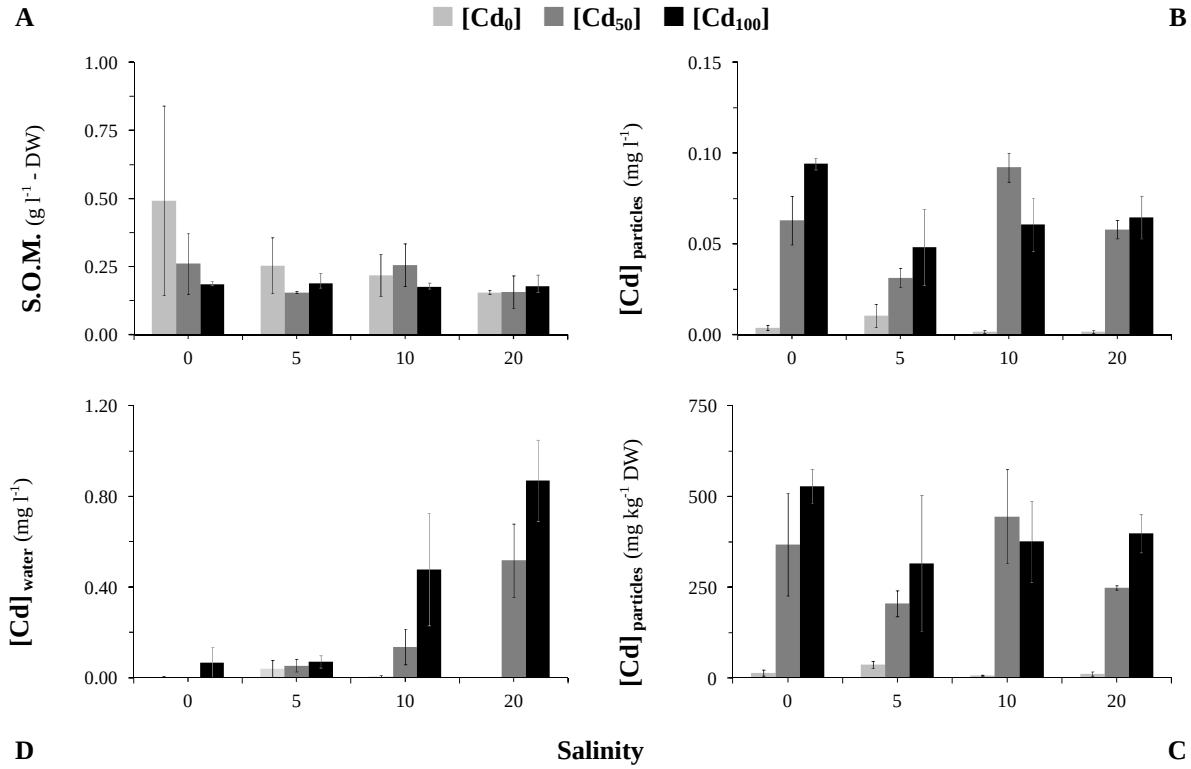

Figure 6 – (**A**) Suspended Organic Matter content (S.O.M.; measured as g l^{-1} DW - Dry Weight) in the trial water vessels, plus concentration of cadmium (**B, C**) in the suspended particles (in mg l^{-1} and mg kg^{-1}, for future comparison with similar works) and (**D**) dissolved in brackish water for each treatment at the end of the experiment. (mean ± standard error; n =3).

During the experiment, some organic matter formed itself in the water (Figure 6A). The values of suspended organic matter (S.O.M.) varied between 0.125 and 0.5 g l^{-1} of suspended organic matter. This matter resulted from microorganisms that live in symbiotic relations with *S. maritimus* and plant decay. In lowest salinity waters (S_0 and S_5), treatments [Cd_0] showed higher organic content, but this tendency tends to faint in higher salinities. No statistically significant differences were noticed between all the treatments (Two-way ANOVA, Table III).

The suspended particles were filtered and analysed for their cadmium content (Figure 6B and 6C). No salinity influence was noticeable, but there was a statistically

significant influence of water cadmium concentration levels. No interaction between these two factors was detected (two-way ANOVA, Table III). Generally, cadmium concentration in the suspended particles increased along with higher cadmium levels in the water. The only exception was the replicates from the S_{10} × [Cd_{50}], which presented more cadmium than the ones from [Cd_{100}].

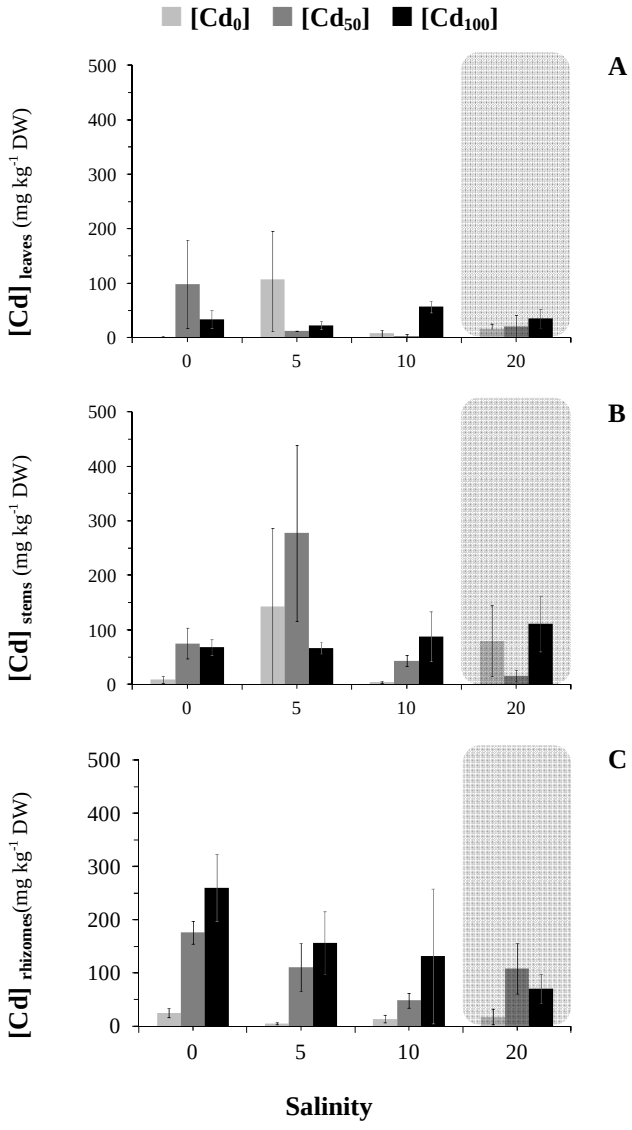

Figure 7 - Cadmium concentration in the (**A**) leaves, (**B**) stems and (**C**) rhizomes of live *S. maritimus* plants collected in the Óbidos Lagoon for this essay, except for those of the treatment with S_{20}.

The concentrations of cadmium dissolved in the water was between 0 to 1 mg l^{-1} (Figure 6D). This heavy metal was found in higher quantities at the highest salinity

treatment S_{20}, for all cadmium concentration treatments. It presented lower values as the salinity values decreased as well. The [Cd_{100}] treatments in all salinities presented the highest values of cadmium, followed by those of [Cd50] and almost no cadmium was found in plants from [Cd_0] treatments. Both factors presented a statistically significant influence over the final concentrations of cadmium dissolved in the water, increasing whenever they increased as well, showing some interaction between them (two-way ANOVA, Table III).

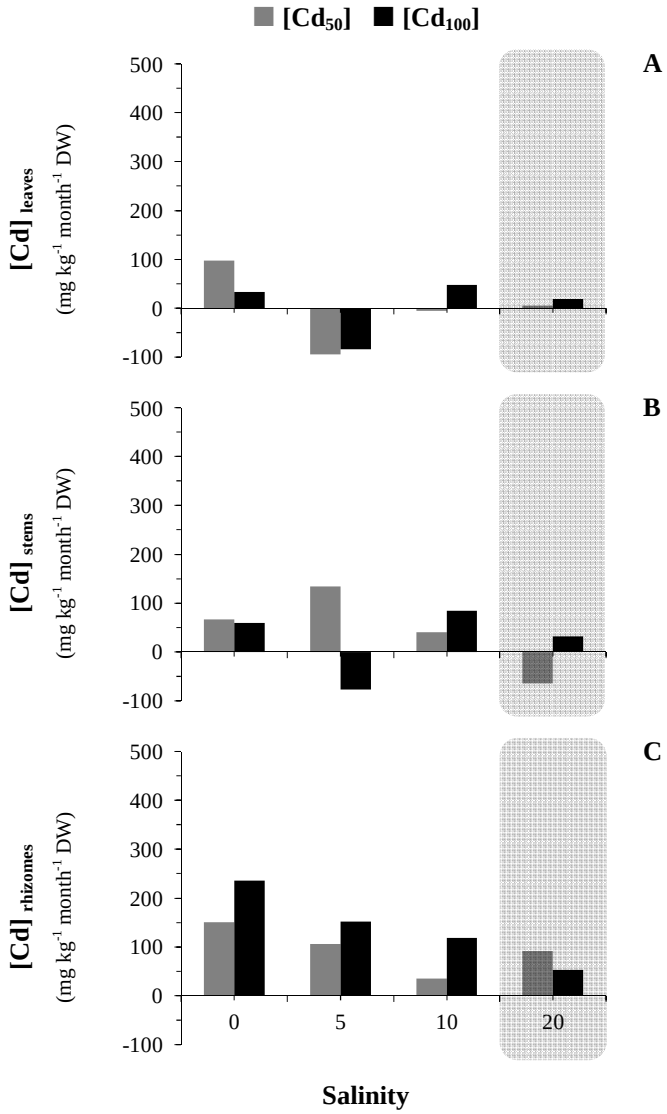

Figure 8 - Cadmium accumulation in the (A) leaves, (B) stems and (C) rhizomes of live *S. maritimus* plants collected in the Óbidos Lagoon for this essay, except for those of the treatment with S_{20}.

The concentrations of cadmium in *S. maritimus* were different in the three parts of the plant (Figure 7). In general, the leaves presented the lowest concentrations of cadmium,

followed by the stems and the rhizomes afterwards. The leaves presented higher values of cadmium at the treatments $S_0 \times [Cd_{50}]$ and $S_5 \times [Cd_0]$, around 100 mg kg^{-1} (Figure 7A). The minimum values registered were close to zero. The stems presented a high variability of cadmium concentrations, but the highest values were found at the treatments $S_5 \times [Cd_0]$ and $S_5 \times [Cd_{50}]$, respectively 150 and 250 mg kg^{-1} (Figure 7B). Both *S. maritimus* leaves and stems should no statistically significant influence of salinity, cadmium contamination levels, or of any interaction between these two factors (two-way ANOVA, Table III). On the other hand, the *S. maritimus* rhizomes did show a positive influence of the water contaminated with cadmium (Figure 7C). Its tissues presented higher concentrations within treatments $[Cd_{100}]$, followed by $[Cd_{50}]$ and, $[Cd_0]$ for last. The rhizomes were also affected by the water salinity, in the inverse order, decreasing as salinity values rouse. These tendencies were statistically supported (two-way ANOVA, Table III), without finding any interaction between the two factors. Therefore, the *S. maritimus* tissues with higher amount of cadmium were the rhizomes of plants subjected to the combined treatment $S_0 \times [Cd_{100}]$, reaching almost 300 mg kg^{-1}. Plants of the $[Cd_0]$ treatments revealed the presence of this heavy metal in their tissues, although in quantities much lower than those subjected to contaminated water during this experiment. The most peculiar case was that of plants belonging to the combine treatment $S_5 \times [Cd_0]$, in which the rhizomes showed almost no cadmium, but the tissues of leaves and mostly of the stems registered the highest concentrations. Despite of surviving only 18 days, the plants of the S_{20} treatments managed to present a fair amount of cadmium.

The *S. maritimus* tissue accumulation of cadmium was evaluated by the difference between the concentrations found within the plant tissues subjected to contaminated water and those of no contamination reference plants, for a given salinity. The leaves where the part of the plants presenting less accumulation (Figure 9A), actually presenting loss of cadmium at the salinity S_5. The stems followed, showing similar accumulation values to those of the leaves. They also presented metal loss in some of the treatments, without any evident pattern (figure 9B). The rhizomes were the part of the plant where the accumulation of cadmium was more evident (figure 9C). For all salinities, plant tissues from the $[Cd_{100}]$ treatment showed more metal accumulation than those from $[Cd_{50}]$ treatments, but it decreased along with the upraising of the water salinity. The only

exception was in the salinity S_{20}, where plants of the [Cd_{50}] accumulated more cadmium than those from [Cd_{100}], as they lived longer

Table III – Two-way ANOVA tests results from the relations between salinity and cadmium concentration treatments ([Cd_0], [Cd_{50}] and [Cd_{100}]) *versus* the amount of suspended organic matter and cadmium concentrations evaluated dissolved in water, within suspended particles, plus *S. maritimus*' leaves, stems and rhizomes. The following symbols stand for: S × [Cd] - salinity and cadmium concentration variable; df - degrees of freedom; MS - Mean Square

Two-way ANOVA	**Source of variation**			
Experiment parameters	**df**	**MS**	**F-statistic**	***p-Value***
Suspended Organic Matter (mg kg^{-1})				
Salinity × organic matter	3	0.037	0.898	0.457
Cadmium × organic matter	2	0.031	0.770	0.474
[S × Cd] × organic matter	6	0.019	0.475	0.820
Suspended particles (mg l^{-1})				
Salinity × suspended particles	3	0.042	1.113	0.363
Cadmium × suspended particles	2	1.361	35.949	0.000
[S × Cd] × suspended particles	6	0.061	1.610	0.188
Dissolved cadmium in water (mg l^{-1})				
Salinity × dissolved cadmium	3	0.79	11.060	0.000
Cadmium × dissolved cadmium	2	0.85	11.938	0.000
[S × Cd] × dissolved cadmium	6	0.027	3.788	0.009
Cadmium concentration in leaves (mg kg^{-1})				
Salinity × cadmium in leaves	3	1883.226	0.478	0.701
Cadmium × cadmium in leaves	2	17.948	0.005	0.995
[S × Cd] × cadmium in leaves	6	5939.472	1.506	2.19
Cadmium concentration in stems (mg kg^{-1})				
Salinity × cadmium in stems	3	20700.796	1.363	0.278
Cadmium × cadmium in stems	2	9307.981	0.613	0.550
[S × Cd] × cadmium in stems	6	21513.177	1.416	0.249
Cadmium concentration in rhizomes (mg kg^{-1})				
Salinity × cadmium in rhizomes	3	18619.999	3.729	0.025
Cadmium × cadmium in rhizomes	2	50241.175	10.061	0.001
[S × Cd] × cadmium in rhizomes	6	6249.198	1.251	0.316

The cadmium transport index indicated that *S. maritimus* accumulated more cadmium in S_5 x [Cd_0] in the leaves comparative with the other results achieved. The presence of cadmium was found at [Cd_0] for S_{10} and S_{20} also, between 0 and 50% (Figure 9).

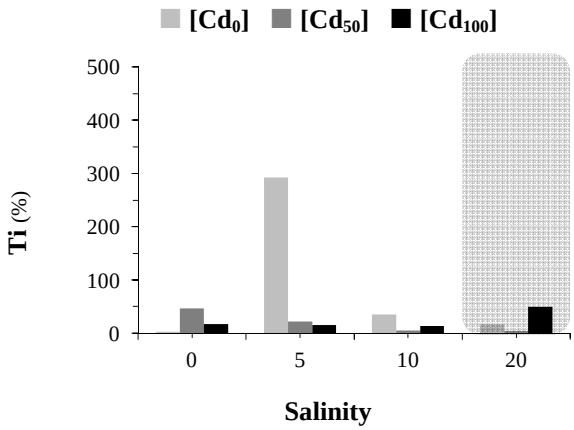

Figure 9 - Cadmium transport index of live *S. maritimus* plants collected in the Óbidos Lagoon for this essay, except for those of the treatment with S_{20}.

Discussion

The mortality of *Scirpus maritimus* showed that salinity influenced the life of this halophyte plant (Figure 4). Ozawa *et al*, (2009) indicated that hyperaccumulator plants are sensitive to high salinity and waterlogged conditions. In this greenhouse experiment, it was possible to verify the same sensitivity of the plants, specially to S_{20}, were the survival only occur for 18 days. The plants subjected to [Cd_0] were the ones that seemed to tolerate salinity better (Figure 4E), although no statistical analyses supported this tendency. The plants' stress, induced by high salinities, was responsible for harmful effects on the plant growth and on retarding the plants shoot germination. (Lillebø *et al* 2003).

The results achieved were consistent with those of other authors. Similar mortality patterns were observed by Lillebø *et al* (2003) for *S. maritimus* collected from Mondego estuary (also located in the western coast of Portugal). Plants went on senescence processes when submersed in brackish water over 15.0 salinity, for long periods.. This agrees with the results obtained in this work.

The sensitive and tolerance of salinity is also influenced by the macrophyte plants natural uptake of nutrients, water and other ions. Salt stress reduces water potential, causes ion imbalance or disturbances in ion homeostasis (Leblebici *et al*, 2011). For this reason *S. maritimus* plant is considered a facultative halophyte of the accumulating type (Kantrud, 1996; Yilmaz, 2007). At high salinity values, halophyte plants require leaching of water 30-50% above consumptive use, as for the plants to be able to flush out the excessive salt below the root zone, preventing plant growth inhibition, a result observed in these plants (Brown *et al*, 1999). High salinities values are responsible for a primary stress, resulting in several cells damages, such as disruption of the cell membrane integrity, induction of structural changes and cytoplasm replacement of Ca^{2+} with Na^{+} (Hegedus ,2010). Besides these damages, facultative halophyte shave specific mechanisms for avoiding them, like the ability to acquire ions and compartmentalise them in vacuoles, or the discrimination in favour of K^{+} over Na^{+} (Flowers *et al*, 2010). These protective functions are some of the possible responses of *S. maritimus*, in order to survive at mild salinities, such as S_5 and S_{10}, where the vessels presented both live and death plants for [Cd_0], [Cd_{50}] and [Cd_{100}] treatments (Figure 4C and 4D).

The acclimation period was realized with a salinity of 2.0, measured *in locco*, at the plants' collection site, in order to ensure similar conditions to those found in the plants' natural environment. All the shoots went senescent during this period, but new ones emerged afterwards. New shoots were also observed by Lillebø *et al* (2003). This was possible because during the life cycle of *S. maritimus*, ramets produce photosynthetic shoots. The experimental trial begun when these turned into grown plants Nevertheless, more new shoots emerged afterwards in some of the vessels (Figure 5A). They surged in highest numbers at the S_0 and S_5 treatments, although no statistical support was found to explain the results observed. But, according to Lillebø *et al* (2003), salinity can be responsible for the number of shoots that appeared in their study. The shoots emerged at salinities below 10.0. The same was observed in this study., On the other hand, senescence was verified in the S_5, S_{10} and S_{20} treatments, with the loss of some leaves. The same senescence was observed in Lillebø *et al* (2003), where it was concluded that continuous exposure to salinities above 15.0 induced the senescence of *S. maritimus*. Ghosh & Singh (2005) also verified presence of new shoots and that the increase of cadmium concentrations lead to the necrosis of leaves, disintegration of cells and to epinasty followed by wilting The leaves appeared dry and yellowish, like observed by Hegedus (2010). For the authors this was a metal detoxification mechanism of the plants. In this study, necroses of the leaves were also observed in S_5, S_{10} and S_{20}.

The shoots length increment was one way to evaluate the difference between the plants' size at the beginning and the end of the experiment (Figure 5B). This parameter was not related with the salinity and cadmium contamination levels in the water (Table II). The negative value observed in the treatment $S_5 \times [Cd_0]$ was a result of the lost of some leaves. That was probably related to the 35 % of mortality achieved in these vessels, as the lost of leaves is one sign of necrosis) (Ghosh & Singh, 2005). The length increase of the plants was highest for S_0 treatments, except for $S_{10} \times [Cd_{100}]$, but there was a high variation among plants, with possible lost of leaves as well in some of them. Nonetheless, there was no statistical support for growth reduction with the factor salinity. The restriction of growth of *S. maritimus* was verified by Lillebø *et al* (2003). For Khan *et al* (2001), the growth reduction at high salinities, verified in the halophyte hyperaccumulator plant *Salicornia rubra*, was caused by the reduction of the plants' osmotic adjustment, due to the saturation of the solute uptake system or the excessive energy requirement demanded by those

systems. According to Bragato *et al* (2006), cadmium did not affect the growth of plants, especially in rhizomatous plants, like *S. maritimus*, which agrees with the results obtained in this study. Nutrients uptake is reduced in the senescent aerial part, because of the resources translocation to the underground over wintering tissues, necessary to the plant growth and survival. Leblebici *et al* (2011) concluded that salt stress entails both osmotic and ionic stress, so the suppression of growth was related to the total concentration of soluble salts. However, this result was not possible to verify for *S. maritimus* in this study

For Yilmaz (2007), plants grown at the highest salinity had significantly lower dried mass than plants grown at lower salinities. The same situation was similar in this study: the fresh weight loss was superior for the S_{10} treatments (Figure 5C). According to Ghosh & Singh (2005), biomass was one of the factors that controlled the total amount of metal accumulation by the plants. *S. maritimus* from the Óbidos Lagoon, presented the largest weight loss in the combined treatment $S_{10} \times [Cd_{100}]$ and the lowest in $S_0 \times [Cd_{100}]$. That first result was compatible with the plants' length increment. It seems to imply that the biomass and the plant length increments must be influenced by the same physical and chemical factors. Kamnev *et al* (2000) concluded, that growth rate is usually low for phytoacumulator plants, as well as biomass increase. Still, in the case of *S. maritimus* from the Óbidos Lagoon, there was no statistical support to fundament the differences between the influence of salinity or cadmium contamination levels to the plants' biomass increase. For Khan *et al* (2001), salinity was considered responsible for the decrease of biomass production, since it causes a lowering of plant water potentials, specific ions toxicity or ionic balance.

No influence of cadmium for the fresh and dry weight of the shoots were also observed in *Salicornia rubra* plants (Ozawa *et al*, 2009). However, the salinity inhibited, at higher salinities, the fresh and dry weight of several parts of *S. rubra* (Khan *et al*, 2011). Another important conclusion for the biomass of halophyte plants was achieved by Flower & Colmer (2008): changes in the water (per dry mass unit) had influence in the biomass fresh weight variations at different salinities (massive ion accumulation can also occur).

The number of algae species in the Óbidos lagoon is elevated and the lagoon has been studied to monitoring eutrophication problems (Pereira *et al*, 2009a). These algae

were washed way from *S. maritimus* plants, both at the collection site and in laboratory, but some algae probably were not removed at all. These algae grew during the experiment, with the conditions created for the development of the *S. maritimus*. Lillebø *et al* (2010) verified that the release of oxygen, carbon dioxide and organic compounds at the rhizosphere resulted mostly by microbial activity, which can also modify the distribution and availability of metals.

Suspended organic matter (Figure 6A) was found present in all experimental vessels. This matter was formed by microorganisms and algae. Their biomass was more evident in $S_0 \times [Cd_0]$. It showed no influence of both salinity and cadmium contamination factors. For example, Pereira *et al* (2009c) reported that *Ulva* sp., a green algae established in the Óbidos lagoon, was capable of lowering f cadmium levels in the lagoon (between 0 and 0.2 µg g^{-1}), during spring at the Barrosa Arm. Coincidently, the same season of *S. maritimus* collection for this experiment, near the same place. Cadmium was present in the form of suspended particles in the brackish water of the experimental vessels (Figure 6B and 6C), like in the Óbidos Lag (authors unpublished data). A significant influence of cadmium levels was evident. Suspended particles presented higher metal concentration within the treatments more contaminated. Still, the results showed that salinity did not present any influence in the concentrations achieved. The combined treatment S_{10} x $[Cd_{50}]$ showed more cadmium, but it also continued more suspended particles of organic matter. These could indicated that S_{10} possibly was the best salinity for the accumulation of cadmium by the organic matter formed in the study, since cadmium factor presented influence in these results (Figure 6B and 6C).

The cadmium dissolved in the brackish water (Figure 6D) was more elevated for the higher initial contamination levels, established for the experimental trial. It presented also higher concentrations at the highest salinities, a possible result from the cytotoxic effects of cadmium for the plants. Also, Vasquez *et al* (1998) concluded that cadmium concentrations in the water lagoon were positively related to salinity. Heavy metals were retained in organic matter due to exchange between the larger number of hydrogen ions on the peats colloidal surface and the metals cations (Manios *et al*, 2003). Thus, it can also be related to the elevated mortality of *S. maritimus* plants at the highest salinities. The rhizomes of the plants that died during the essay were only removed from the sediment at

the end of the study. This procedure avoided possible damages to the other surviving plants, or cause disruption of their normal physiology. As the plants revealed some cadmium accumulation from field conditions, especially in the rhizomes, it is acceptable to hypothesise that this cadmium may have been dissolved to the water, in which the plants were submerged. It may have occurred by tissue degradation and disruption. This may have been potentiated as well by the plants' symbiotic organisms and those from the suspension biofilm, some of which may have promoted tissue decomposition and bioavailability of dissolved cadmium.

The concentration of cadmium was different in the several parts of the plant (figure 7). If heavy metals are absorbed by the plant, they may be stored away from metabolically active compartments, to prevent phytotoxic effects (Carranza-Álvarez *et al*, 2008). Ozawa *et al* (2009) indicated that most of the hyperaccumulator plants developed cell vacuoles, in which metals are accumulated, to be segregated from the cytosol. Thus, the metal uptake by plants was made mainly in the form of cations into the cells. This segregation of cadmium could also be a protection response of *S. maritimus* in this study, being also a response to the several different concentrations found between the three parts of the plants analysed: leaves, stems and rhizomes.

The cadmium concentration in leaves (Figure 7A) showed no influence of salinity or cadmium contamination The cadmium concentrations measured at treatments S_{20} were a representation of the ability of the plants to accumulate cadmium even at elevated salinities, plus the transportation of cadmium to the leaves, due to the elevated mobility of this metal (Reboreda & Caçador, 2007). It occurred in a short period of time, since almost all *S. maritimus* died after a few days, mostly by salinity influence. Reboreda & Caçador (2007) concluded that the cadmium content in leaves was due also to the fact that the presence of leaves was very dependent on the plants' life cycle. The hottest seasons induce the production of more leaves in most plants, thus metals' accumulation becomes dependent on this factor. (Almeida *et al*, 2006). Shupping (2008) found values between 0 and 0.6 mg kg^{-1} within leaves of *S. maritimus*. These values were inferior to the results achieved in this present study (between 0 and 100 mg kg^{-1}), with no influence of cadmium and salinity in the presence of cadmium in leaves. The plants used by Shupping (2008), were collected in a local with low values of salinity and cadmium contamination.

The values of cadmium in stems (Figure 7B) were between 0 and 300 mg kg^{-1}. The concentration of cadmium within *S. maritimus* from the Óbidos Lagoon presented higher values than in those collected from other ecosystems (Madejón *et al*, 2006; Shupping *et al* 2008). Seasonal variations should be considered. For *S. maritimus*, spring and summer are the seasons were plant growth is best developed (Almeida *et al*, 2006). For this trial, the plants were collected in the spring. This can be a factor for the appearance of elevated plant growth at the lowest salinities. Since the uptake of cadmium was lower on the stems, the toxicity in this part of the plants was potentially lower. The stems include mostly vascular tissues with lower metabolic activity and, consequently, the accumulation of metals is expected to be lower (Madejón *et al*, 2006). Still, at $S_5 \times [Cd_{50}]$ the values of cadmium were very elevated. These values appear to be influenced by salt stress induced in plants, interfering in the uptake of water and heavy metal, even though the salinity and cadmium showed no statistically influence in these results.

In accordance with other similar studies, *S. maritimus* rhizomes (Figure 7C) were the part of the plant where cadmium accumulated the most (Madejón *et al*, 2006; Shupping *et al* 2008). The concentration of cadmium in rhizomes was strongly influenced by both salinity and cadmium contamination levels, individually and combined together. There was a clear presence of cadmium within plants from the reference trial situation of no contamination $[Cd_0]$, which indicates that there was a prior cadmium accumulation at the Óbidos Lagoon. Madejón *et al*, 2006, registered cadmium concentrations of 0.25 ± 0.08 mg kg^{-1} in tubers (rhizomes) from "Entremuros", a heavy metal polluted salt march area at the South of Spain. Almeida *et al* (2006) observed the same in the Douro Estuary (Portugal), where the authors concluded that cadmium was easily mobilised and available for plants.

For Ololade & Ologundudu (2007), the increase in metal content within certain parts of the plants can be informative on sublethal responses, indicating the increased metal availability and the potential metal stress for the plant. Particular species of halophyte plants have a natural capacity to absorb cadmium through the rhizomes and accumulate it within the aerial parts (Ozawa, 2009). This absorption could indicate if salinity presented or not a general influence in the metal accumulation at all the parts of plants. That was possible to see in the cadmium uptake results (Figure 8). It was possible to conclude, that

salinity did not interfere with the presence of cadmium in a specific part of the plant, except for the rhizomes. These were the plant structure that accumulated more cadmium during this experiment (Figure 8C). The negative accumulation in the leaves, observed at S5, was an indication of the small amounts present in the tissues. With these results would be possible to assume that, in salt stress, plants subjected to the [Cd_{50}] treatment accumulated cadmium better within their rhizomes. Especially considering that the treatments S_0 and S_5 were close to the salinity value measured at the collection point.

The transport index (Figure 9) showed a highest cadmium transportation with no salt flow, as this can cause bounded/complexed substances in the conducting vascular system of the plants (Gosh & Singh, 2005). The cell walls and different apoplastic structures are similar to filters, responsible for limiting the movements of substances within the symplast and aerial parts. The root system of *S. maritimus* has the capacity to collect water for the plant, if it becomes impaired this will be indicated by the wilting of leaves (Gosh & Singh, 2005). The fact that 80% of Na^+ is found in the leaves could be associated to the elevated values of cadmium, also found in this part of the plant. The elevated cadmium of the plants from the combined treatment $S_5 \times [Cd_0]$ could be a result of some these interferences during the experiment, or the fact that, being this salinity may be ideal for the accumulation of cadmium by the plant.

Conclusion

Heavy metals remain available in the environment for long time and their specification and bioavailability might change over time. According to Kamnev & Lelie (2000), plants with complex rhizomes systems are promising candidates for phytoremediation.

The factor salinity was responsible for the mortality of plants, and this was the principal factor of disadvantage for the use of *S. maritimus* plant as a good candidate for phytoremediation of coastal lagoons, since the variations of salinity are divergent during the year, especially over 15.0. For the number of new shoots, length and biomass increments, the influence of salinity and cadmium were not significant, but according to studies from other authors, the salt stress is responsible for their inhibition and decrease.

The formation of organic matter indicated that rhizomes present microorganisms and algae's symbioses, brought from Óbidos Lagoon, that could alter the concentration of cadmium results in the brackish water. The concentration of dissolved cadmium and the suspended particles were influenced by cadmium initial contamination factor in this study.

The results obtained in this study presented some disadvantages that could indicate that *S. maritimus* is not an indicated halophyte plant for the phytoremediation for contaminated coastal lagoons, because the relation between salinity and cadmium concentrations could be too stressful for the growth of the plants. The *S. maritimus* population present in salt marshes are constantly n submitted to e stress induced salinity changes, not only in the water, but also in the soil. The soil physical and chemical properties are important (Kamnev & Lelie, 2000). In locals were salinity was closer to freshwater values, *S. maritimus* stress will not be such problem and plant mortality would be lower.

For phytoremediation is important to evaluate the growth rate and the biomass of the target plants. For an effective phytoremediation of a large contaminated ecosystems, like the Óbidos Lagoon , is important that the plant biomass (*S. maritimus*) must be elevated, in order to remove the metals discharged in the area, particularly at the Barrosa

Arm. (Cheraghi *et al*, 2011). As for the mortality, salinity's influence of was important, but the effect of cadmium cannot be disassociated. The toxicity of the heavy metal, in large quantities, can lead also to the mortality of *S. maritimus.* According to Stout & Nussle (2010), the seasonal growth and the contaminated biomass are disadvantage factors for phytoremediation success.

Rhyzofiltration is one of the techniques applied in aqueous environments for heavy metals remediation. This has been studied before, for removing contaminants from solutions. The metal hipperacumulation is a adaptative process between microbes exposed to metals and plants. The interaction created indicated the presence of a rizhosphere microbial population, adapted to heavy-metal polluted locals and is important to enhance roots of hyperaccumulator plants, like *S. maritimus*. Algae and fungus cannot be forgotten. This interaction could be responsible for the plant growth (needed for the phytoremediation techniques) and for the increase in the plants' surface area for absorvation, which can lead to a better uptake of nutrients and pollutants (influencing the food chains) (Stout & Nusslein, 2010).

Actually, the *S. maritimus* population from the Óbidos Lagoon was not able to detoxify cadmium from the water. The values found for cadmium were above the values established in Portuguese directives for conservation of several ecosystems, in the last years (Pereira *et al*, 2009 b,c). According to Vasquez *et al* (1998), the shallow depth of the lagoon promotes resuspension of the sediments, by constant wind driven waves and tidal mixing. This activity promotes the release of the metals entrapped within the sediments into the water column. The continuous decomposition of organic matter can also release incorporated metals into the lagoon system.

Future Perspectives

The halophyte plant *S. maritimus* was not efficiency for bioremediation of cadmium in elevated conditions of salinity, some other studies could be realized for a much better knowledge of their capabilities as a possible phytoacumulator in coastal Lagoons.

1) *S. maritimus* was study for phytoacumulation of other heavy metals, like lead (Shupping *et al*, 2011). These other metals in the presence of the salinities (inferior or superior to salinity 15.0) could present a different results in terms of accumulation in a similar greenhouse experiment.

2) The presence of cadmium in sediments of 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. maritimus*, more close to the MAC established for surface waters (but impossible to apply in brackish or marine water systems).

4) The analysis of the rhizomes of *S. maritimus* 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. maritimus* associated with other hyperaccumulator halophyte plants (such as *Salicornia ramossisima*, also found in Óbidos Lagoon), could be interesting to analyse if the potential of bioremediation is more successfully achieved together, or if exists a competition for heavy metal accumulation.

6) The study of *Scirpus maritimus* decomposition/mineralization, plus the possible influence of salinity on the heavy metals concentrations resulting from those

processes, would be important to analyse for optimising phytoremedation techniques, in order to verify if it could result in increased metal bioavailability.

Given the above, there is still plenty to be known in the interaction of salt marshes organisms and their potential for bioremediation, where natural native organisms in a given ecosystems

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Annexes

Annex I- Post-hoc tests results for the greenhouse experiment with *S. maritimus* to test the relations between salinity, cadmium concentrations ([Cd_0], [Cd_50], [Cd_{100}]) and plant parameters as factors. The following symbols stand for: [S × Cd] - salinity and cadmium concentration variable; df - degrees of freedom; MS - Mean Square.

Post Hoc Test			
Dependent variable and factors tested		**Test**	***p-value***
Mortality (%)			
Salinity	Tukey HSD	Comparation: S_0 and mortality	0.503
		Comparation: S_5 and mortality	0.503
		Comparation: S_{10} and mortality	0.175
		Comparation: S_{20} and mortality	0.175
	LSD	Comparation: S_0 and S_5	0.170
		Comparation: S_0 and S_{10}	0, 000
		Comparation: S_0 and S_{20}	0.000
		Comparation: S_5 and S_{10}	0.009
		Comparation: S_{10} and S_{20}	0.44
	Bonferroni	Comparation: S_0 and S_5	1.000
		Comparation: S_0 and S_{10}	0.002
		Comparation: S_0 and S_{20}	0.000
		Comparation: S_5 and S_{10}	0.056
		Comparation: S_{10} and S_{20}	0.267
Cadmium	Tukey HSD	Comparation: cadmium and mortality	0.451
	LSD	Comparation: Cd_0 and Cd_{50}	0.233
		Comparation: Cd_0 and Cd_{100}	0.233
		Comparation: Cd_{50} and Cd_{100}	1.000
	Bonferroni	Comparation: Cd_0 and Cd_{50}	0.698
		Comparation: Cd_{50} and Cd_{100}	0.698
Shoots			
Salinity	Tukey HSD	Comparation: salinity and shoots	0.552
	LSD	Comparation: S_0 and S_5	0.511
		Comparation: S_0 and S_{10}	0.195
		Comparation: S_0 and S_{20}	0.511
		Comparation: S_5 and S_{10}	0.511
		Comparation: S_{10} and S_{20}	1.000
		Comparation: S_{10} and S_{20}	0.511
	Bonferroni	Comparation: S_0 and S_5	1.000
		Comparation: S_0 and S_{10}	1.000
		Comparation: S_0 and S_{20}	1.000
		Comparation: S_5 and S_{10}	1.000
		Comparation: S_5 and S_{20}	1.000
		Comparation: S_{10} and S_{20}	1.000
Cadmium	Tukey HSD	Comparation: cadmium and shoots	0.214
	LSD	Comparation: Cd_0 and Cd_{50}	0.096
		Comparation: Cd_0 and Cd_{100}	0.775
		Comparation: Cd_{50} and Cd_{100}	0.162
	Bonferroni	Comparation: Cd_0 and Cd_{50}	0.288
		Comparation: Cd_0 and Cd_{100}	1.000
		Comparation: Cd_{50} and Cd_{100}	0.486

Stem elongation (cm)			
Salinity	Tukey HSD	Comparation: salinity and stem elongation	0.822
	LSD	Comparation: S_0 and S_5	0.549
		Comparation: S_0 and S_{10}	0.711
		Comparation: $S_5$and S_{10}	0.816
	Bonferroni	Comparation: S_0 and S_5	1.000
		Comparation: S_0 and S_{10}	1.000
		Comparation: S_5 and S_{10}	1.000
Cadmium	Tukey HSD	Comparation: cadmium and stem elongation	0.543
	LSD	Comparation: Cd_0 and Cd_{50}	0.962
		Comparation: Cd_0 and Cd_{100}	1.000
		Comparation: Cd_{50} and Cd_{100}	0.869
	Bonferroni	Comparation: Cd_0 and Cd_{50}	0.962
		Comparation: Cd_0 and Cd_{100}	1.000
		Comparation: Cd_{50} and Cd_{100}	0.869
Plant Fresh weight (g FW)			
Salinity	Tukey HSD	Comparation: salinity and plant fresh weight	0.463
	LSD	Comparation: S_0 and S_5	0.337
		Comparation: S_0 and S_{10}	0.259
		Com paration: S_5 and S_{10}	0.779
	Bonferroni	Comparation: S_0 and S_5	1.000
		Comparation: S_0 and S_{10}	0.776
		Com paration: S_5 and S_{10}	1.000
Cadmium	Tukey HSD	Comparation: cadmium and plant weight	0.549
	LSD	Comparation: Cd_0 and Cd_{50}	0.304
		Comparation: Cd_0 and Cd_{100}	0.529
		Comparation: Cd_{50} and Cd_{100}	0.680
	Bonferroni	Comparation: Cd_0 and Cd_{50}	0.911
		Comparation: Cd_0 and Cd_{100}	1.000
		Comparation: Cd_{50} and Cd_{100}	1.000

Annex II - Post-hoc tests results from the relations between salinity, cadmium concentrations ([Cd_0], [Cd_{50}], [Cd_{100}]) and the cadmium concentrations *versus* the amount of suspended organic matter and cadmium concentrations evaluated dissolved in water, within suspended particles, plus S. maritimus leaves, stems and rhizomes. The following symbols stand for: [S × Cd] - salinity and cadmium concentration ($\mu g\ l^{-1}$) variable; df - degrees of freedom; MS - Mean Square

Post Hoc Test				
Dependent variable and factors tested		**Test**	**Condition**	***p-value***
Cadmium concentration in rhizomes ($mg\ kg^{-1}$)				
Salinity		Tukey HSD	Comparation: salinity and rhizomes	0.949
		LSD	Comparation: S_0 and S_5*	0.023
			Comparation: S_0 and S_{10}*	0.007
			Comparation: S_0 and S_{20}*	0.012
			Comparation: S_5 and S_{10}	0.595
			Comparation: S_5 and S_{20}	0.776
			Comparation: S_{10} and S_{20}	0.804
		Bonferroni	Comparation: S_0 and S_5	0.141
			Comparation: S_0 and S_{10}*	0.041
			Comparation: S_0 and S_{20}	0.074
			Comparation: S_5 and S_{10}	1.000
			Comparation: S_5 and S_{20}	1.000
			Comparation: S_{10} and S_{20}	1.000
Cadmium		Tukey HSD	Comparation: cadmium and rhizomes	0.564
		LSD	Comparation: Cd_0 and Cd_{50}*	0.003
			Comparation: Cd_0 and Cd_{100}*	0.000
			Comparation: Cd_{50} and Cd_{100}	0.312
		Bonferroni	Comparation: Cd_0 and Cd_{50}*	0.010
			Comparation: Cd_0 and Cd_{100}*	0.001
			Comparation: Cd_{50} and Cd_{100}	0.935

*The mean difference is significant at the 0.05 level