UNIVERSIDADE DE ÉVORA

Mestrado em Gestão e Conservação de Recursos Naturais

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Metal Biogeochemical Cycling in Tagus Estuary Salt Marshes: from Halophytes to Microbes

Bernardo Afonso de Aranha Alhandra Duarte

Orientador:
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“The Alchemy has three essential steps: Comprehension, Decomposition and Reconstruction.”

Nicolás Flamel (1330 – 1418)

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CICLO BIOGEOQUÍMICO DOS METAIS NOS SAPAIS DO ESTUÁRIO DO TEJO: DOS HALÓFITOS AOS MICRO-ORGANISMOS

RESUMO

As zonas de sapal recebem habitualmente elevadas quantidades de poluentes provenientes das zonas urbanas circundantes, através da circulação estuarina e da inundação destas zonas pela maré. Por esta razão são frequentemente considerados sumidouros de poluentes, nomeadamente metais pesados.

Quando se consideram os efeitos tóxicos dos metais pesados no ecossistema sapal e também na saúde pública, a sua concentração total não é tão importante como a forma química em que estes metais surgem. Esta forma vai ser determinante na a sua biodisponibilidade para serem tomados pelas plantas e consequentemente entrar na cadeia trófica. A actividade do sistema radicular das plantas e os microrganismos a elas associados alteram as propriedades físicas e químicas dos sedimentos, influenciando o fraccionamento químico dos metais e consequentemente a sua biodisponibilidade.

Em zonas altamente poluídas, as espécies vegetais desenvolveram mecanismos de sobrevivência a concentrações elevadas de metais pesados, tornando-se tolerantes à contaminação por estes elementos. A absorção de metais depende de vários factores como o pH, a especiação química do elemento, a matéria orgânica do sedimento, entre outros. Adicionalmente, as raízes das plantas sintetizam, acumulam e secretam inúmeros compostos, como os ácidos orgânicos, modificando as características físico-químicas do sedimento e dos metais, influenciando a tomada de nutrientes e na desintoxicação de metais pesados.

Durante este trabalho, pretendeu-se estudar como as diferentes espécies vegetais absorvem os metais, os incorporam na sua biomassa e como os libertam para o ambiente circundante durante a senescência dos seus órgãos. Pretende-se também investigar os processos microbianos de decomposição desta matéria vegetal contaminada e como estes vão afectar a especiação dos metais e consequentemente a sua disponibilidade para serem novamente absorvidos pelas plantas.

A quantidade total de Zn, Cu, Cd e Co presente nas folhas, caules e raízes de *Sarcocornia fruticosa*, *S. perennis*, *Halimione portulacoides* e *Spartina maritima* foram analisados de dois em dois meses, num sapal do estuário de Tejo. Para todos os

elementos as concentrações mais elevadas foram encontradas nas raízes, sendo as concentrações nos órgãos aéreos negligenciáveis no que diz respeito ao balanço de metais no sedimento, como é evidente, devido às baixas taxas de translocação raíz-parte aérea. Foram também avaliados outros parâmetros como a acumulação de metais, taxas de "turnover" radicular e coeficientes de reciclagem. Os elevados "turnovers" radiculares e coeficientes de reciclagem observados em *S. maritima* para a maioria dos metais tornam esta espécie, uma espécie fito-estabilizadora. Pelo contrário, o baixo "turnover" radicular e baixa geração de necromassa observada para *S. perennis*, tornam esta espécie a mais indicada para processos de fitorremediação. Assim, apesar das elevadas quantidades de metais devolvidos aos sedimentos, devidas à senescência radicular, os sapais continuam a poder ser considerados sumidouros de metais pesados, reciclando-os principalmente entre os sedimentos e as raízes dos halófitos.

Considerando este mecanismo de tomada, reciclagem e senescência de biomassa contaminada, tornou-se de grande importância estudar também a decomposição desta mesma necromassa. Como caso de estudo, dirigiu-se a investigação destes mecanismos aos sedimentos de *H. portulacoides*. Numa base sazonal, avaliou-se a influência de enzimas extracelulares presentes nos sedimentos, na especiação química dos metais e na reciclagem da matéria orgânica do sedimento, de forma a melhor compreender a ligação entre estes mecanismos de reciclagem orgânica e as espécies químicas de metais associadas. A especiação metálica em sedimentos de *H. portulacoides* foi avaliada usando o método de Tessier, tendo evidenciado um padrão sazonal comum no que diz respeito à fracção de metais associados à matéria orgânica, apresentando valores elevados durante o Outono, ou seja, na estação do ano em que os valores de matéria orgânica se apresenta mais elevados. As fracções de metais associados à matéria orgânica, bem como a fracção residual, apresentam-se sempre dominantes, sendo as variações sazonais verificadas devidas principalmente ao intercâmbio de metais entre as duas fracções. As enzimas fenol oxidase e β-*N*-acetilglucosaminase apresentam valores elevados de actividade durante a Primavera e Verão, contrariamente à peroxidase, que exibe o seu pico de actividade durante o Inverno. A enzima protease apresenta os seus valores de maior actividade durante a Primavera e Inverno. Estes períodos diferenciais de elevada degradação de matéria orgânica levam à existência de dois períodos de decréscimo de metais associados à matéria orgânica. Os picos de actividade da enzima sulfatase (Primavera e Inverno) coincidem com a depleção de metais sob a forma disponível, provavelmente devido à formação de sulfuretos e consequente mobilização dos

metais. Todos estes factores mostram uma evidente interacção entre diversas enzimas microbianas, afectando a especiação metálica.

Estes resultados levam à inevitável conclusão de que as plantas de sapal actuam como “sink” temporário de metais pesados. Apesar desta característica temporária, as plantas de sapal continuam exercer uma grande influência na biogeoquímica do ambiente circundante. Este ciclo é completado durante o período de senescência, geração de necromassa e consequente reintrodução de metais no ambiente circundante, em formas químicas diferentes. Durante este processo, outro factor que assume uma elevada importância será a reciclagem biogeoquímica mediada pela comunidade microbiana existente na rizosfera, decompondo a matéria orgânica e desta forma actuando sobre as ligações estabelecidas entre esta e os metais pesados, afectando novamente a especiação química dos metais. Devido a este mecanismo, a nova tomada de metais por parte das plantas poderá ser potenciada ou inibida, reiniciando desta forma o ciclo dos metais. Num ambiente de tão grande complexidade, com todos estes processos a ocorrer, torna-se evidente que todas estas variáveis actuam em conjunto, tornando os sapais ecossistemas ideais para o estudo da fitorremediação e das suas possibilidades de aplicação em zonas contaminadas.

ABSTRACT

Salt marshes usually receive high inputs of pollution from nearby urban areas, river transport and tidal inundation. They are considered to act as sinks for pollutants, namely heavy metals.

When we consider possible toxic effects of metals to the marsh ecosystem and also to human health, the total amount of metal is not as important as the chemical form in which it is present. This form will be responsible for the bioavailability to the plant uptake and consequently to the introduction in the food web. The activity of plant roots and associated microbes can alter the physical and chemical properties of the sediment, influencing the chemical fractionation of metals and thus bioavailability.

In highly contaminated sites plant species have developed mechanisms to survive with highly toxic concentrations, becoming therefore tolerant to heavy metal pollution. The absorption of metals depends on varying factors such as pH, metal speciation and soil organic matter, among others. Plant roots can synthesize, accumulate and secrete many compounds, such as organic acids that will modify the sediment and metals physical and chemical characteristics, influencing nutrient uptake and heavy metal detoxification.

In this study was investigated how the different plant species uptake heavy metals, incorporate them in their biomass and how these metals will return back into the surrounding environment, due to the plant organ senescence. It was also an objective of this study to investigate the microbial decomposition processes of this contaminated vegetable material and how this affects the metal speciation and consequently their availability to be uptake back into the plants.

Pools of Zn, Cu, Cd and Co in leaf, stem and root tissues of *Sarcocornia fruticosa*, *S. perennis*, *Halimione portulacoides* and *Spartina maritima* were analysed every two months, in a Tagus estuary salt marsh. All the major concentrations were found in the root tissues, being the concentrations in the aboveground tissues/organs neglectable for sediment budget proposes, as seen by the low root-aboveground translocation. Metal annual accumulation, root turnovers and cycling coefficients were also assessed. *S. maritima* showed the higher root turnovers and cycling coefficients for most of the analysed metals, making this a phyto-stabilizer species. By the contrary the low root turnover, cycling coefficient and low root necromass generation makes *S. perennis* the most suitable species for phytoremediation processes. Although the high amounts of metal return to the sediments, due to root senescence, salt marshes can

still be considered sinks of heavy metals, cycling heavy metals mostly between sediment and root.

With this it became of great importance to study also the decomposition of the highly contaminated root necromass by the microbial community existent in the rhizo-sediment. For this, *H. portulacoides* rhizosediment microbial processes were evaluated, as a case study. The influence of salt marsh sediment extracellular enzymatic activity (EEA) on metal fractions and organic matter cycling was evaluated on a seasonal basis, in order to study the relation between organic matter cycling and the metal species associated. Metals in the rhizosediment of *H. portulacoides* were fractioned according to the Tessier's scheme and showed a similar pattern concerning the organic bound fraction, being always high in autumn, matching the season when sediment organic matter presented higher values. Both organic bound and residual fractions were always dominant, being the seasonal variations mostly due to interchanges between these fractions. Phenol oxidase and β-N-acetylglucosaminidase had higher activities during spring and summer, contrarily to peroxidase, which had it higher activity during winter. Protease showed high activities in both spring and winter. These differential periods of high organic matter degradation caused two periods of organic bound metals decrease. Sulphatase peaks (spring and winter) matched the depletion of exchangeable forms of metals, probably due to sulphides formation and consequent mobilization. This showed an interaction between several microbial activities affecting metal speciation.

These findings lead to the inevitable conclusion that vascular plants may act as temporary sinks for heavy metals. Although this temporal sinking characteristic, they continue to influence the biogeochemistry of the surrounding environment. The cycle becomes complete with senescence, necromass generation and consequent re-input of metals to the surrounding environment in a different chemical form. Another key factor at this point will be the biogeochemical cycling by the microbial community inhabiting the rhizosphere, decomposing organic matter, acting this way on the bonds established with heavy metals and again the chemical speciation is affected. With this a new uptake is enhanced or delayed and the process restarted. In such a complex environment with all these processes acting, it becomes evident that all the variables are gathered to make salt marshes ideal ecosystems to study phytoremediation and its possibilities of application in other contaminated areas.

INDEX

ABBREVIATIONS

ANOVA	Analysis of variance
CAM	Crassulacean Acid Metabolism
Cd	Cadmium
Co	Cobalt
Cr	Chromium
Cu	Copper
DW	Dry Weight
EEA	Extracellular Enzymatic Activity
EU	European Union
FW	Fresh Weight
G.A.	Galic Acid
HA	Humic Acids
MPA	Metal Primary Accumulation
Ni	Nickel
NPP	Net Primary Production
Pb	Lead
p-NP	p-nitrophenol
TW	Transitional Waters
WFD	Water Framework Directive
Zn	Zinc

CHAPTER 1

General Introduction

GENERAL INTRODUTION

Salt marshes have a great ecological value for the estuarine ecosystem, namely in terms of nutrient regeneration, primary production, wildlife habitat and as shoreline stabilizers. Periodical tidal flooding of salt marshes provides large amounts of pollutants to the marsh ecosystem. Thus, salt marshes are considered to be important sinks (Caçador *et al.,* 1993; Davy, 2000; EU, 2000; Reboreda and Caçador, 2007; Caçador *et al.,* 2009; Duarte *et al.,* 2007; Salgueiro and Caçador, 2007). The approval of the Water Framework Directive (WFD) forces the member states of the European Union (EU) to monitor the biological quality of all the water bodies and elaborate a management plan for the river basins, in order to solve the potential problems (EU, 2000). The Guidance Document No. 5 (Working Group 2.4, COAST, 2003) advises that, in transitional waters (TW), the intertidal areas from the highest tide limit to the lowest tide limit to be included in the monitoring program, including this way salt marshes. The WFD states that for this propose a reference condition for angiosperms, corresponding totally or nearly totally to undisturbed conditions, where there are no detectable changes in the angiosperm abundance due to anthropogenic activities. As it is now known the major carbon sink of the planet are the oceans (38 630 Pg C), followed by the terrestrial zones. Considering the terrestrial sink (1 400 Pg C), the more productive and more important zones retaining carbon are the wetlands retaining about 1/2 to 1/3 of the carbon (455-700 Pg C) (Sousa *et al.*, 2008). Vascular plants in salt marshes are crucial to the dynamics of the estuarine ecosystem, strongly influencing the processes of retention of heavy metals, reduction of eutrophication and mitigation of carbon (Caçador *et al*., 2004). These salt marsh plants are characterized, among other characteristics, by being extremely productive. The striking zonation of marsh plant communities has been explained to be the product of competitively superior plants dominating physical mild habitats and displacing competitively subordinate plants to physical marsh habitats (Mendelssohn and Morris, 2000; Caçador *et al*., 2007a). Recent studies however, have suggested that both nutrient supply and thermal stress can influence this simple scenario increasing nutrient availability, which may alleviate below ground competition for nutrients and lead to above ground competition for light dictating competitive dominance among marsh plants. Similarly, climate may have important, but largely unrecognized effects on marsh plant community organization. Tagus salt marshes are majorly colonized by the pioneer *S. maritima* (a C4 metabolism species), two species of *Sarcocornia* (*S. fruticosa* and *S. perennis*, both CAM) and by *H. portulacoides* (a C3 metabolism species). The knowledge on plant

distribution, colonization and seasonal biomass changes, is well known for Tagus salt marshes, providing a baseline for species dynamics studies (Braun-Blanquet, 1979; Mendelssohn and Morris, 2000; Caçador *et al*., 2007b; Costa *et al*., 2007; Duarte *et al.*, 2008). These metabolic differences, makes these species growth rates and CO2 fixation abilities very different from species to species, having as consequence of the ecosystem overall, different spatial carbon accumulation as well as alterations at the provided services level. There are also significant differences in the growth periods, due to seasonality. All the four referred species present distinct seasonal growth patterns, not only in biomass production peaks but also in senescence periods (Caçador *et al*., 2009), leading to a seasonality in the accumulation of elements withdrawn from the sediment and allocated in the biomass differential generated from the atmospheric carbon. Not only the biomass production is an important aspect to be taken in account but also their senescence periods and differential introduction of organic matter back to the sediment and surrounding environment must be considered in a holistic view of the biogeochemical cycles. In the last years there have also been developed several studies in sediment microbiology. It is known that salt marsh plants due to its submersion adaptations, have developed a very functional aerenchyma system in their roots (Duarte *et al.,* 2007) allowing to pump oxygen from the atmosphere to the rhizosphere, turning these sediments oxidized, although their long submersion periods. This promotes an intense microbial activity in these sediments, also stimulated by organic matter inputs (Duarte et al., 2008). Studies on the metabolic diversity of these communities showed that there are a large number of carbon-based eco-substrates, degraded by these microorganisms (Costa, 2007). Together with the recent enzymatic data from microbial extracellular enzymes (Costa *et al.,* 2007), this indicates an intense decomposition of organic molecules generating respiratory substrates. This extracellular enzymatic activity (EEA) has as main objective the degradation of large and complex organic molecules into simpler and more easily assimilated ones by the microbial community. This degradation will not only release less complex forms of carbon, nitrogen, phosphorous and sulphur molecules, but also other components that were associated to the organic matter in decomposition, such as heavy metals. As consequence the binding forms of these heavy metals will be affected, and with this a new concept is introduced in the biogeochemical cycles, elemental speciation in this case heavy metal speciation. Heavy metal speciation refers to the forms in which heavy metals are bound with other elements or constituents of the sediments, such as salts, organic matter, complex ions and others (Tessier, 1979). This speciation is directly related with the mobility and availability of the heavy metals not only to be uptake by plants but also to their availability to enzymatic action. Also

this speciation can be related to metal toxicity, since the more stabilized forms of bounds established by metals and other components are usually inert and armless forms (Duarte *et al.,* 2008). Along the last years several sequential extraction schemes have been developed (Stover *et al.,* 1976; Tessier, 1979; Mossop and Davidson, 2003, Hullebusch *et al.,* 2005) with some differences in which concerns the fractions defined. These chemical extractions schemes are based in extractants of increasing strength for dissolving/destroying a specific group of molecules (salts, carbonates, organic matter, oxides). Considering this, from the first extracted fraction to the last one, the metals released by each extractant are originated in molecules with increasing strength bounds (Tessier, 1979), which can also be interpreted in terms of less bioavailability of the metals in the last fractions. With this, becomes of great importance the consideration of the microbial loop in the biogeochemical cycling of metals, as it was previous considered for the carbon cycle (Raynaud and Lata, 2006). Not only the action of microbes on the organic matter, consequent degradation and metal releasing due to organic bounds breakdown, but also the action of microbes on the sulphidization reactions (e.g. throughout the action of sulphate reducing bacteria) play a very important role on metal biogeochemistry throughout alterations of their speciation. These changes will have impacts on metal bioavailability and consequent uptake by plants and other organisms, closing back the cycle. In the present thesis these processes of pant uptake, translocation, senescence and necromass decomposition and consequent impact on metal biogeochemistry are addressed and integrated as a complex process with several distinct mechanisms occurring in two main compartments: sediment and plant. In the second chapter, the net primary production and senescence of several halophytes is evaluated and compared with the heavy metal uptake, accumulation and exports to the surrounding medium. In the third chapter, the influence of the microorganisms on the decomposition of the contaminated necromass is focused as well as it role on the modifications of the metal speciation in the rhizosphere sediments.

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CHAPTER 2

Heavy Metal Accumulation and Biological Cycling: The Halophyte Perspective

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ABSTRACT

Pools of Zn, Cu, Cd and Pb in leaf, stem and root tissues of *S. fruticosa*, *S. perennis*, *H. portulacoides* and *S. maritima* were analysed on a bimonthly basis, in a Tagus estuary salt marsh. All the major concentrations were found in the root tissues, being the concentrations in the aboveground organs neglectable for sediment budget proposes, as seen by the low root-aboveground translocation. Metal annual accumulation, root turnovers and cycling coefficients were also assessed. *S. maritima* showed the higher root turnovers and cycling coefficients for most of the analysed metals, making this a phyto-stabilizer species. By contrast the low root turnover, cycling coefficient and low root necromass generation makes *S. perennis* the most suitable species for phytoremediation processes. Although the high amounts of metal return to the sediments, due to root senescence, salt marshes can still be considered sinks of heavy metals, cycling heavy metals mostly between sediment and root.

KEYWORDS: Metal cycling; Salt marshes; Root, Turnover periods

1. INTRODUCTION

Salt marshes are natural deposits of heavy metals in the estuarine system (Doyle and Otte, 1997; Williams *et al.,* 1994). When located near polluted areas, these ecosystems receive large amounts of pollutants from industrial and urban wastes, that either drifts downstream within the river flow or of waste dumping from the near industrial and urban areas (Reboreda and Caçador, 2007a). When metals enter salt marshes they spread along with the tides and periodic floods and interact with the sediment and the biotic community (Suntornvongsagul *et al.,* 2007). Most salt marsh plants accumulate large amounts of metals in their aerial and belowground organs (Caçador *et al.,* 1996). Among these are the most common plants of south European salt marshes: *S. fruticosa*, *S. perennis*, *H. portulacoides* and *S. maritima*. Their ability to phytostabilize those contaminants in the rhizosediment, is an important aspect in the ecosystem self-remediative processes and biogeochemistry (Weis and Weis, 2004). Because these species are characterized by a high biomass production, they generate large amounts of litter (Zawislanski *et al.,* 2001). Salt marsh species greatly influence the inputs and outputs of metals and nutrients in the marsh (Brune *et al.,* 2000; Caçador *et al.,* 2004; Reboreda and Caçador, 2007a; Deborde *et al.,* 2008; Sousa *et al.,* 2008; Caçador *et al.,* 2009), due to their different ability of uptake these elements.

Several studies have shown that plant litter decomposition occurs in three different stages, independently of the species (Valiela *et al.,* 1985; Wilson *et al.,*1986; Benner *et al.,*1991). There is a rapid leaching process consisting in a fast loss of the most soluble fractions of the plant material. A second stage consisting in microbial decomposition is slower and ends in a refractory phase (third stage) where the decomposition rate is almost null (Zawislanski *et al.,* 2001). During this decaying process, the metals associated with the decomposing matter stay bounded to the more resistant fraction of the organic matter, remaining in the nearby sediment. Other factors like rhizosphere dissolved oxygen, redox state, temperature and microbial community will also influence the rate of the decomposing process (Pereira *et al.,* 2007). Being the highest metal accumulations found in the root tissues (Caçador *et al.,* 2000; Reboreda and Caçador, 2007a), the decomposition of root litter may also be a source of metals released to the surrounding sediment, throughout leaching (Weis and Weis, 2004; Pereira *et al.,* 2007). The metals released may form complexes with organic acids, bind to organic matter (organic complexes with sulphur radicals) or stay soluble in their ionic form in the sediment (Duarte *et al.,* 2008 and 2009). The sediment components and solution interacts with heavy metals throughout adsorption and absorption reactions but also precipitation and solubilization processes (Tessier *et al.,* 1979). These reactions are very important concerning the metal cycling in this environment, as metals can be trapped in the sediment or exported to the water column, altering greatly the ecosystem metal balance. In Tagus estuary, a large fraction of salt marshes are located near industrial areas, becoming highly contaminated being the sediments the major sink for these inputs of heavy metals (Caçador *et al.,* 2000). With the evaluation of the different species of halophytes and their differential ability to retain elements, comes a great interest in investigating the decomposition and metal releases driven by this decaying process and possible metals exportation. In this work, we investigate the role of the more abundant halophyte species at Tagus estuary in metal cycling throughout the year. The marsh is characterized by a typical zonation with homogeneous stands of *S. maritima* as a pioneer species, colonizing the lower marsh area. Across the elevation transect pure stands of *H. portulacoides* follow *S. maritima*, while *S. fruticosa* and *S. perennis* are found in the middle and upper salt marsh. During this work, we have focused our research on the underground processes, since several works have pointed to high metal stocks in belowground biomass and rhizosediment (Vale *et al.,* 1990; Caçador *et al.,* 2000; Reboreda and Caçador, 2007b). Aiming towards these underground compartments, this work focused on the evaluation of the primary production of several halophytes and how this biomass production and senescence influence the metal concentrations in the halophyte tissues.

2. MATERIAL AND METHODS

2.1. Study site and sampling

Pure stands of *S. fruticosa* (Caryophyllalles, Chenopodiaceae), *S. perennis* (Caryophyllalles, Chenopodiaceae), *H. portulacoides* (Caryophyllalles, Chenopodiaceae) and *S. maritima* (Poales, Poaceae) were sampled bimonthly at Rosário salt marsh (Tagus estuary, Portugal, SW Europe) from October 2001 to June 2002. This species selection was carried out in order to sample the more abundant species in the marsh. As described before (Caçador *et al.,* 1999), the selected period comprises the beginning and the end of the growing season, allowing samplings in the maximum and minimum biomass period for all the studied species. The aboveground biomass was assessed for each species by clipping out five squares of 0.3×0.3 m. Samples were stored in plastic bags and quickly transported to the laboratory. The collected aboveground plant biomass was washed with Milli-Q water to remove dust and sediment, and separated into photosynthetic and non-photosynthetic organs. *S. fruticosa* and *S. perennis* do not have a true shoot system with leaves and stems. From these plants the swollen photosynthetic stems (referred to as "leaves" hereafter) were separated from the dry perennial shoots (referred to as "stems" hereafter). The similar procedure was followed for *S. maritima* where the tillers were divided in leaves and in non-photosynthetic organs (referred to as "stems" hereafter). Otherwise, the shoot system of *H. portulacoides* allows separating leaves from stems. For all species, no flowering plants were sampled. Five sediment cores were also taken at each study site (pure stands of each species) using a tubular probe with 7 cm diameter for sampling the first 25 cm, which contains the majority of belowground components (Gross *et al.,* 1991; Caçador *et al.,* 2004). The belowground plant material was separated from the sediment, carefully under a flux of Milli-Q water (18.2 MΩ.cm) and using a sieve with 212 µm mesh size to remove any adhering particulate matter. Plant organs were oven dried at 60 ºC and powdered in a grinding ball mill (Glen CrestomMM2000) (Gross *et al.,* 1991). Sediment samples were also oven dried at 60 ºC until constant weight, cleaned of roots with tweezers, passed through a 0.25 mm mesh, homogenised and ground with an agate mortar.

2.2. Sediment physical-chemical characteristics.

For all parameters five replicate measurements were made between 10 and 15 cm depth, which are usually characterized by the maximum of root biomass. Redox

potential (Eh) and pH of the sediment between the roots were measured in situ in all sampling periods except October 2001. This sediment obtained in the root zone of the four considered species will be referred as rhizosediment hereafter. pH was measured using a combined glass electrode with one Ag/AgCl reference electrode while for Eh measurements a platinum electrode with a calomel reference electrode was used. Values of redox potential were corrected for the reference potential of H_2. Calibration of redox potential measurements was done using a standard redox solution (Crison, Eh=468 $\pm$ 5 mV at 25ºC). The pH calibration was performed using buffer solutions of pH 4 and pH 7. Pore water salinity was measured using a refractometer (Atago, S/Mill-E). The sediment organic matter content was determined in dried samples by loss of ignition (LOI) at 600 ºC for 2 hours following the method described by Caçador *et al.* (2000). For particle size evaluation, samples were dried to constant weight at 60 ºC for about five days and then homogenised. Particle size was determined using two distinct methods: for fractions larger than 50 mm a column of five sieves with calibrated mesh size (AFNOR type) was used, while for particle size fractions less than 50 mm the pipette method was applied (Gee and Bauder, 1986). All samples were primarily sieved through a 500-mm mesh size and no particles were retained. A total of six grain size classes were considered: 100–500 mm (medium sand), 50–100 mm (fine sand), 20–50 mm, 5–20 mm, 2–5 mm (silt) and 0–2 mm (clay) (USDA Soil Texture Classification System, Buol et al., 1997). For sediment metal accumulation rates several data from previous works were used. The average metal deposition per year could be estimated by considering a sediment density of 1.2 $g.cm^{-3}$ (Reboreda and Caçador, 2007a), a vertical accretion rate of 0.9 cm y^{-1} (Caçador *et al.,* 2007b) and the sediment metal concentrations evaluated during this work.

2.3. Elemental Analysis.

Sediment samples (≈100 mg) were mineralized with 10 mL of HNO_3/HCl (3:1 v/v) at 130ºC. This procedure was repeated twice, as described by Otte (1991). Plant samples were digested with 10 mL of HNO_3/$HClO_4$ (7:1 v/v) at 130ºC according to the method described by Otte (1991). Metal concentrations (Cu, Zn, Pb and Cd), were determined by flame atomic absorption spectrometry (FAAS) with an air-acetylene. International certified reference materials (CRM 145, CRM 146 and BCR 62) were used to ensure accuracy and precision was determined by analysing replicate samples. Trace metal concentrations in the reference materials determined by FAAS were not statistically different from their certified ones (t-student; $\alpha = 0.05$). Control blanks consisting only in

acid mixture were also preformed to ensure that there were no external metal contaminations.

2.4. Litterbags field experiment.

Belowground biomass of the four considered species was collected at Rosário salt marsh. The samples were rinsed and the belowground biomass dried. Approximately 5 g of this material were placed in twenty-four 10 x 10 cm nylon mesh bags with 450 μm diameter holes. The bags were buried at 10 cm depth in their respective environments in order to mimic as closely as possible their natural habitat. A set of three bags was collected bimonthly for each plant species October 2001 to June 2002. In the laboratory, the plant material was removed from the litterbags, rinsed with distilled water, dried at 80 ºC for 48 h, weighted and analysed.

2.5. Statistics and Data Analysis.

Above and Belowground Net Primary Production (NPP, grams) for each species was calculated according to Caçador *et al.* (2007a), using the formula:

$$\mathrm{NPP} = \mathrm{Maximum\ Biomass} - \mathrm{Minimum\ Biomass} \quad (1)$$

The root decomposition was calculated as percentage of NPP (grams) and determined during the litterbags experiment:

$$\mathrm{Root\ decomposition} = \left(1 - \frac{\mathrm{Minimum\ Root\ Biomass}}{\mathrm{Maximum\ Root\ Biomass}}\right) \times \mathrm{Root\ NPP} \quad (2)$$

Aboveground biomass losses (grams) were assessed considering the biomass lost due senescence, using the biomasses recorded as described above for root decomposition (Eq. 2).

Metal pools (mg) were determined multiplying the biomass of a sample at the time t by the metal content per mg at the time t to assess the effective amount of metal retained in the plants.

$$\mathrm{Metal\ Pool} = [\mathrm{Metal}]_t \times \mathrm{Biomass}_t \quad (3)$$

Metal Primary Accumulation (MPA, mg) was calculated according to the following equation, considering the maximum and minimum verified during the sampling season:

$$\text{MPA} = \text{Maximum Metal Pool} - \text{Minimum Metal Pool} \quad (4)$$

Metal Exports (mg) were attained as a percentage of MPA (Eq. 5 and 6), as described above, considering the percentage of mass losses due to decomposition of the belowground (Metal export_{dec}) or due to senescence of the aboveground organs (Metal export_{snc}).

$$\text{Metal Export}_{\text{dec}} = \text{Root Decomposition} \times \text{Root MPA} \quad (5)$$

$$\text{Metal Export}_{\text{dec}} = \text{Aboveground Senescence} \times \text{Abovegroud MPA} \quad (6)$$

In order to make an annual balance between the vegetation and the sediment was considered an average metal concentration in the sediment. Metal returns to the sediment due to biomass losses (root decomposition and aboveground organs decomposition) were determined as (Wenjiao *et al.,* 1997):

$$\text{Metal Return} = \frac{\text{Export Rate} \times \text{MPA}}{\text{Metal Concentration in Sediment}} \quad (7)$$

The cycling coefficient and the turnover period were also calculated according to Wenjiao *et al.,* 1997 and Válega *et al.,* 2008.

$$\text{Cycling coefficient} = \frac{\text{Metal returned due to litter fall}}{\text{MPA}} \quad (8)$$

For determining the turnover rate of each plant part the biomass parameters were used (NPP and Maximum Biomass), while for assessing the turnover rate of each metal pool the metal accumulation parameters were used (MPA and Metal Pool):

$$\text{Turnover rate} = \frac{\text{NPP or MPA}}{\text{Maximum Biomass or Metal Pool}} \quad (9)$$

Both these two last parameters are very used in elemental cycling works, as they give important information about the role of the species in these processes. The turnover period of an element in a specific matrix (e.g. plant species or plant part)

Statistical analysis was performed using Statistic Software version 7.0 from Statasoft Inc. Five replicates were always considered and the data was analysed using Kruskal-Wallis non-parametric test. The correlation coefficient between plant biomass and metal content was also assessed.

3. RESULTS

3.1. Sediment physical-chemical characteristics.

Sediments from the analysed layer (10 – 15 cm) of all sampling points along all the studied period were formed mainly of silt (60 ± 0.4 %) and clay (38 ± 0.4 %). Pore water salinity varied from the upper marsh to the lower marsh. Pore waters from sediments of the upper marsh colonized by *H. portulacoides* and *S. fruticosa* (42 ± 1.0 PSU and 36 ± 0.5 PSU) and the middle marsh sediments colonized by *S. perennis* (41 ± 1.7 PSU) presented higher salinities than the sediments in the lower marsh colonized by *S. maritima* (31 ± 0.2 PSU). This difference was always present, although lower values were observed in spring. Sediment pH presented lower values in the winter (Table 1), however, no statistically significant differences were found between each plant species. The redox potential (Table 1) varied irregularly between plants and throughout the studied period with no distinct pattern. Comparing the organic matter content of the sediments colonized by the considered species, it was found that the sediments of *S. maritima* contained less organic matter (13 ± 1.0 %) than the sediments from areas colonised by the other plant species (*S. fruticosa* = 21 ± 1.4 %; *S. perennis* = 20 ± 1.6 %; and *H. portulacoides* = 20 ± 1.5 %).

Table 1. Average (n=5) pH and redox potential (Eh, mV) in *S. fruticosa*, *S. perennis*, *H. portulacoides*, and *S. maritima* rhizosediments.

		pH	Eh (mV)
S. fruticosa	Dec.	6.9 ± 0.0	17 ± 11
	Feb.	7.3 ± 0.1	-6 ± 10
	Apr.	7.4 ± 0.1	-25 ± 9
	Jun.	7.9 ± 0.0	34 ± 13
S. perennis	Dec.	6.3 ± 0.3	43 ± 10
	Feb.	7.7 ± 0.0	34 ± 14
	Apr.	7.5 ± 0.1	73 ± 7
	Jun.	7.9 ± 0.0	125 ± 19
H. portulacoides	Dec.	7.0 ± 0.0	44 ± 10
	Feb.	7.9 ± 0.0	67 ± 8
	Apr.	7.7 ± 0.0	84 ± 7
	Jun.	8.0 ± 0.0	0 ± 10
S. maritima	Dec.	6.6 ± 0.1	91 ± 7
	Feb.	7.8 ± 0.0	98 ± 7
	Apr.	7.7 ± 0.0	93 ± 8
	Jun.	7.8 ± 0.0	90 ± 12

3.2. Biomass production (NPP) and losses.

Changes in the biomass dry weight (DW) of the four studied species are shown in Table 2. *S. fruticosa* was the most productive species, with highest biomass values both for the aboveground organs and for the root biomass, although the major peaks of

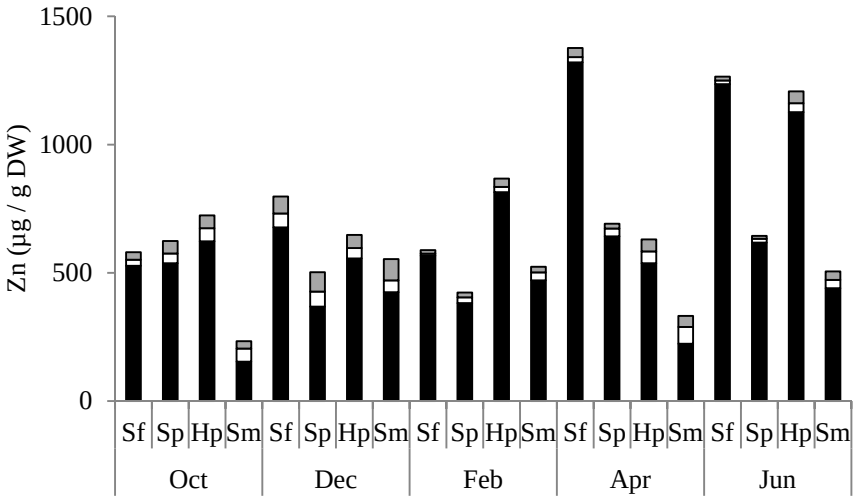
biomass production differ between these organs. The major peaks of aboveground biomass production were detected in October 2001, being this the month where the highest values of leaves and stems biomass were assessed. The exception was found for *S. maritima* that had its highest value of stem biomass in February 2002. This month is also the period where the highest values of root biomass were assessed for all species but *S. perennis*. All four species showed the majority of the biomass allocated in the belowground. The major losses of aboveground biomass were found in *S. fruticosa*, followed by *S. perennis* and *H. portulacoides*, with *S. maritima* presenting the lowest losses of aboveground biomass. As observed for the belowground biomass the highest value of losses was detected in *S. fruticosa*, being *H. portulacoides* and *S. perennis* the species with the lowest losses in root biomass. Except for *S. maritima,* all the other species showed higher turnover rates for the aboveground organs than for the roots. Similar turnover rates were found for leaves and stems in both *S. fruticosa* (0.98 ± 0.01 and 0.93 ± 0.05 y^{-1}, respectively) and *H. portulacoides* (0.78 y^{-1} ± 0.06 for both organs). Higher turnover rates were calculated for *S. perennis* in stems than leaves, while the opposite was verified in *S. maritima*. Root turnovers showed rather low values in both *Sarcocornia* species (0.21 ± 0.05 and 0.43 ± 0.05 y^{-1}) and in *H. portulacoides* (0.23 ± 0.06 y^{-1}), contrarily to what was calculated for *S. maritima*, which showed a turnover rate of 0.99 ± 0.20 y^{-1}.

Table 2. Mean Biomass (n = 5) ± standard deviation values along the considered period and corresponding NPP and biomass losses (*p*-values corresponding biomass seasonal differences are shown in superscript: [1] $p < 0.05$, [2] $p < 0.02$ and [3] $p < 0.01$).

		Oct	Dec	Feb	Apr	Jun	NPP	Losses (%)	Turnover (y-1)
S. fruticosa									
	Leaves [3]	2.83 ± 0.97	0.63 ± 0.15	0.05 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	2.79 ± 0.96	98.23 ± 1.11	0.98 ± 0.01
	Stems [3]	1.62 ± 0.70	0.70 ± 0.21	0.21 ± 0.07	0.12 ± 0.05	0.11 ± 0.03	1.61 ± 0.50	92.90 ± 4.76	0.93 ± 0.05
	Roots [3]	4.37 ± 0.50	4.57 ± 0.18	7.18 ± 0.37	5.35 ± 0.47	4.25 ± 0.16	3.01 ± 0.50	40.65 ± 3.36	0.42 ± 0.05
S. perennis									
	Leaves [2]	0.17 ± 0.04	0.06 ± 0.02	0.04 ± 0.00	0.04 ± 0.00	0.06 ± 0.01	0.13 ± 0.04	77.38 ± 6.66	0.77 ± 0.07
	Stems [3]	0.42 ± 0.04	0.15 ± 0.06	0.08 ± 0.00	0.04 ± 0.02	0.08 ± 0.02	0.38 ± 0.04	89.80 ± 4.02	0.90 ± 0.04
	Roots [3]	3.90 ± 0.33	4.17 ± 0.43	4.55 ± 0.21	4.86 ± 0.09	4.09 ± 0.15	1.01 ± 0.25	16.35 ± 2.33	0.21 ± 0.05
H. portulacoides									
	Leaves [3]	0.21 ± 0.06	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.00	0.06 ± 0.02	0.17 ± 0.05	78.22 ± 3.97	0.78 ± 0.04
	Stems [3]	0.73 ± 0.12	0.32 ± 0.05	0.19 ± 0.05	0.18 ± 0.05	0.19 ± 0.03	0.57 ± 0.15	77.58 ± 8.07	0.78 ± 0.08
	Roots [3]	4.13 ± 0.11	3.58 ± 0.28	4.48 ± 0.27	4.42 ± 0.07	4.39 ± 0.23	1.02 ± 0.29	16.84 ± 8.64	0.23 ± 0.06
S. maritima									
	Leaves [3]	0.15 ± 0.06	0.06 ± 0.02	0.00 ± 0.00	0.10 ± 0.02	0.08 ± 0.02	0.15 ± 0.06	97.33 ± 1.09	0.97 ± 0.01
	Stems [3]	0.28 ± 0.07	0.15 ± 0.03	0.34 ± 0.07	0.27 ± 0.05	0.18 ± 0.08	0.22 ± 0.04	55.01 ± 19.17	0.65 ± 0.07
	Roots [3]	2.56 ± 0.60	1.51 ± 0.06	3.68 ± 0.49	3.39 ± 0.62	3.21 ± 0.37	2.83 ± 0.35	38.10 ± 16.06	0.99 ± 0.20

3.3. Heavy metal concentrations in the different species and plant organs.

The variation observed in biomass production along the survey, did not follow the same pattern that the metal uptake and translocation variations in all cases. Over viewing the metal concentrations along the studied period within the considered species (Figure 1), it is possible to notice that the higher fraction of metal accumulations was found in roots. The preferential order of metal accumulation in this organ was: Pb < Zn < Cu < Cd. Except for Pb, all the other metals accumulations were statistically different from species to species and within plant organs ($p < 0.05$). In December there were found the greater similarities between the metal accumulations either between organs or plant species. The major metal pools were always assessed for the belowground organs, due to the preferential metal accumulation in these organs and higher root biomass. Considering only the aboveground organs, it was possible to notice that the major metal pools were verified in the stems of the considered species. Overall, Pb pool in both aboveground and belowground organs was one of the major pools calculated in this study. Pb root accumulation was found to be very similar in all species in October ($p > 0.05$), although *S. maritima* showed the lowest values. The same trend was verified for the Pb concentrations in the leaves of all species, in February. The temporal variation of the metal pools showed that only the Pb pool in the leaves of *S. maritima* didn't exhibit any defined temporal pattern ($p > 0.05$). As for Cd, it was found that only in February the concentrations of this element were similar in all species roots and leaves. The temporal variation of Cd concentration evidenced that only the root concentrations didn't show a statistically significant pattern ($p > 0.05$). The lowest pools were calculated for this metal, as expected, due to the lower abundance and availability in the salt marsh sediments. Only in *S. perennis* root Cd pool showed a significant variation along the studied period ($p < 0.01$). For Cu only it's concentration in stems didn't show temporal variation ($p > 0.05$). In April all species presented similar accumulations of Cu. This was more evident when considering this metal concentration in the aboveground organs with identical values for all species ($p > 0.05$). Overlooking Cu pools, it was observed that their pool in the stems of *S. fruticosa* and in the roots of *S. perennis* have no statistically significant temporal variation ($p > 0.05$). Unlike the other metals, Zn concentrations in both aboveground plant organs showed no statistically differences between species ($p > 0.05$). As for the root accumulation, in February and April the concentrations were identical in all the considered species.


Zn (µg / g DW)
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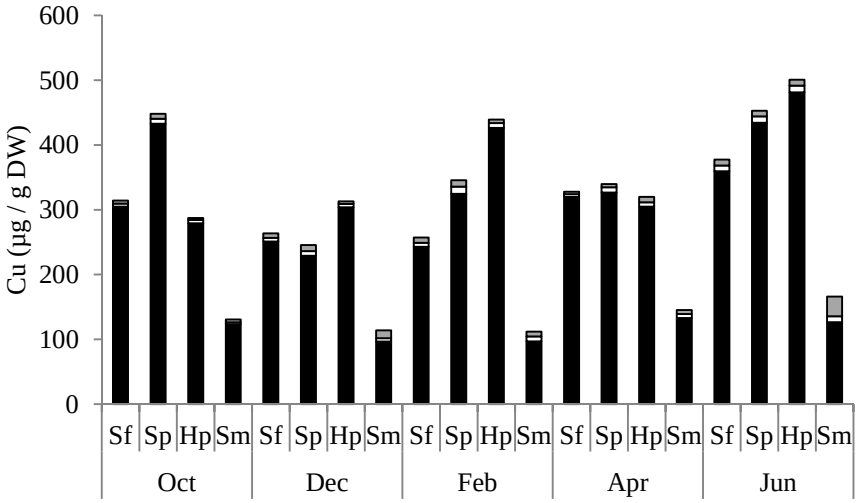
1000
500
0
Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm
Oct
Dec
Feb
Apr
Jun


Cu (µg / g DW)
600
500
400
300
200
100
0
Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm
Oct
Dec
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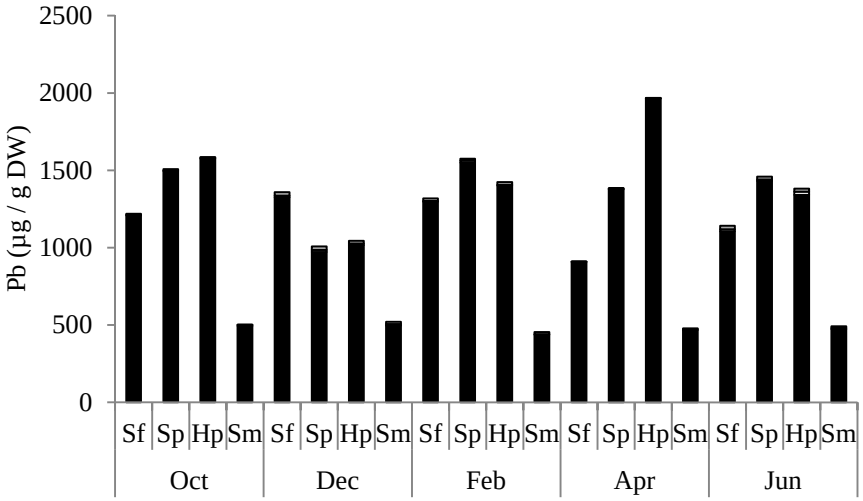
Apr
Jun


Pb (µg / g DW)
2500
2000
1500
1000
500
0
Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm Sf Sp Hp Sm
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Dec
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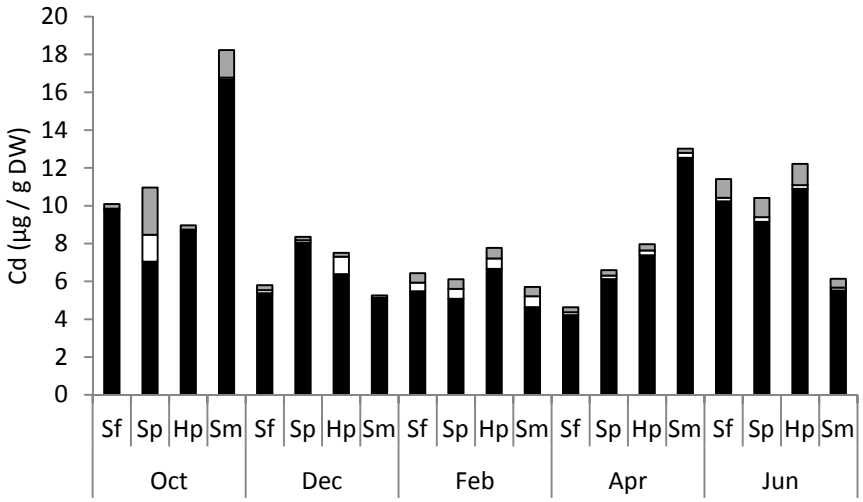

Figure 1. Above- and belowground metal accumulation (n = 5) values for *S. fruticosa* (Sf), *S. perennis* (Sp), *H. Portulacoides* (Hp) and *S. maritima* (Sm) measured between October 2001 to June 2002 (Roots ■ Stems □ Leaves ■).

Table 3 presents the correlation coefficients between metal concentrations in plant part and biomass. Non-significant correlations were found for most of the cases, suggesting that metal accumulations in the different organs do not depend on the biomass production. Exceptions were found for Cd, with two opposite patterns. While in *S. fruticosa* metal levels and biomass production were inversely correlated, for *S. perennis* and *S. maritima* strong positive correlations were found in aboveground organs. By concerning the differences between the metal pools of the four plant species, it is noticed that in December and April all the metal pools were statistically different both, between species and between organs ($p < 0.02$ in December and $p < 0.05$ in April). The same trend was found in February ($p < 0.01$) with the exception of Pb pool in stems, as already stated. October and June were the periods with fewer differences found between the metal pools ($p > 0.05$).

Table 3. Correlation coefficients between plant parts biomass and metal content (* $p < 0.05$, ** $p < 0.02$ and *** $p < 0.01$).

		Zn	Cu	Pb	Cd
S. fruticosa					
	Stems	0.28	-0.27	-0.08	-0.44 *
	Leaves	0.00	-0.25	-0.30	-0.39
	Root	0.01	-0.22	0.09	-0.47 *
S. perennis					
	Stems	0.30	-0.26	-0.49 **	0.90 ***
	Leaves	0.29	-0.05	-0.16	0.90 ***
	Roots	0.03	-0.23	0.02	-0.33
H. portulacoides					
	Stems	0.46 ***	-0.38	-0.24	-0.29
	Leaves	0.24	-0.54 ***	-0.40 *	-0.32
	Roots	0.20	0.27	0.25	0.15
S. maritima					
	Stems	-0.10	0.09	-0.20	0.20
	Leaves	-0.05	-0.11	-0.74 ***	0.59 ***
	Roots	0.20	0.19	-0.15	-0.17

3.4. Heavy metal concentrations in sediments.

Analysing metal concentrations in the sediment surrounding the roots of the four species (Fig. 2) it was found that for Cu the highest annual concentration was found in the rhizosediment of *S. perennis* while the lowest was assessed for *S. fruticosa*. Higher Zn concentrations were measured in the rhizosediment of *S. maritima*, while in the remaining species rhizosediment the concentrations were very similar. Lead and cadmium concentrations exhibited the same pattern, being higher in *S. fruticosa* rhizosediment and lower in *H. portulacoides*.

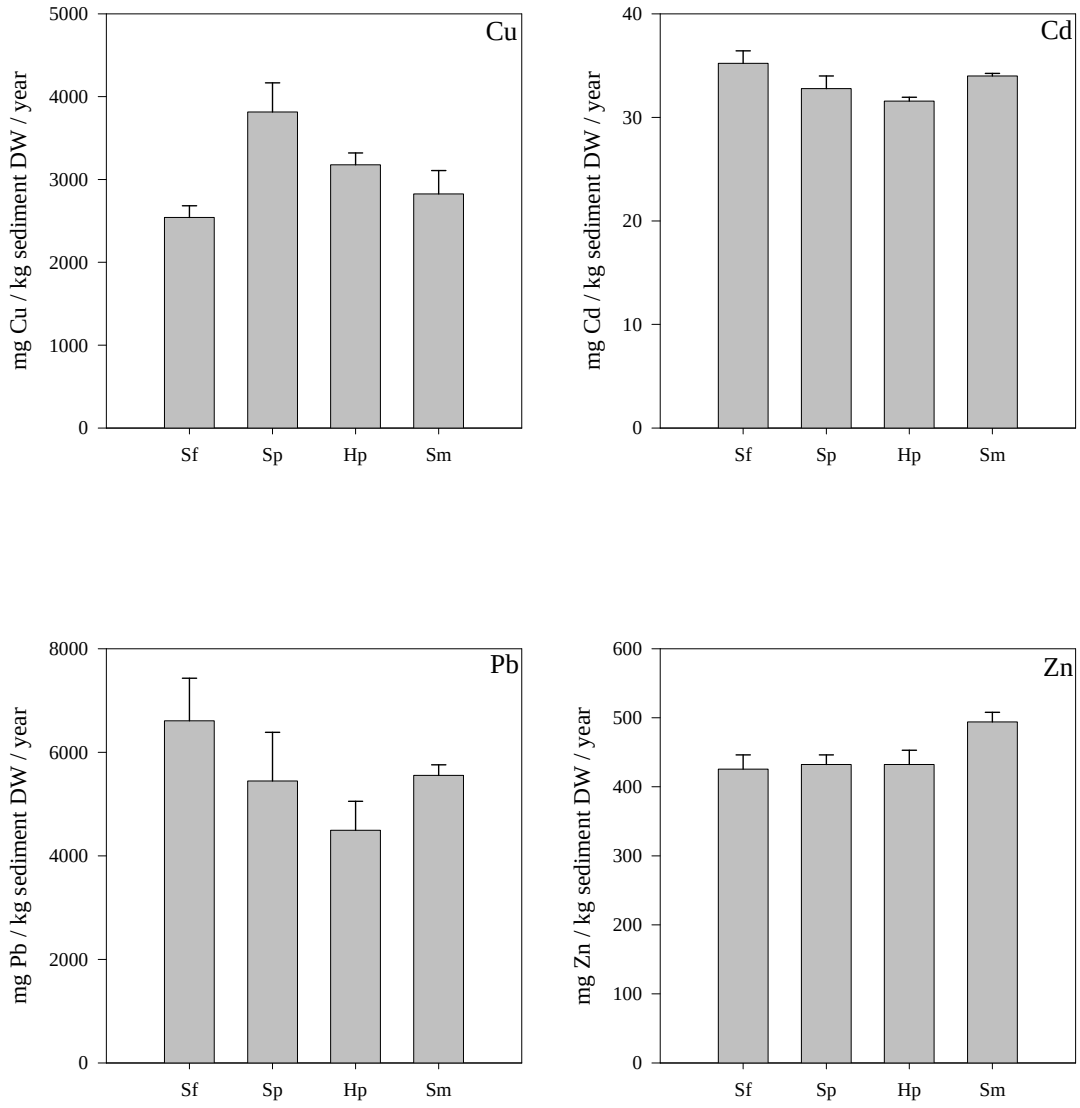

Figure 2. Average metal concentrations (n = 5) in sediments colonized by the four studied species (Sf – *S. fruticosa*, Sp – *S. perennis*, Hp – *H. portulacoides*, Sm – *S. maritima*), calculated for a one-year period.

3.5. Biological cycling of heavy metals in the salt marsh vegetation.

Metal primary accumulation (MPA) and exportations due to senescence are shown in Fig. 3. The differential metals accumulation for each plant species result in distinct accumulation budget (MPA). Only the annual accumulation of Zn in the stems and Cd has showed fewer differences between species ($p > 0.05$). As observed for metal pools, the highest values of MPA were always found for Zn, followed by Pb and Cu. Since the MPA calculations take into account the metal pools, the variation of MPA between metals and species mirrors their metal pools. Overlooking to all data of the MPA is possible to notice that the annual budget of the aboveground is very low compared to the large metal primary accumulation of the bellow ground. This difference

results from the small amount of metals translocated from roots to the aerial plant organs.

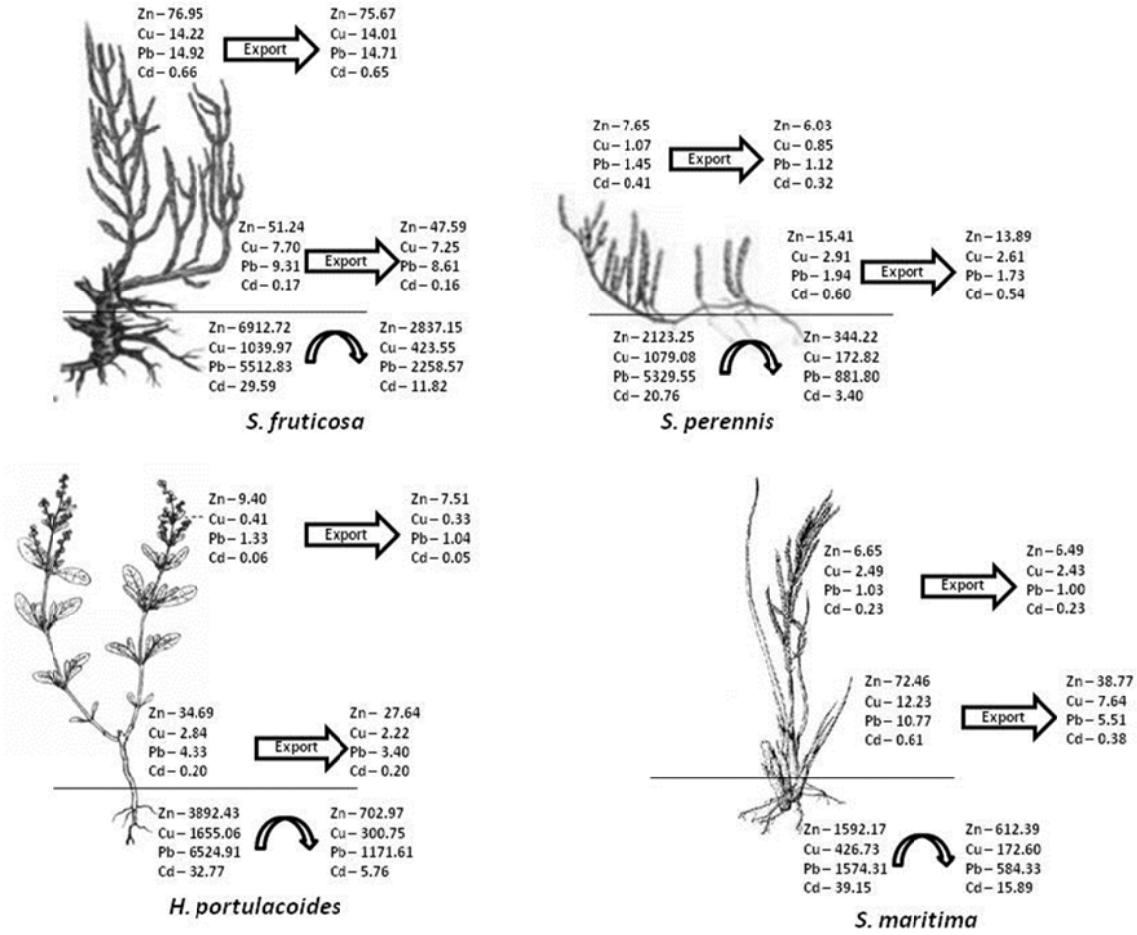

Figure 3. Metal Primary Accumulation (MPA, mg) and losses due to litter generation for the studied species and plant organs (Sf – *S. fruticosa*, Sp – *S. perennis*, Hp – *H. portulacoides*, Sm – *S. maritima*).

To better understand which were the major sinks of metals, translocation ratios were assessed for the studied species (Table 4). Only Zn did not show any significant differences between species ($p > 0.05$). In general it is possible to observe that all the translocation ratios are below 9%, being the majority of the values lower than 1%. This indicates that, for this salt marsh plants, aboveground organs have a minor contribution in metal storage. Thus, our attention focused in the root and sediment recycling processes, which are the major sinks of metal accumulation. The metal remobilization due to root biomass losses was also analysed, throughout the root turnover for each metal (Table 4).

Table 4. Translocation ratios, cycling coefficient and root turnover (n = 5, average ± standard deviation) for all the considered species.

		Zn	Cu	Pb	Cd
S. fruticosa	Translocation ratio (%)	2.14 ± 0.56	2.83 ± 0.78	0.46 ± 0.12	2.31 ± 0.43
	Cycling Coefficient	0.42 ± 0.03	0.42 ± 0.03	0.41 ± 0.03	0.42 ± 0.04
	Root Turnover	0.80 ± 0.08	0.50 ± 0.14	0.59 ± 0.06	0.59 ± 0.13
S. perennis	Translocation ratio (%)	1.15 ± 0.38	0.36 ± 0.14	0.07 ± 0.01	4.03 ± 0.99
	Cycling Coefficient	0.17 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.20 ± 0.06
	Root Turnover	0.64 ± 0.12	0.55 ± 0.24	0.65 ± 0.17	0.51 ± 0.23
H. portulacoides	Translocation ratio (%)	1.27 ± 0.64	0.21 ± 0.10	0.10 ± 0.05	1.06 ± 0.36
	Cycling Coefficient	0.39 ± 0.15	0.41 ± 0.15	0.37 ± 0.16	0.41 ± 0.16
	Root Turnover	0.67 ± 0.10	0.65 ± 0.08	0.64 ± 0.14	0.63 ± 0.12
S. maritima	Translocation ratio (%)	8.94 ± 4.84	3.29 ± 2.57	1.01 ± 0.25	0.75 ± 0.18
	Cycling Coefficient	0.39 ± 0.15	0.41 ± 0.16	0.37 ± 0.15	0.41 ± 0.16
	Root Turnover	0.79 ± 0.07	0.73 ± 0.06	0.72 ± 0.11	0.83 ± 0.07

Considering all the studied species, it was found that only Cu losses were not statistically different between species ($p > 0.05$). Considering that the more accumulated metals in the root tissues were always Zn and Pb, the calculated metal remobilization into the sediment, showed that these metals present the highest remobilized amounts. Considering the different root biomass losses and also different root MPAs, it is possible to observe that the major amounts of metal remobilized into the sediment was assessed for *S. fruticosa*, in opposition to *S. maritima* that only returned a small amount of metals to the sediment. This is in agreement to what was verified before for the metal pools. The calculated root decompositions showed higher decomposition percentages for *S. fruticosa* and *S. maritima* (≈ 40 %) than for *S. perennis* and *H. portulacoides* (≈ 18 %). Due to the low translocation rates from the root to the aboveground (Table 4), we focused mainly in the root turnovers. Although root turnover (Table 4) differed between species ($p < 0.01$), only the turnover of Cd concentration in the roots was statistically different between species ($p < 0.02$). Overall, the highest root turnover of Cu, Pb and Cd was found for *S. maritima*, while for zinc the highest value was for *S. fruticosa*. As for the cycling coefficients (Table 4) assessed for

the four studied species it was found that the lowest values for Zn were in *S. perennis*, indicating a great mobilization of metal, while coefficients below 0.5 for *S. fruticosa* and *S. maritima* are indicative of moderate mobilization. Analysing Cu and Pb cycling it was found that *S. perennis* has the lowest value of cycling coefficient mobilizing great amounts of these metals. *S. fruticosa* has a comparatively high value of this parameter for Cu and Pb, although below 0.5. Again in the case of Cd, *S. maritima* shows an extremely high cycling coefficient, while *S. fruticosa* shows a value around 0.5 being this value the lowest. Both, *H. portulacoides* and *S. maritima,* showed values above one indicating a return of Cd proportional to the mobilized by these species.

4. DISCUSSION

Wetlands, in particular salt marshes, are generally considered to be suitable for retaining heavy metals. The rhizosediment and vegetation metal concentrations assessed in this study for Rosário salt marsh, are of the same magnitude as the results obtained in previous studies (Reboreda and Caçador, 2007a; Caçador *et al.,* 2000). Being a polluted salt marsh the metal cycling throughout the sediment and the vegetation becomes of great interest. According to our results it is possible to observe that the two major pools of heavy metals in the salt marshes are the sediment and the root system of resident species. The pools of metals bioaccumulated by the four studied species were much lower in the aboveground organs, as seen by the lower translocation rates and by the metal concentrations, especially for Pb, probably due to its low solubility in less oxic environments (Sundby *et al.,* 2005). The higher metal budgets in roots corroborate their increased ability for heavy metal accumulation. The calculated root decomposition rates suggest that these metal pools are quite mobile in particular in *S. fruticosa* and in *S. maritima*. This mobility is very important since creates a cycle of metals between the sediment and the root system. Although it was found a higher fraction of biomass losses in the aboveground organs, their low metal concentration makes the detritus generated by the aboveground less contributing for the metal budgets. Conversely, the comparatively low losses of biomass in the root system generate less necromass, but with very high concentrations of metals. This necromass becomes important to de metal budget of the sediment, not only due to its input of heavy metals, but also due to the increase of organic matter content of this matrix. Therefore, the metal cycling between the two major sinks (roots and sediments) was also evaluated throughout the root turnovers and cycling coefficients. The root turnover values were above 0.5, being the highest detected in *S. maritima*, indicating that the cycling of metals between roots and sediments occur at a rather slow rhythm.

Sediments colonized by *S. maritima*, have different characteristics from those in the upper marsh where the other species are dominant. Low marsh sediments are subjected to larger periods of submersion that could have potentially prevailing anoxic conditions. These conditions are only counteracted by the active oxygen pumping from the atmosphere to the rhizosphere, by *S. maritima* (Duarte *et al.,* 2009). In these sediments the metals are highly bound to sulphides and/or organic matter, being easily stabilized in the solids (Reboreda and Caçador, 2007a). Moreover, root system of *S. maritima* has lower specific area than the other plant species, which leads to minor root-sediment interaction (Lee *et al.,* 1999). Consequently, less metal sulphides are oxidised and transported towards their roots (Caetano *et al.,* 2008). Although low metal turnover rates from root to sediment were found in the other salt marsh plants, roots are the most important biological sink of metals in salt marshes. The bioaccumulation of these elements in the belowground organs is rather mobile, being able to return to the sediment matrix due to necromass generation and mineralization processes subdue. The return of metals due to these decaying processes and consequent input of metals into the sediments, although in rather lower concentrations comparatively to the existent in this matrix, is very important to be considered not only due to the amount of metal released through this process, but also by the metal forms it introduces into the sediment. These organic bound metals were already reported as being one of the most important fractions of metals present in these sediments, being subjected to microbial degradation processes (Duarte *et al.,* 2008), that can lead to more bioavailable metal forms and contamination of the pore waters. This process will be focus of attention in Chapter 3 of this work.

5. CONCLUSIONS

Metal budgets can be very important while assessing remediation projects. Both the high root turnovers and cycling coefficients verified in *S. maritima* for the majority of the analysed of metals can be useful for understanding metal cycling. The low cycling of the elements from sediment to plant clearly points out that this species act as phyto-stabilizer. For phytoremediation proposes it should be considered the use of species with low root turnovers and low cycling coefficients. Coupled with both these characteristics, the low root necromass generation makes *S. perennis* the more suitable of the studied species, for remediation projects. From the ecosystem point of view, these findings help understanding the role of plant detritus in the trophic transport of metals and also the processes involved in the contamination of a salt marsh and the adjoining estuarine areas. This study should be regarded as a starting point to better

understand the metals cycling within the salt marsh, not only the flux between plant and sediment, but also through the trophic chain. Furthermore, the results presented here support the observations from previous studies that point out salt marshes as a sink of heavy metals, clarifying the major cycles of metals between the two more important metal retaining matrixes.

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CHAPTER 3

Seasonal Variation of Extracellular Enzymatic Activity (EEA) and its Influence on Metal Speciation in a Polluted Salt Marsh.

The results and conclusions within this chapter are published in two scientific papers published as “Duarte, B., Reboreda, R. and Caçador, I., 2008. Seasonal variation of Extracellular Enzymatic Activity (EEA) and its influence on metal speciation in a polluted salt marsh. Chemosphere 73, 1056-1063” and as “Duarte, B., Raposo, P., Caçador, I., 2009. *Spartina maritima* (cordgrass) rhizosediment extracellular enzymatic activity and its role on organic matter decomposition and metal speciation processes. Marine Ecology 30, 65-73”.

ABSTRACT

In order to study the relation between organic matter cycles and the metal species associated, the influence of salt marsh sediment extracellular enzymatic activity (EEA) on metal fractions and organic matter cycling was evaluated on a seasonal basis. Metals in the rhizosediment of *Halimione portulacoides* were fractioned according to the Tessier's scheme and showed a similar pattern concerning the organic bound fraction, being always high in autumn, matching the season when organic matter presented higher values. Both organic bound and residual fractions were always dominant, being the seasonal variations due to interchanges between these fractions. Phenol oxidase and β-N-acetylglucosaminidase had higher activities during the spring and summer, contrarily to peroxidase, which had it higher activity during winter. Protease showed high activities in both spring and winter. These differential periods of high organic matter hydrolysis caused two periods of organic metal bound decrease. Sulphatase peaks (spring and winter) matched the depletion of exchangeable forms of metals, probably due to sulphides formation and consequent mobilization. This showed an interaction between several microbial activities affecting metal speciation.

KEY WORDS: Salt marsh, extracellular enzymatic activity, sediment, metal speciation

1. INTRODUCTION

Salt marshes located in estuaries are frequently the recipient of high nutrient runoff (Tobias *et al.,* 2001), but also of particulate and dissolved organic matter as well as plant litter. This nutrient load makes salt marshes some of the most productive ecosystems on the planet. Being highly productive there are large amounts of biomass produced that will generate large amounts of decaying litter. Along with this, in highly industrialized estuaries, there is also a great input of metals that are accumulated in the salt marsh sediments (Doyle and Otte, 1997), making this ecosystems key zones not only for the biogeochemistry of the estuary, but also for metal cycling (Weis and Weis, 2004). Sediment microbial communities play an important role in these processes, being the base of the ecosystem as they are the major decomposers of organic matter releasing nutrients into more phyto-available forms (Ravit, 2005). Wetlands, in particularly salt marsh sediments are often very organic (Richert *et al.,* 2000), being a good support for the microbial activity by providing large amounts of belowground litter as well as organic compounds exudated by living plants (Duarte *et al.,* 2007). This kind of sediment is often waterlogged and shows low levels of oxygen, being adverse to

plant growth (Richert *et al.,* 2000). However salt marsh plants are well known for pumping oxygen from the atmosphere to the sediment, turning the redox conditions of the root zone oxidative and having as consequence the stimulation of the aerobic microbial activity (Ludemann *et al.,* 2000). This kind of plant-microflora interaction is very variable, depending not only on the plant species but also the plant growth period and physiological state.

Microbial enzymes are involved in several biogeochemical cycling processes, catalyzing the conversion of multiple complex molecules into smaller ones, either by redox reactions (catalyzed by oxidoreductases) or by organic matter breakdown (promoted by hydrolases). Being these ecosystems very productive, they are also of great importance in which concerns the organic matter recycling by the microbial decomposers, in order to maintain the ecological balance. There are several extracellular enzymes that are very important for this process, like proteases, phenol oxidases, peroxidases and β-*N*-acetylglucosaminidases, contributing for the breakdown of several organic compounds (Kang *et al.,* 2005; Oyekola and Pletschke, 2006; Acosta-Martínez *et al.,* 2007). There are also evidences that sulphur linked reactions also play an important role on the salt marsh sediments decomposition processes, either by reactions of sulphidisation of organic matter (Passier *et al.,* 1999) or by leaching of labile organic sulphur present in tissues, during the early stages of decomposition (Brüchert and Pratt, 1996 in Passier *et al.,* 1999).

Tidal flooding of the salt marsh supplies considerable amounts of heavy metals from nearby urban and industrialized areas, which tend to accumulate in sediments and plant tissues. These metals retained in the sediment present various forms (Tessier, 1979) depending on the bounds they establish with the different sediment components. This is also a very dynamic process mostly influenced by the sediment biogeochemistry and by external factors (hydrodynamics, weather and seasonal variation) but also by the vegetation that colonizes the area (Reboreda and Caçador, 2007). There are also evidences that microbial activity can greatly influence metal speciation, throughout interactions with metal ligands (Gadd, 2001 and 2004; Tabak *et al.,* 2005). These transformations include reactions of metal precipitation by metallic sulfides and redox reactions with changes on the metal species and associations (Hullebusch *et al.,* 2005). Along with this, the environmental conditions of this ecosystems and the important role of salt marsh plants introducing great amounts of organic matter, enhances these microbial processes (Otero and Macías, 2002). Caçador *et al*. (2000) showed a strong seasonal variation of plant biomass in these ecosystems together with a variation in metal concentrations in plant tissues, indicating a possible similar variation in the metal biogeochemistry. Studies on metal-microbe

interactions are scare, focusing mainly on enzymatic inhibition processes, rarely considering seasonal patterns either of extracellular enzymatic activity (EEA) profiles or metal speciation. There is also an evident lack of bibliography, concerning studies of extracellular enzymatic activities in salt marsh sediments in its role in the ecosystem biogeochemistry. Great part of the bibliography focuses in inhibition processes driven by heavy metals in sediments (Smejkalová *et al.,* 2003; Renella *et al.,* 2004; Mikanova, 2006 and others from the same authors). This paper brings a new point of view of the role of microbial driven recycling of organic matter and how it affects the metals bound to this organic matter, and ultimately the sediment metal speciation.

2. MATERIALS AND METHODS

2.1. Site description and sampling

Rosário (38º40'N, 9º01'W) is a mature salt marsh (Valiela, *et al.,* 2000) located in the southern part of the Tagus estuary, in the vicinity of various urbanized and industrialized zones. The upper marsh is mainly colonized by *H. portulacoides* (Chenopodiacea), and *S. fruticosa* (Chenopodiacea) and undergoes short submersion episodes during the high tide. Between October 2006 and July 2007, four samplings were made: October (autumn), January (winter), April (spring) and July (summer). For each sampling, five sediment cores (50 cm depth) were taken in pure stands of *H. portulacoides*. The stands were located along the marsh always with a minimum distance of 10 meters from each stand. All the collections were made during the low tide. The cores were transported in refrigerated bags to the laboratory, were the sediment was sliced. According to previous studies (Reboreda and Caçador, 2008) the depth between 5-10 cm proved to have high EEA and it was used for analysis. These sediment samples surrounding the rhizosphere of *H. portulacoides* are referred hereafter as rhizosediment.

2.2. Sediment physical-chemical characteristics

Redox potential (Eh) and pH values were measured in the fresh selected sediment slice using HANNA pH/mV (HI 9025). Calibration of redox potential measurements was done using a standard redox solution (Crison, Eh=468 ± 5 mV at 25ºC). The pH calibration was performed using buffer solutions of pH 4 and pH 7. Organic matter was determined by the loss on ignition (LOI) method by burning 1 g of sediment at 600 ºC

for 2 h. Humic acids were extracted and quantified according to Adani *et al.* (1995) with some modifications concerning the sediment to extractant ratio. To 5 g of dried and sieved sediment were added 25 ml of a solution 0.1 M NaOH + 0.1 M $Na_4P_2O_7$. The extraction was carried out in an end-to-end shaker for 24 h at 65 ºC. After extraction the samples were centrifuged at 45.880 x g for 20 min at 4 ºC. The supernatant was recovered totally and to the solid residue was added distilled water, re-suspended and centrifuged again. This operation was repeated until the supernatant was clear. The supernatant solutions were combined and acidified with 50% sulfuric acid to pH < 1.5 in order to precipitate the humic acids. These were separated by centrifugation as above, the supernatant evaporated completely at 60 ºC until constant weight and the humic acids weighted. Phenolic content in the sediment samples was determined according to Folin and Ciocalteu (1927) modified by Waterman and Molle (1994). Briefly, 5 grams of fresh sediment were mixed with 50 mL distilled water after which, 10 mL of the slurry was centrifuged at 6.53 x g for 2 min at 10 ºC. The supernatant (0.5 mL) was added with 2.5 ml of Folin-Ciocalteu's phenol reagent (0,2 N) and 2.5 ml of alkali reagent, and left to stand for 2 h. After this period the absorbance is read in a Shimadzu UV-1603 spectrophotometer at 760 nm and compared with a calibration curve made with galic acid. Phenolic content was expressed as galic acid (GA) equivalents per gram sediment fresh weight.

2.3. Extracellular enzyme activity in the sediment.

All enzymatic determinations were carried out with colorimetric methods and the absorbances recorded using a TECAN Absorbance Microplate Reader (SPECTRA Rainbow). Phenol oxidase, peroxidase, β-*N*-acetylglucosaminidase and sulphatase were assayed according to Ravit *et al.,* (2003) with a modification in the incubation temperature and without dilution of the supernatant. Briefly, 75 ml of sodium acetate buffer (pH 5) was added to 5 g of fresh sediment, and mixed for 1 min in order to obtain the sediment slurry. The substrates (5 mM) used were *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-sulphate, respectively for β-*N*-acetylglucosaminidase and arylsulphatase. Two ml of each substrate were added to 2 ml of slurry and incubated at 30 ºC with gentle agitation for 60 min (sulphatase) and 2 h (β-*N*-acetylglucosaminidase). After the incubation, samples were centrifuged at 6.530 x g for 15 min, at 4ºC and 0.2 ml of 1 N NaOH was added in order to stop the reaction and reveal the *p*-nitrophenol (*p*NP) formed. Absorbance of the supernatant was read at 410 nm and compared with the calibration curve for (*p*NP). The activity was expressed as

μg of *p*NP released per gram of sediment dry weight per hour. Phenol oxidase and peroxidase were assayed using 5 mM L-DOPA (L-3,4-dihydroxyphenylalanine) as substrate. Two ml were added to 2 ml of slurry (adding 0.1 ml of 0.3 % H_2O_2 for peroxidase assay) and incubated for 60 min for both enzymes. After incubation samples were centrifuged ate 6.53 x g for 15 min, at 4 ºC. Absorbance of supernatant was read at 460 nm and the absorbance of phenol oxidase was subtracted from the absorbance of total peroxidase in order to obtain the real value for peroxidase activity alone. The activity was expressed as μmol L-DOPA oxidized per gram sediment dry weight per hour, by comparison with the L-DOPA standard curve. Protease activity was assayed according to Ladd et al., (1976). Briefly, 1 g of fresh sediments was incubated with 5 ml of Tris (Trishydroxymethyl-aminomethane) buffer (0.05 M, pH 8.1) and a 2% (w/v) casein solution, for 2 h at 50 ºC. After incubation the reaction was stopped with 1 ml of thichloroacetic acid 17.5 % (w/v) and centrifuged at 14.690 x g for 15 min, at 4 ºC. For photometric analysis, 1 ml of supernatant was added to 1 ml of Folin-Ciocalteu's phenol reagent (0.2 N) and 2.5 alkali reagent, and left to stand for 90 min. The color developed was measured at 700 nm and compared with a calibration curve for tyrosine. Activity was expressed as μg tyrosine equivalents per gram of sediment dry weight per hour.

2.4. Metal sequential extraction and elemental analysis.

In order to determine chemical fractioning of Cu, Cd, Cr, Ni, Zn, Co and Pb in sediments, a sequential extraction procedure was used. In previous works (Sousa *et al*., 2008) it was verified that these were the more abundant metals and with higher ecological relevance at Tagus salt marshes. For metal determinations all labwares were soaked for two days in hydrochloric acid (10%) and rinsed with distilled water to avoid contaminations. The metal sequential extraction scheme adopted in this study was the described by Tessier (1979) and modified by Hullebush *et al.,* 2005. Briefly, 1 g of air dried sediment was sequentially extracted by 1 M ammonium acetate (exchangeable/available fraction, corresponding to the most labile fraction of the metal weakly bound to sediment constituents), 0.6 M acetic acid (carbonate bound fraction, more susceptible to changes in pH), 30 % hydrogen peroxide (organic bound fraction, comprehending living organisms, detritus, peptidic molecules and coatings) and *aqua regia* (residual fraction, mainly primary and secondary minerals containing metals in their crystal structure). Between all steps the sediment was centrifuged 20.4 x g for 10 min at 4º C and the supernatant filtered by Whatman No. 42 filters (2.5 μm of pore

diameter) and stored at 4 ºC until analysis. A total digestion was performed with *aqua regia* in a Teflon reactor at 110 ºC for 3 h, in order to evaluate the efficiency of the sequential procedure. All efficiencies were between 95-110%. Concentrations of Cu, Cd, Cr, Ni, Zn, Co and Pb were determined by Flame Atomic Absorption Spectrometry (SpectraAA 50, VARIAN) or Graphite Furnace ASS (932 plus, GBC). The accuracy of the results was checked by processing reference material CRM 145 and CRM 146. Control blanks consisting only in extracting solution, were also preformed at each extraction step to ensure that there were no external metal contaminations.

2.5. Statistics and data analysis

Statistical analysis was performed using Statistica Software version 7.0 from Statasoft Inc. Due to the lack of normality and homogeneity of the environmental values obtained, the significance of the results was evaluated using Kruskal-Wallis non-parametrical tests.

3. RESULTS

3.1. Sediment physical-chemical characteristics

Both organic matter content, determined as LOI, and humic acid concentrations (Table 1) showed a very marked seasonal variation in the sediments colonized by *H. portulacoides* ($p < 0.02$ for LOI and $p < 0.03$ for humic acid concentration), increasing from spring to autumn and with a substantial decrease during winter. Redox potential and pH (Table 1) were found to be no season related in *H. portulacoides* rhizosediments ($p > 0.05$), having some fluctuations along the considered period. The more reduced sediments were collected in the cold seasons (autumn and winter) while during spring and summer the rhizosediment collected was always more oxidized. Sediment phenolic content (Table 1) was always very low during most of the year, except in winter, were there was an evident increase of this content, showing a significant seasonal dependence ($p < 0.05$).

Table 1. Sediment characteristics along the studied period (mean value ± standard error)

	Autumn	Winter	Spring	Summer
Eh (mV)	- 12.73 ± 5.28	- 0.32 ± 9.96	43.77 ± 1.83	16.35 ± 6.91
pH	7.21 ± 0.10	6.86 ± 0.20	6.21 ± 0.04	6.71 ± 0.12
LOI (%)	31.83 ± 1.34	19.00 ± 0.04	18.10 ± 0.26	21.42 ± 0.31
Humic Acids (g HA / g DW)	0.30 ± 0.02	0.11 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
Phenolics (meq GA / g DW)	12.16 ± 1.17	918.56 ± 36.78	0.00 ± 0.00	20.81 ± 12.01

3.2. Metal content and speciation

Overlooking the metal speciation results it was possible to notice a similar behavior in all metals concerning the organic bound fraction (Fig. 1). This metal fraction was always higher in autumn, matching the season when organic matter presented higher values. Cadmium concentration in the sediment presented values rather low in comparison with the other studied metals. This metal is very mobile although it was mainly present in the residual fractions, which were dominant along almost all the year (Fig. 1). In autumn, this fraction was reduced and it was verified an increase in the organic fraction, indicating a seasonal pattern, although it wasn't statistically significant ($p > 0.05$). In winter the carbonate bound fraction disappeared (Fig. 1). Cobalt fractioning showed similar distribution, although with lower values for the organic bound fraction. The available fraction in the rhizosediment of *H. portulacoides* showed a seasonal pattern ($p < 0.05$) increasing during summer and autumn and decreasing in winter and spring, while for the residual and organic fraction the inverse trend was observed. Most of the differences between fractions were found in the warm seasons. As for Cu and Cr, both elements were only found in considerable amounts in the organic and residual fractions in different proportions (Fig. 1). Both these elements showed an increase of the organic bound fraction during summer and autumn, along with a depletion of the residual fraction, while it was possible to notice the inverse behavior during spring and winter. Copper was present in almost equal parts in both these fractions while for Cr the major concentration (65-70%) was found in the residual fraction. As for Cu the two most representative fractions proved to be seasonally affected ($p < 0.05$). When analyzing Cr, it was possible to notice a seasonal variation, being this variation well marked in the residual fraction ($p < 0.02$). Lead distribution among the sediment fractions was found to be in greater amounts in the residual and

organic fractions, being the most available forms almost absent (Fig. 1). This metal concentration along the studied period showed a seasonal pattern influenced by fluctuations in the residual fraction ($p < 0.05$). Zinc is highly abundant (Table 2) and more mobile than the previously metals (high concentration in the available and carbonate bound fractions), and there wasn't an evident seasonal variation of this metal concentration ($p > 0.05$), being the concentrations rather stable along the studied period (Fig. 1). The small fluctuations were majorly due to interchanges in the organic bound and residual fractions. Observing Ni behavior it was possible to verify that all metal fractions assessed by the Tessier extraction scheme, showed a strong seasonal pattern in *H. portulacoides* rhizosediment ($p < 0.03$ for the available, carbonated bound and residual fractions and $p < 0.05$ for the organic bound fraction). During summer and autumn the carbonate bound and available fractions prevailed, while in spring and winter these fractions were very low, and a very significant increase in the organic bound and residual fractions could be verified (Fig. 1).

Table 2. Sediment total heavy metal concentrations in μg metal . g-1 sediment DW (mean value ± standard error).

	Autumn	Winter	Spring	Summer
Cd	2.78 ± 0.55	1.52 ± 0.22	1.67 ± 0.08	1.87 ± 0.09
Co	16.09 ± 2.19	18.37 ± 3.13	11.72 ± 0.49	15.60 ± 0.56
Cu	80.35 ± 7.47	42.25 ± 2.57	40.41 ± 1.02	80.95 ± 2.27
Pb	237.61 ± 31.59	117.70 ± 3.26	117.70 ± 3.26	429.18 ± 53.49
Zn	424.21 ± 86.21	396.48 ± 55.61	284.40 ± 41.78	358.37 ± 16.96
Cr	39.24 ± 1.05	28.65 ± 0.46	25.25 ± 0.90	44.02 ± 0.66
Ni	25.03 ± 5.06	18.05 ± 0.23	19.75 ± 0.62	57.69 ± 11.85

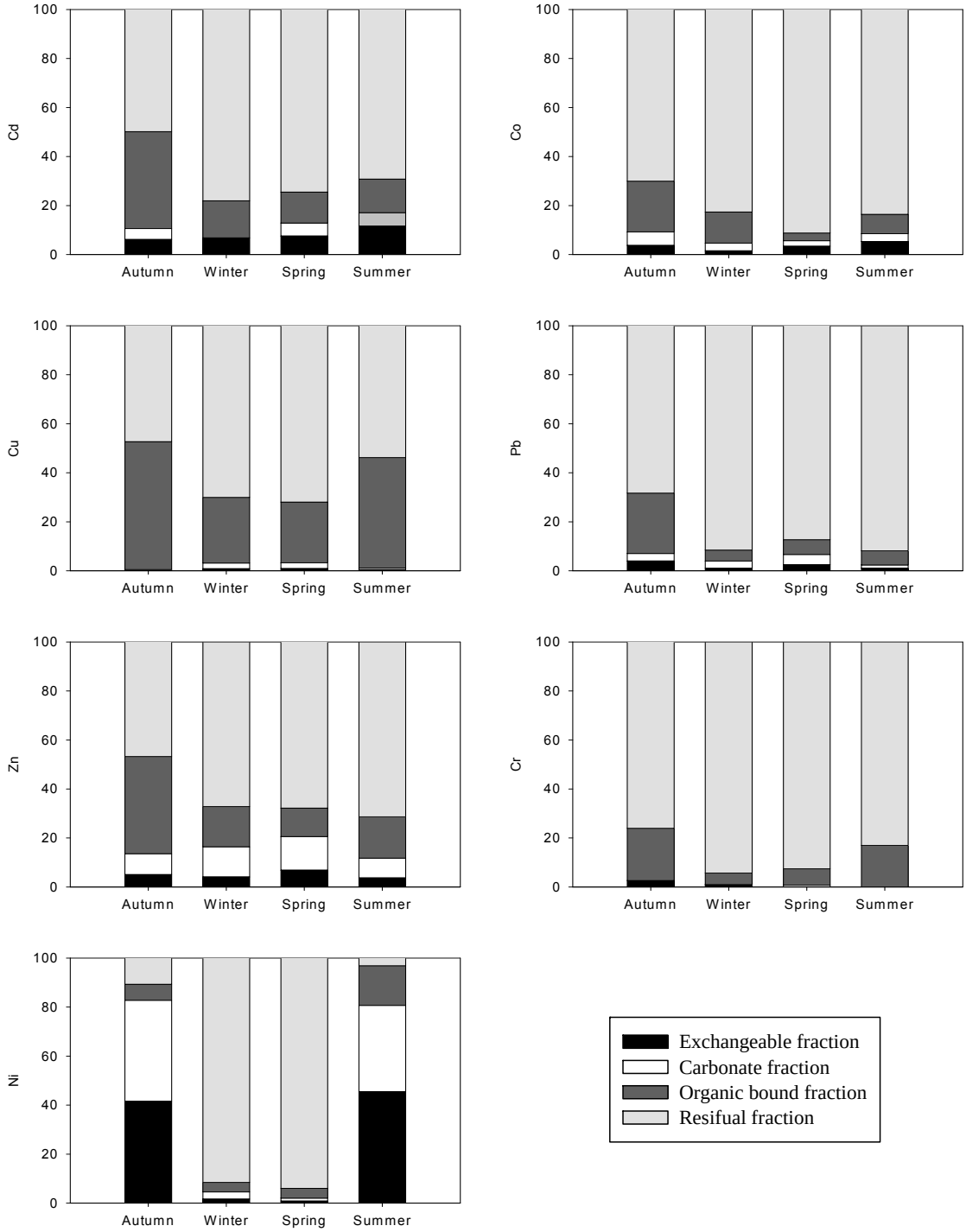

Figure 1. Metal speciation in the rhizosediments of *H. portulacoides* (n = 5), along the studied period.

3.3. Extracellular Enzymatic Activities

Analyzing sulphatase enzyme extracellular activity (Fig. 2) it didn't appear to have a strong seasonal variation ($p > 0.05$). Its activity in the sediment underneath *H. portulacoides* stands, showed two peaks of activity in spring and winter, being the highest in this last. For the rest of the year this activity was very low. This was also verified for β-*N*-acetylglucosaminidase. This enzyme also showed an absence of detectable activity in autumn, being very low from spring until this season (Fig. 2). Although the absence of seasonal pattern ($p > 0.05$) it was observed a peak of activity during the warmer seasons. On the other hand, peroxidase activity exhibited a strong seasonal pattern in the sediment colonized by *H. portulacoides* ($p < 0.05$). It was observed that peroxidase activity maintained rather low during the warmer seasons having a peak of activity during autumn and winter (Fig. 2). This enzyme showed a different pattern of activity along seasons ($p < 0.05$). Contrarily to peroxidase, phenol oxidase showed a minimum of activity during the cold seasons (Fig. 2), while during spring and summer it presented higher activities ($p < 0.05$). Protease EEA showed a similar pattern to the observed for sulphatase, having two major peaks of activity during spring and winter and an activity depletion during the middle seasons ($p < 0.05$). Overall two distinct periods of differential enzymatic influence can be distinguish: one during the warm seasons (summer and spring) and other during the cold seasons (autumn and winter). It was also possible to verify differences in the activity values assessed, showing phenol oxidase, peroxidase and protease with high values of activity compared to the lower values detected for sulphatase and β-*N*-acetylglucosaminidase.

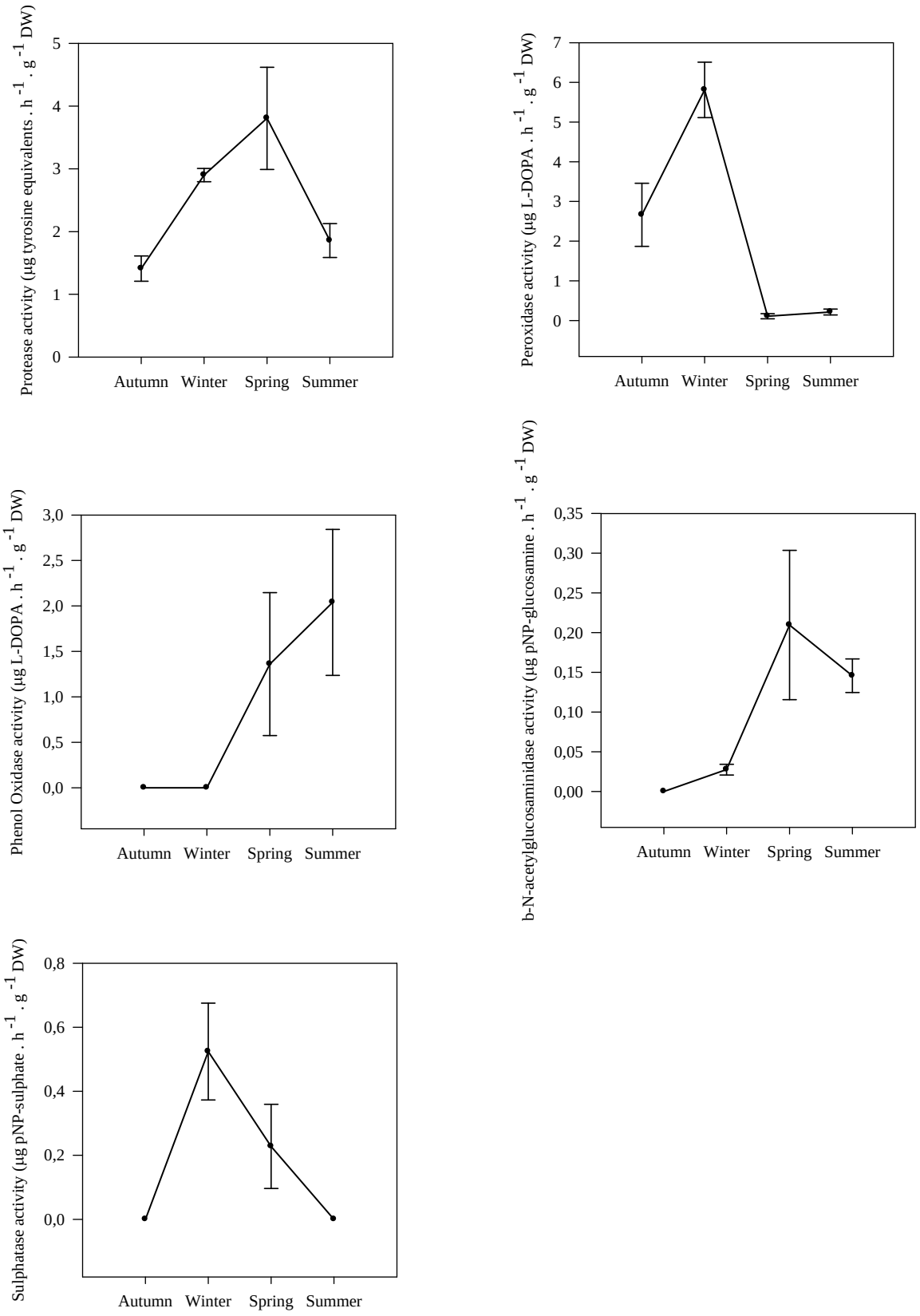

Figure 2. Extracellular enzymatic activites assessed for the rhizosediment of *H. portulacoides*, along the studied period (n = 5, standard error bars represented).

4. DISCUSSION

Considering the organic-bound fraction of all the analyzed metals, sediment organic matter and humic acid content, it was possible to observe a similar seasonal pattern for these three parameters (Fig. 3). Based on this, was possible to infer that a depletion of the organic-bound metals is caused by a decrease in the organic matter content of the sediment, and not by desorption of these metals from this organic matter. As it was stated before, there are several enzymes involved in the decomposition and breakdown of organic matter. These enzymes exhibit different patterns of activity along the seasons, with activity peaks in different periods of the year, having as a consequence a differential decomposition of the organic components of the sediment, at different year periods. As all enzymes their activity is greatly affected by medium conditions, in this case the pH, Eh and salinity of the sediment. From spring to autumn there was an increase of the organic matter content in the sediments colonized by *H. portulacoides* as it was showed previously (Caçador *et al.,* 2000). It seems to exist two different periods of organic matter cycling during the year. In both periods there were verified high protein degradation activities. In the first period during the warm seasons (spring and summer) there was also observed high β-*N*-acetylglucosaminidase activity along with high phenol oxidase activities. β-*N*-acetylglucosaminidase degrades chitin and this enzyme release is associated with the ecdysis process. As for phenol oxidase, it catalyzes the degradation of recalcitrant phenolics materials, such as lignin (Freeman *et al.,* 2004). Both this enzymes degrade large polymers that are structural components of animals and plants. Chitin exoskeletons are known to be able of accumulate toxic metals as it was reported before (Bergey and Weis, 2007). There are also reports (Sousa *et al.,* 2008) that lignin from *H. portulacoides* can accumulate small amounts of heavy metals. As chitin is a protein its degradation is due not also by β-*N*-acetylglucosaminidase but also by protease, explaining the simultaneous high activity of both this enzymes. Comparing the activity peaks of these two enzymes with the organic-bound metals found, it is possible to notice that along with this degradation process there is also a high depletion of the organic-bound metals, which indicates that these elements were probably bound to chitin like proteins. Phenol oxidase presented its maximum activity during summer and a total inhibition in the following seasons. This inhibition is due to the obligatory need of molecular oxygen (Freeman *et al.,* 2004) for this enzymatic activity, which during the cold seasons is scare in the rhizosediments, as it can be seen by the negative redox values verified during these seasons (Table 1).

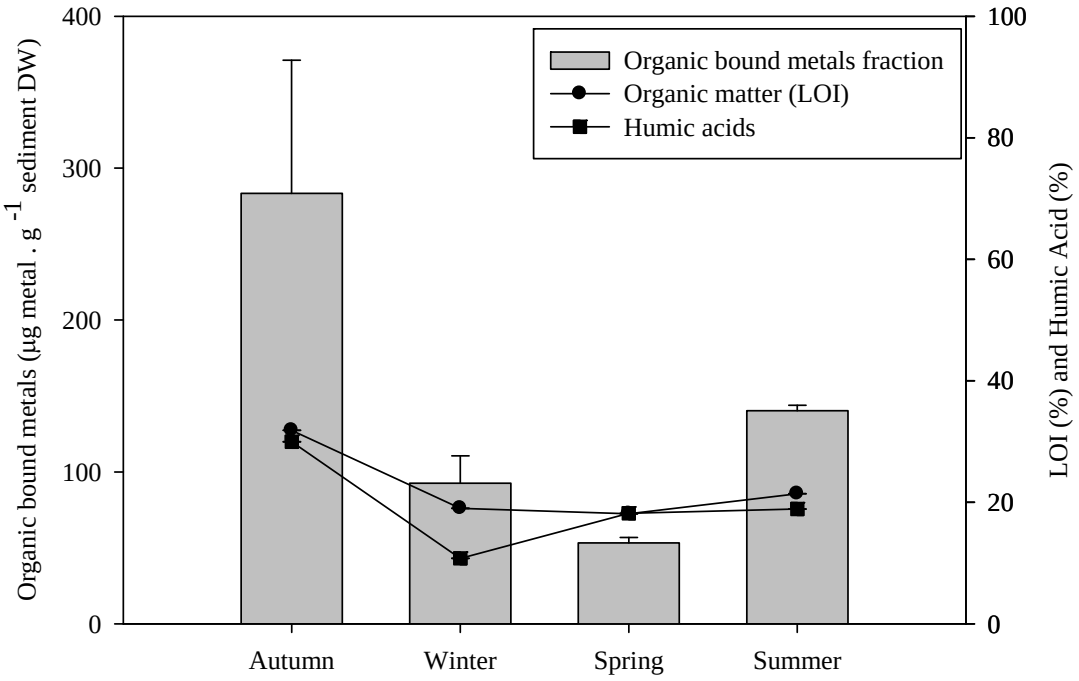

Figure 3. Comparasion between organic matter content (LOI), humic acids and the total heavy metals present in the organic bound fraction.

Along with the decrease of activity of protease and β-*N*-acetylglucosaminidase there is a peak of phenolic degradation in summer, keeping the organic-bound metal fractions with low values, when compared with autumn, when all enzymes are found to be inactive (Fig. 4). This indicates a degradation of plant residues, releasing the percentage of metals associated to phenolics, as it can be observed by the increase in the labile metals fraction. This is also supported by the data relative to phenolic content, where it can be seen that only in winter there is a considerable increase in the concentration of phenolic substances (Table 1), which is also the season where there is no phenol oxidase activity. While for spring and winter there was an increase of metals in the residual fraction accompanying the depletion of metals in the organic bound fraction, this was not observed in summer.

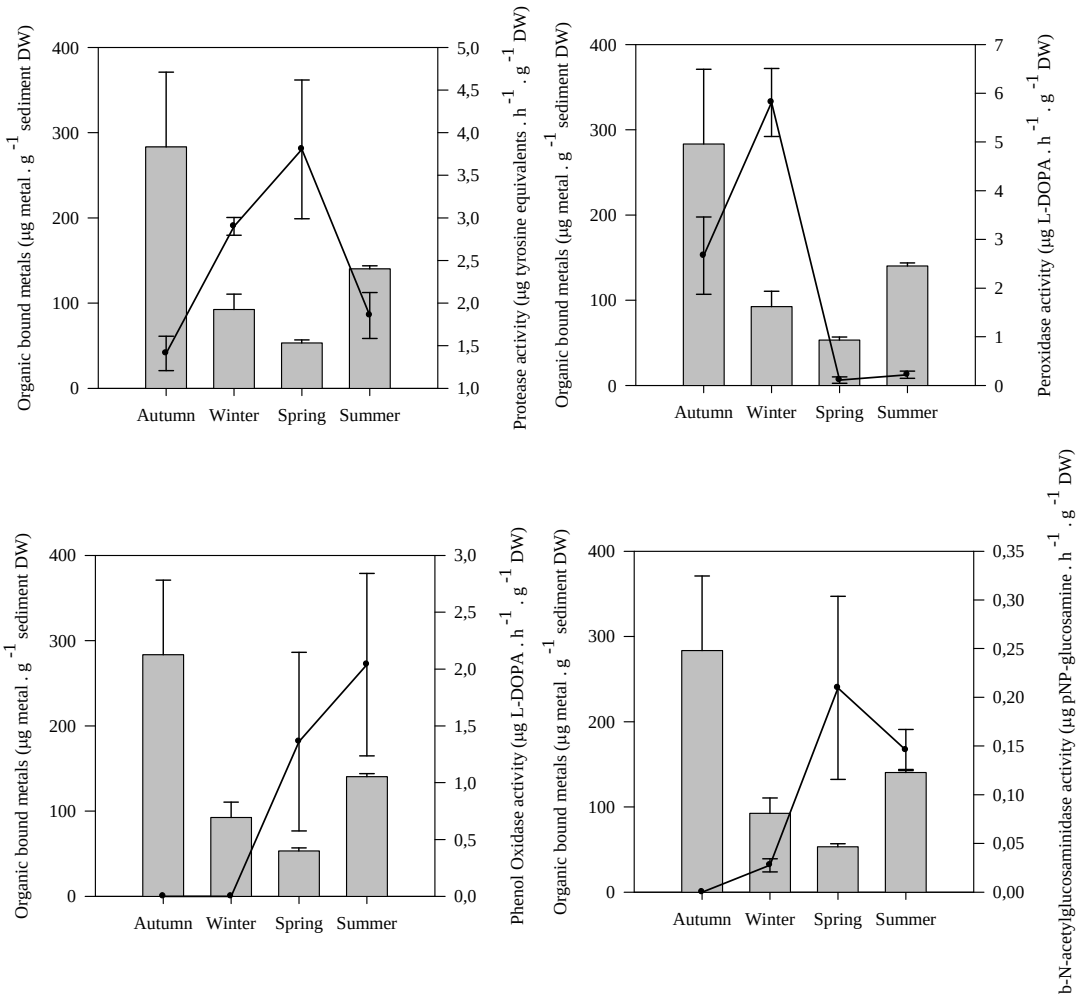

Figure 4. Influence of extracellular enzymatic activities of Protease, Peroxidase, Fenol Oxidase and β-*N*-acetylglucosaminidase (line) on total organic bound metals (bars).

This may be due to the peaks of sulphatase activity detected in spring and winter. Some authors (Hullebusch *et al.,* 2005) pointed out that high sulphatase activities can lead to the conversion of the sulphate produced by this enzyme into sulphides, by sulphate reducing bacteria. Sulfides can chemically reduce metals into a stable form for extended periods of time (Tabak *et al.,* 2005), increasing therefore the metal concentrations in the residual fraction of this extraction scheme. This could be observed while comparing the amount of metals in the more available form with sulphatase activity (Fig. 5). This leads us to the second major period of organic matter depletion, most significantly observed in winter. In this season all enzymes except sulphatase, peroxidase and protease are inactive or inexistent (Fig. 4). Extracellular peroxidase is known to be produced mostly by ligninolytic fungi in order to degrade plant litter (Johnsen and Jacobsen, 2008). In the presence of hydrogen peroxide this enzyme catalyzes the degradation of ligninocellulosic litter. Due to this mechanism this enzyme can operate even when the Eh values are very reduced as it was verified in

winter, without needing of molecular oxygen contrarily to phenol oxidase. It is also known that, in salt marshes saprophytic fungi are majorly present in leaves and stems as epiphytes (Castro and Freitas, 2000). As it was stated before, in this season, the low Eh values do not allow the activity of phenol oxidase, being this peroxidasic activity the principal source of organic matter recycling during winter. Also in this season, there are great inputs of plant litter due to leaf and stem senescence, and with this the decaying parts with retained metals in an organically bound form, become available for peroxidasic activity. The degradation of these compounds release phenolic substances of lower molecular weight, which due to the absence of phenol oxidase activity are accumulated in the sediment, has it was verified by the phenol concentrations. Along with this intense activity there is also a high protease activity, due to the degradation of lignin and associated components, leaving protein content more accessible to proteasic degradation. The breakdown of this bounds release metals to the surrounding environment and as it was verified in spring, the high sulphatase activity detected could be responsible the depletion of the labile metal pool contrarily to which would be expected.

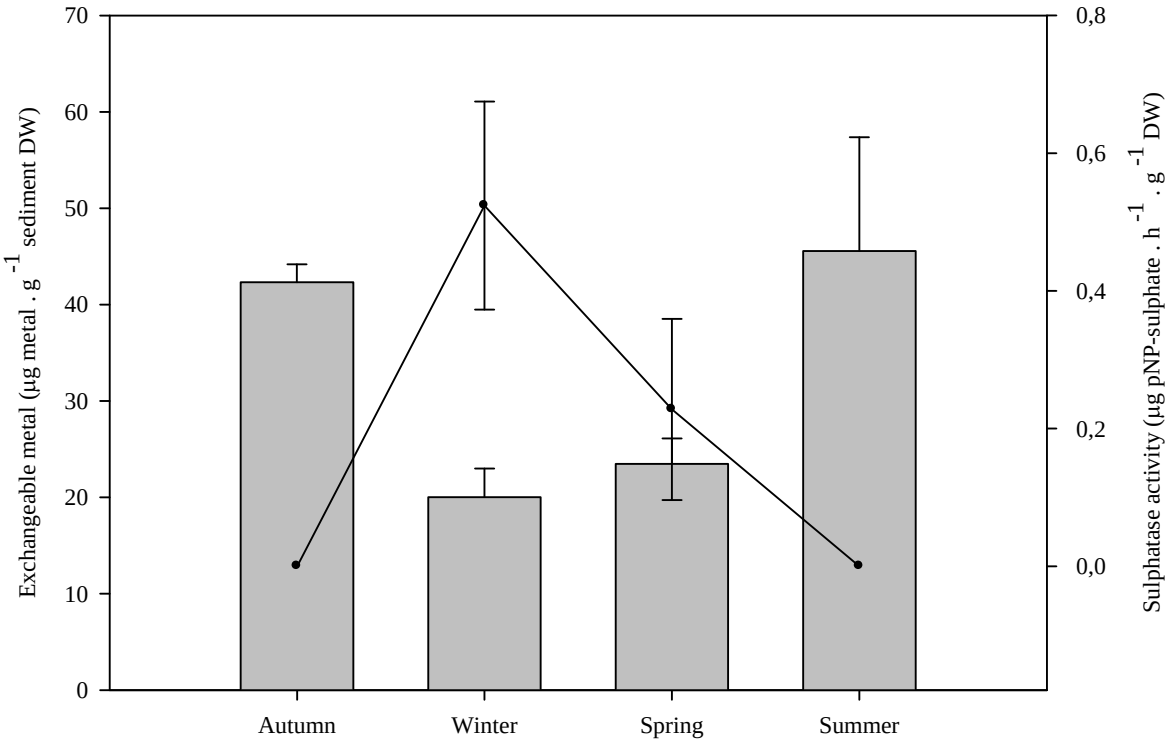

Figure 5. Influence of extracellular enzymatic activities of Sulphatase (n = 5, line) on total exchangeable metals (n = 5, bars).

5. CONCLUSION

Organic matter pools are known to be highly efficient sinks of heavy metals, constituting an important fraction of metal species, found in salt marsh sediments. Organic matter cycles are supported by microbial enzymatic degradation mechanisms and consequently affect the organic bound metals. Along with this, microbial driven sulphidisation is also an important factor when considering speciation processes. Throughout this work, it became evident the importance of considering microbial EEA as a key factor affecting not only, for organic matter biogeochemical recycling, but also in heavy metal speciation models. Without ignoring physical and chemical factors, as pH, Eh and oxygen profiles, microbial speciation processes assume a very important role in metal speciation, unconsidered in most studies. In conclusion, these sediment characteristics not only influence directly metal speciation throughout chemical processes but also indirectly by its effect in the activity of several extracellular enzymes. It becomes clear that the EEAs studied in this paper are important to be taken in consideration in future in situ bioremediation projects, as well in batch reactors or constructed wetlands and water treating plants.

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CHAPTER 4

Final Remarks

FINAL REMARKS

Salt marshes are very important areas in terms of provided services and biodiversity. Among these services they are often considered the “kidneys of the landscape”, due to its water filtration capacity and contaminant retention. This process is mostly achieved throughout the retention capacity of the salt marsh vegetation and their sediments. Salt marsh vegetation influences the dynamics of the estuarine ecosystem and efficiently retains anthropogenic metals discharged to the system, strongly influencing the processes of accumulation and retention of heavy metals there. Typically, these influences include in summary the taking up metals from contaminated sediments during the growth season and accumulating them in the plant tissues, mostly within the root system. These findings lead to inevitable conclusion that vascular plants may act as temporary sinks for heavy metals. By releasing oxygen and organic compounds to the rhizosphere salt marsh plants critically impact the biogeochemistry of the sediments, modifying dramatically the soil characteristics and with this metal speciation and bioavailability. This will lead us to the starting point with possible increases or decreases of uptake. The cycle becomes complete with the senescence and necromass generation and consequent re-input of metals to the surrounding environment in a different chemical form from the uptake. Although this temporal sinking characteristic, they continue to influence the biogeochemistry of the surrounding environment. Tagus salt marshes are no exception to this case. Although the floristic richness of these salt marshes, they are mostly colonized by *H. portulacoides, S. fruticosa*, *S. perennis* and *S. maritima*. Four species with four different metal uptake and accumulation behaviors. While for example *S. perennis* has a typical phyto-extractor behavior up taking high amounts of heavy metals, *S. maritima* has a phyto-stabilizer role retaining these metals in their sediment in less bioavailable forms. Not only this capacity for uptaking heavy metals influences the metal cycling in a determined stand, but also how these metals are incorporated in the biomass, allocated and exported in terms of necromass. This way the high exportation of necromass by *S. maritima* during the senescence period re-introduces the metals in the estuarine system, while although with some contaminated necromass exportation, *S. perennis* contributes less for the estuarine metal re-introduction. All these biodiversity aspects as well as plant morphology, biomass allocation and senescence while greatly influence the salt marsh services, in terms of contaminant retention.

Another key factor at this point will be the biogeochemical cycling by the microbial community inhabiting the rhizosphere, decomposing organic matter and this way acting on the bonds established with heavy metals and again the chemical

speciation is affected. With these speciation alterations, a new uptake is enhanced or delayed and the process restarted. In such a complex environment with all these processes acting, it becomes evident that all the variables are gathered to make salt marshes ideal ecosystems to study phytoremediation and its possibilities of application in other contaminated areas. Salt marsh two major sinks (vegetation with a special focus on their root system and sediment) are the key players on the metal cycling of the estuary. The sediment microbial community although without a direct action as metal accumulators, is a key element on the speciation processes occurring in the salt mash major metal sink and are without doubt one of the more important aspects to be considered while interpreting metal cycling within such a complex system.