UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Salt marshes role in Phosphorus cycling: Importance to natural remediation of estuarine systems

Joana Fernandes de Sousa Freitas

Dissertação

MESTRADO EM BIOLOGIA CELULAR E BIOTECNOLOGIA

2012

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Salt marshes role in Phosphorus cycling: Importance to natural remediation of estuarine systems

Joana Fernandes de Sousa Freitas

Dissertação Orientada pela Professora Doutora Isabel Caçador

Centro de Oceanografia

Faculdade de Ciências, Universidade de Lisboa

MESTRADO EM BIOLOGIA CELULAR E BIOTECNOLOGIA

2012

Acknowledgments

I would like to express my gratitude to everyone that contributed to the realization of this master thesis.

I would like to thank Professor Isabel Caçador for her supervision and support throughout the entire work, as well as for her friendship and continuous efforts to keep me in my best performance.

I would also like to thank Bernardo Duarte for his friendship, constant help and support together with crucial advice in every step of the way.

To all my colleagues and friends: Sílvia Pedro, Laíse Gomes, Dinis Santos, Gilda Silva, Teresa Silva, Lourenço Ribeiro, João Paulo Medeiros, Ana Brito, Elsa Cabral and Mafalda Mascarenhas thanks for the support and cheerful company.

To all my friends that are always available to help me in the way they can and specially because you always have a way to get me in a good mood, Teresa, Ritinha, Catarina, Andreia, Ana, Patrícia, Pedro, Rita, Marta, Yao, Luís, Nádia, Manuel João, thank you so much for always being by my side.

I would like to thank my family for total and unconditional support, especially to my mother who always knows how to help me, even when I think there is no way. Thanks to my sister and Luís for being so supportive and patient with me. I thank my grandmother, my great-aunt and my aunt Isabel for always being interested on and supportive of my work. I would like to thank, my father, Estela and Tatiana for helping more than they realize, in spite of the distance between us. And to the memory of my uncle and my grandfather António for their love and to the memory of my grandfather Rogério for his unconditional friendship and love, but mostly for being my role model.

I would like to thank to the “Fundação para a Ciência e Tecnologia (FCT)” for funding the research in the Centre of Oceanography throughout the project PEst-OE/MAR/UI0199/2011 and this specific work throughout the projects ECOSAM (PTDC/AAC-CLI/104085/2008).

Resumo

Os sapais são zonas intertidais onde ocorre acumulação de sedimentos finos devido à sua localização em ambientes protegidos como os estuários. Apesar de já terem sido considerados zonas sem qualquer valor, o seu papel na defesa da linha costeira, tal como conservação da biodiversidade, funcionando como abrigo, zona de nidificação e fonte de alimento para vários animais é extremamente relevante. Para além destas funções os sapais também têm a capacidade de reter poluentes, nomeadamente metais pesados ou nutrientes em excesso que possam levar à eutrofização. Tanto o Azoto como o Fósforo podem ter efeitos negativos por levarem ao aumento excessivo da produção primária e consequentemente à depleção de oxigénio dos sistemas.

As plantas halófitas que colonizam os sapais têm a capacidade de impedir a passagem de fósforo para a coluna de água através da oxigenação das camadas superficiais dos sedimentos. Estas plantas constituem uma importante ferramenta na remediação da eutrofização nestes sistemas já que, para o seu crescimento, necessitam de tomar fósforo do sedimento e também porque a sua presença aumenta a capacidade do sedimento para adsorver fósforo.

O sistema radicular das plantas promove a criação de um ambiente favorável ao crescimento da comunidade microbiana devido à oxigenação do sedimento e à produção de exsudados radiculares. O estado da comunidade microbiana pode ser avaliado através da determinação de atividades enzimáticas como é o caso da desidrogenase que é uma enzima intracelular que reflete a respiração microbiana. A comunidade microbiana existente no sedimento é crucial no ciclo de nutrientes já que produz enzimas envolvidas na mineralização da matéria orgânica. É o caso das fosfatases que são um grupo de enzimas responsáveis por hidrolisar o fósforo orgânico de forma a obter ortofosfato que pode ser absorvido pelas células.

Os objetivos principais desta tese são a determinação dos fatores biogeoquímicos que influenciam a atividade da comunidade microbiana como um todo e em particular os fatores que influenciam a atividade da fosfatase tal como o seu papel no ciclo biogeoquímico do fósforo em sedimentos de sapais do estuário do Tejo.

Para tal, as variações sazonais na atividade da desidrogenase foram avaliadas, assim como as diferenças de atividade nos sedimentos colonizados pelas espécies vegetais mais abundantes no estuário do Tejo (*Halimione portulacoides, Sarcocornia fruticosa* e *Spartina maritima*). Nestes sedimentos avaliou-se o conteúdo em matéria orgânica, pH, humidade relativa e salinidade de forma a perceber quais os parâmetros que influenciam a atividade microbiana. A atividade da desidrogenase varia ao longo

do ano, sendo possível observar um pico de atividade no Inverno e é influenciada pelos vários fatores avaliados, em particular pelo pH e pela humidade relativa dos sedimentos. A espécie de halófito que coloniza o sedimento também mostrou ser um fator importante na atividade microbiana.

A atividade das diferentes isoformas de fosfatase presentes no sedimento colonizado por plantas (*Halimione portulacoides, Sarcocornia fruticosa, Sarcocornia perennis* e *Spartina* maritima) e sedimento não vegetado também foi avaliada de forma a perceber os processos de mineralização do fósforo que ocorrem no sedimento. Com o mesmo fim, também foram avaliados os fatores biogeoquímicos que podem influenciar estes processos.

Desta forma, foram encontradas diferenças entre sedimentos colonizados e não colonizados por vegetação superior, nomeadamente na atividade das fosfatases e o conteúdo de fósforo, indicando que os halófitos testados aumentam a capacidade do sedimento para reter o fósforo. No entanto, não se encontraram diferenças significativas entre sedimentos colonizados pelas diferentes espécies.

A atividade total da fosfatase no sedimento depende sobretudo da fosfatase ácida que depende sobretudo do pH e do conteúdo em ácidos húmicos do sedimento. A quantidade de fósforo inorgânico presente no sedimento também influencia a atividade da fosfatase.

Deste modo, a atividade microbiana tem um papel importante no ciclo biogeoquímico de fósforo, especialmente devido à atividade da fosfatase ácida. A atividade microbiana do sedimento tanto no seu todo, como no caso da fosfatase, é influenciada pelo pH e pelo coberto vegetal. Assim, quando se considera os sapais como fatores-chave na remediação natural da eutrofização, é importante ter em conta a sazonalidade e a cobertura vegetal que influenciam a comunidade microbiana do sedimento.

Abstract

Salt marshes have been shown to act as a sink for pollutants, such as excess nutrients that cause eutrophication, like nitrogen and phosphorus. Halophytes have been known to block phosphorus efflux. Sediment microbial communities are involved in nutrient cycle due to the action of extracellular enzymes such as phosphatase. Microbial community status can be assessed through the measurement of dehydrogenase that is an intracellular enzyme that provides information about microbial community status.

The main objectives of the present thesis are to understand the biogeochemical factors underlying sediment microbial community activities on its hole and in particular the phosphatase activity and its role on phosphorous biogeochemical cycle in salt marsh sediments from the Tagus estuary.

The possible seasonal effects on and the biogeochemical factors influencing dehydrogenase activity in salt marsh sediments were assessed. A peak in dehydrogenase activity was found during the winter and dehydrogenase activity was found to be influenced by several factors, mainly water content and pH.

The biogeochemical sediment drivers that control phosphatase activities, how the different isoforms are controlled and its influence on the enzyme-mediated phosphorous cycling processes in salt marshes were studied. Bare sediments differed significantly from vegetated sediments in respect to several characteristics such as pH, total and acid phosphatase activities, water content, humic acids and organic matter contents, however, no significant differences were found among vegetated sediments. Acid phosphatase was the most active pH-isoform and its activity was driven mostly by pH and humic acid content, although inorganic phosphorus was also found to have a clear relation with phosphatase activity.

Salt marshes may have a key role to achieve ecosystem natural remediation being important to take seasonal variability and plant coverage into account when considering the efficiency of these remediation processes.

Index

Chapter 1

General Introduction

Salt marshes are defined as intertidal areas of accumulation of fine sediments, due to their location in low-energy environments such as embayments and estuaries. During tidal flooding, these fine sediments enter the salt marsh network and are trapped by the vegetation, leading to salt marsh elevation (Boorman, 2003; Best, 2007). Although salt marshes have been previously regarded as intertidal wastelands, they are currently widely recognized for their important role in coastal defense, wildlife conservation (for e.g. as shelter, nursing and feeding areas for several animals, but also as a biodiversity pool) and as a fundamental source of organic material as well as nutrients for marine communities (Boorman, 1999).

Salt marshes have also been shown to act as a sink for pollutants such as heavy metals or excess nutrients. On the other hand, salt marshes can also act as sources of pollutants, nutrients and organic matter in general, due to exportation of dead biomass (Duarte et al., 2008; Caçador *et al.*, 2009). Eventually this sink could also become a source of pollutants, although the amount of contaminants exported is always far lower than the amount retained in salt marsh sediments, allowing to these ecosystems to maintain their important remediative service for the estuary (Duarte et al., 2008). This way it becomes of great importance to to determine in what extent salt marshes act as sources or sinks of specific components (Reboreda and Caçador, 2007; Coelho, *et al.,* 2004; Guené and Winett, 1994; Boorman, 1999).

Nutrients such as nitrogen and phosphorus can also have damaging effects at high concentrations, causing eutrophication of coastal waters, leading to the increase of aquatic primary productivity and thus to the rapid growth of algae (Andrieux-Loyer *et al.,* 2008; Howarth *et al.,* 2011). Eutrophication in coastal waters has become a worldwide problem since it is one of the major stresses to marine environment. It is mainly caused by the discharge of excess nutrients from municipal and industrial wastewaters and urban and agricultural runoff (Meyer-Reil and Köster, 2000).

Spartina maritima marshes have an important role on phosphorus bioavailability decreasing total P, probably due to plant uptake for growth purposes. Also, salt marsh plants have the ability to oxygenate the sediments surrounding the root sediments, due to radial oxygen loss (ROL). At this point halophytes acquire an important role blocking phosphorous efflux due to the oxygenation of the sediments. Other macrophytes, such as ulvaceans have been shown to favor phosphorus storage in the sediment of eutrophic estuaries. *Spartina* marshes have been described as a useful tool in ecosystem recovery from eutrophic status, due not only to plant P-uptake but also by

an increase of the phosphorous adsorption capacity in *Spartina maritima* sediments (Lillebo *et al.,* 2007; Palomo, 2004).

Sediment microbial community is responsible for decomposition processes and has a major role in biogeochemical cycles, being involved in the cycling of nitrogen, carbon, phosphorus and sulphur (Ceccanti and Garcia, 1994; Caravaca *et al.,* 2005). Microorganisms present in the sediment produce extracellular enzymes involved in the mineralization of organic matter. They, in particular hydrolases such as proteases, ureases, phosphatases, are responsible for the breakdown of complex molecules. Microorganisms are also involved in redox reactions catalyzed by another major group of extracellular enzymes, the oxidoreductases, such as phenol oxidase and peroxidase (Acosta-Martínez *et al.,* 2007, Alef *et al., 1998*).

Plant root systems have a direct effect on microbial communities due to root exudates input into the rhizosphere (Hartman, 2009) but also due to their capacity to diffuse oxygen to the surrounding environment (Lüdemann, 2000; Lillebo *et al.*, 2006; Caetano *et al.,* 2011). Plant type has been shown to alter microbial community structure and function, altering microbial activity (Garbeva, 2008).

Extracellular enzyme activities (EEA) have been used in several studies as proxies of the microbial activity in soils and sediments (Duarte *et al.,* 2008; Pascaud *et al.*, 2012). Efforts have been directed to the conception of a simple index of soil and sediments quality through the use of measurements of several microbial community parameters including EEA or other enzymes such as dehydrogenase (Duarte *et al*., 2012). This last is an intracellular enzyme directly related to the microbial substrate respiration, providing information about microbial community status. Extracellular enzymes are able to persist in the sediment and remain active even after changes on microbial community, although its activity might change; on the other hand dehydrogenase is more sensitive to changes in microbial community, since it is an intracellular enzyme. Microbial activities have been shown to be sensitive to environmental changes, being affected by wastewaters and heavy metals and thus constituting a good indicator of sediment quality status (Duarte *et al*., 2012). Microbial community has also a role in the stabilization of pollutants (Beazley, 2011; Huang, 2011; Duarte et al., 2008).

The main objectives of the present thesis, are to understand the biogeochemical factors underlying sediment microbial community activities on its hole and in particular the phosphatase activity and its role on phosphorous biogeochemical cycle, in salt marsh sediments from the Tagus estuary.

References

Acosta-Martinez, V., Cruz, L., Sotomayor-Ramírez, D. and Pérez-Alegría, L. 2007. Enzyme activities as affected by soil properties and land use in a tropical watershed. *Applied Soil Ecology*, 35:35-45.

Alef, K., Nannipieri, P., Trazar-Cepeda, C., 1998. Phosphatase activity. In: Alef, K. and Nannipieri, P. (Eds.), Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London.

Andrieux-Loyer, F., Philppon, X., Bally, G., Kérouel, R., Youenou, A, and Le Grand, J. 2008. Phosphorus dynamics and bioavailability in sediments of the Penzé Estuary (NW France): in relation to annual P-fluxes and occurrences of Alexandrium Minutum. *Biogeochemistry*, 88:213-231.

Beazley, M.J., Martinez, R.J., Webb, S.M., Sobecky, P.A. and Taillefert, M. 2011. The effect of pH and natural microbial phosphatase activity on the speciation of uranium in subsurface soils. *Geochimica et Cosmochimica Acta*, 75:5648-5663.

Best, M., Massey, A., Prior A., 2007. Developing a saltmarsh classification tool for the European water framework directive. *Marine Pollution Bulletin* 55, 205–214.

Boorman, L.A., 1999. Salt marshes – present functioning and future change. *Mangroves and Salt Marshes* 3, 227-241.

Boorman, L.A., 2003. Saltmarsh Review. An overview of coastal saltmarshes, their dynamic and sensitivity characteristics for conservation and management. JNCC, Peterborough. On-line version at http://www.jncc.gov.uk/pdf/jncc334.pdf Accessed 15 Oct 2012

Caçador, I., Caetano, M., Duarte, B., Vale, C., 2009. Stock and losses of trace metals from salt marsh plants. *Marine Environmental Research*, 67:75-82.

Caetano, M., Bernárdez, P., Santos-Echeandia J., Prego, R., Vale C., 2011. Tidally driven N, P, Fe and Mn exchanges in salt marsh sediments of Tagus estuary (SW Europe). *Environmental Monitoring Assessment*, 184(11):6541-52.

Caravaca, F., Alguacil, M.M., Torres, P. and Roldán, A., 2005. Plant type mediates rhizospheric microbial activities and sediment aggregation in a semiarid Mediterranean salt marsh. *Geoderma*, 124:375-382.

Coelho, J.P., Flindt, M.R., Jensen, H.S., Lillebø, A.I. and Pardal M.A. 2004. Phosphorus speciation and availability in intertidal sediments of a temperate estuary: relation to eutrophication and annual P-fluxes. *Estuarine, Coastal and Shelf Science*, 61:583–590.

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*, 73:1056-1073.

Duarte, B., Freitas, J. and Caçador, I. (2012) Sediment microbial activities and physic-chemistry as progress indicators of salt marsh restoration processes. *Ecological Indicators*, 19:231–239.

Garbeva, P, van Elsas, J.D. and van Veen, J.A. 2008. Rhizosphere microbial community and its response to plant species and soil history. *Plant Soil*, 302:19–32

Hartman, A., Schmid, M., van Tuinen, D. and Berg, G. 2009. Plant-driven selection of microbes. *Plant Soil,* 321:235-257.

Howarth, R., Chan, F., Conley, D.J., Garnier, J., Doney,S.c., Marino, R. and Billen, G. 2011. Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. *Front Ecol Environ*, 9(1): 18–26.

Huang, L., Du, S., Fan, L., Lin, X., Wang, H. and Zang, Y. 2011. Microbial activity facilitates phosphorus adsorption to shallow lake sediment*. Journal of Soils and Sediments*, 11:185-193.

Lillebø, A.I., Coelho, J.P., Flindt, M.R., Jensen, H.S., Marques, J.C., Pedersen, C.B. and Pardal, M.A. 2007. Spartina maritima influence on the dynamics of the phosphorus sedimentary cycle in a warm temperate estuary (Mondego estuary, Portugal). *Hydrobiologia*, 587:195–204.

Lillebø, A.I., Flindt, M.R., Pardal, M.A. and Marques, J.C., 2006. The effect of Zostera noltii, Spartina maritima and Scirpus maritimus on sediment pore-water profiles in a temperate intertidal estuary. *Hydrobiologia* 555:175–183.

Ludemann, H., Arth, I., Wiesack, W., 2000. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy sediment cores. *Applied Environmental Microbiology*, 66:754–762.

Meyer-Reil, L.A. and Köster, M. 2000. Eutrophication of Marine Waters: Effects on Benthic Microbial Communities*. Marine Pollution Bulletin*. 41(1-6): 255-263.

Palomo, L., Clavero, V., Izquierdo, J.J., Avilés, A., Cecerra, J. and Niel, F.X. 2004 Influence of macrophytes on sediment phosphorus accumulation in a eutrophic estuary (Palmones River, Southern Spain). *Aquatic Botany*, 80:103–113.

Pascaud, A., Soulas, ML., Amellal, S. and Soulas, G., 2012. An integrated analytical approach for assessing the biological status of the soil microbial community. *European Journal of Soil Biology*, 49:98-106

Reboreda, R. and Caçador, I., 2007. Halophyte vegetation influences in salt marsh retention capacity for heavy metals. *Environmental Pollution*, 146:147-154.

Gueuné, Y. and Winnett, G. 1994. The transport of the pesticide Atrazine from the fresh water of the wetlands of Brittany to the salt water of the bay of Mont St. Michel (France), Journal of Environmental Science and Health. *Environmental Science and Engineering and Toxicology*, 29(4):753-768.

Chapter 2

Abiotic factors influencing dehydrogenase in salt marsh sediments (Tagus estuary).

Abstract

Dehydrogenase has been used as a measure of sediment microbial activity in several studies (e.g. Pascaud *et al*., 2012) and reflects the total oxidative activity of the sediment microbial community, being an almost exclusively intracellular enzyme that degrades rapidly when released from cells, and due to that does not accumulate in the sediments. In the present work were assessed the possible variations in dehydrogenase activity due to seasonality and which biogeochemical factors influence dehydrogenase activity in salt marsh sediments. A peak in dehydrogenase activity was found during the winter and dehydrogenase activity was found to be influenced by several factors, mainly water content and pH.

Key words: Dehydrogenase activity, salt marsh, microbial activity.

Introduction

Sediment is a complex and dynamic ecosystem composed by a mixture of minerals, organic matter, living animals and microorganisms, gases and water, with all of these components providing important ecosystem services (Daily *et al*., 1997). Sediment large biodiversity, comprises organisms from the macrofauna, mesofauna, microfauna and microflora, being fundamental in fuelling the food chain with a crucial role in both terrestrial and aquatic systems (Gardi *et al*., 2009; Nannipieri *et al*., 2003). Sediment ecosystem services include buffering and moderation of the hydrological cycle, physical and chemical support for plants, retention and delivery of nutrients to plants, disposal of wastes and dead organic matter, renewal of sediment fertility and regulation of major element cycles. This way sediment governs plant productivity and maintains biogeochemical cycles due to the microbial activity that degrades organic compounds (Daily *et al*., 1997; Nannipieri *et al*., 2003). Microbial communities are key players in all these processes (Nannipieri and Badalucco, 2003).

There are several factors that may put sediment quality at risk, including heavy metal pollution, acidification and climate change. Being the microbial community recognized as an essential living component of the sediment, any decreases in microbial community capacity to maintain its functions is seen as a sign of decreasing sediment quality (Chapman *et al*., 2007).

Salt marsh plants favour microbial growth around the root system due to their capacity to release oxygen from the roots (Caetano *et al*., 2011; Lillebo *et al*., 2006) turning the redox conditions of the root zone oxidative and consequently stimulatind the aerobic microbial activity (Lündemann *et al*., 2000). Microbial enzymes are involved in several biogeochemical cycling processes, catalysing the conversion of multiple complex molecules into smaller ones, either by redox reactions (catalysed by oxidoreductases) or by organic matter breakdown (hydrolases). Due to the high productivity of these ecosystems, the organic matter recycling by the microbial decomposers plays also a major role in maintaining an ecological balance.

Dehydrogenase can be used as an inderect measurement of basal respiration (Alef and Nannipieri, 1995) and it has been used as a proxy of sediment microbial activity in several studies (e.g. Pascaud *et al*., 2012; Xie *et al*., 2009; Pandey and Singh, 2006; Murata *et al*., 2005; Doi and Ranamukhaarachchi, 2008). A measurement of total oxidative activities of the cell is possible through the measure of the activity of one or more enzymes from the respiratory chain. Electrons funnel to the electron transfer chain through the action of several dehydrogenases according to the substrate available and then NAD^+ and ubiquinone collects reducing equivalents from oxidized

substrates transferring the electrons to the cytochrome system and oxidized by O_2 (Alef, 1998). Dehydrogenase reflects the total oxidative activity of the sediment microbial community, and it is an almost exclusively intracellular enzyme that degrades rapidly when released from cells, thus not accumulating in sediments as an extracellular enzyme (Pascaud *et al*., 2012). This feature is an important since dehydrogenase activity estimation can be used as a proxy of the microbial community activity that was present in the sediment during sampling.

The main objectives of the present work were to assess possible variations in dehydrogenase activity due to seasonality and which biogeochemical factors influence dehydrogenase activity in salt marsh sediments, and evaluate possible differences across sediments colonized by different plant species and/or from different salt marshes.

Material and Methods

Site description and Sampling

One sampling per season was carried out at each of two salt marshes of the Tagus estuary (Rosário and Alcochete) between April 2010 and August 2011 during the low tide. Three sediment cores were collected in pure stands of each of the three most abundant species, using PVC tubular cores (9 cm diameter, 50 cm long) and transported to the lab in refrigerated bags. Only the sediment surrounding the root system of the halophytes was used in the analysis, and are hereafter referred as rhizosediments. In both marshes of the Tagus estuary the upper marsh is mainly colonized by *Halimione portulacoides* (Chenopodiaceae) and *Sarcocornia fruticosa* (Chenopodiaceae) and the lower marsh is colonized mainly by *Spartina maritima* (Poaceae)..

Sediment physical-chemical characteristics

Sediment pH was measured using a HANNA pH/mV (HI 9025) electrode directly in the sediment. 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°C during 2h. Sediment water content was determined by drying sediment samples at 60°C until constant weight. Pore water salinity was measured after pore water extraction by centrifugation at 14,000 g for 15 min at 4 °C with a hand refractometer.

Dehydrogenase activity

Dehydrogenase activity (DHA) was determined using the TTC method according to Thalmann (1968). Briefly, approximately 5g of frozen sediment were incubated with 5 ml of TTC solution (1 %), samples without the substrate were also prepared with 5 ml Tris-HCl buffer (100 mM) instead of the TTC solution. Incubation was carried ou at 30 ºC for 24 hours. After the incubation 40 ml of acetone were added to each tube and shaken. The tubes were kept in the dark for 2 h and then centrifuged at 14 000 x g for 15 minutes, at 4 ºC. The clear supernatant absorbance was read on a TECAN Absorbance Microplate Reader (SPECTRA Rainbow) at 546 nm.

Statistical analysis

Statistical analysis was performed using Statistica Software version 10 from Statasoft Inc. The lack of normality and homogeneity of the data package lead to the

application Kruskal-Whallis non-parametrical tests for significance analysis. To understand the influence of biogeochemical factors in DHA a Similarity Percentage test (SIMPER), a Multi-Dimensional Scalling (MDS) projection and a Canonical Analysis of Principal coordinates (CAP) test were performed using Primer 6 software (Clarke and Gorley, 2006) .

Results

Rhizosediment physical chemical characteristics

Rosário salt marsh rhizosediments showed a significantly higher content in organic matter than Alcochete salt marsh rhizosediments ($p < 0.0001$) and SIMPER analysis showed that organic matter content was the factor that contributed the most to the diffences between Alcochete and Rosário salt marsh. MDS (Figure 1) projection forms two clear groups, separating samples collected in Alcochete salt marsh from samples collected in Rosário salt marsh ($p = 0.001$). Although there are no significant differences in organic matter content across plant species, in spring and summer *H. portulacoides* rhizosediments had the highest organic matter content, while *S. fruticosa* rhizosediments had the highest organic matter content during summer and winter in both Rosário and Alcochete salt marshes. *Spartina maritima* rhizosediments presented the lowest organic matter content in both salt marshes across all seasons, except for Alcochete salt marsh during the spring, when *S. fruticosa* rhizosediments presented the lowest organic matter content (Figure 2 and 3). Rhizosediment organic matter content was significantly lower in autumn than in spring and summer ($p < 0.05$) and SIMPER analysis showed that organic matter content was the factor that contributed the most for the dissimilarity between summer and autumn, and it was the second most important factor contributing to the differences between spring and autumn.

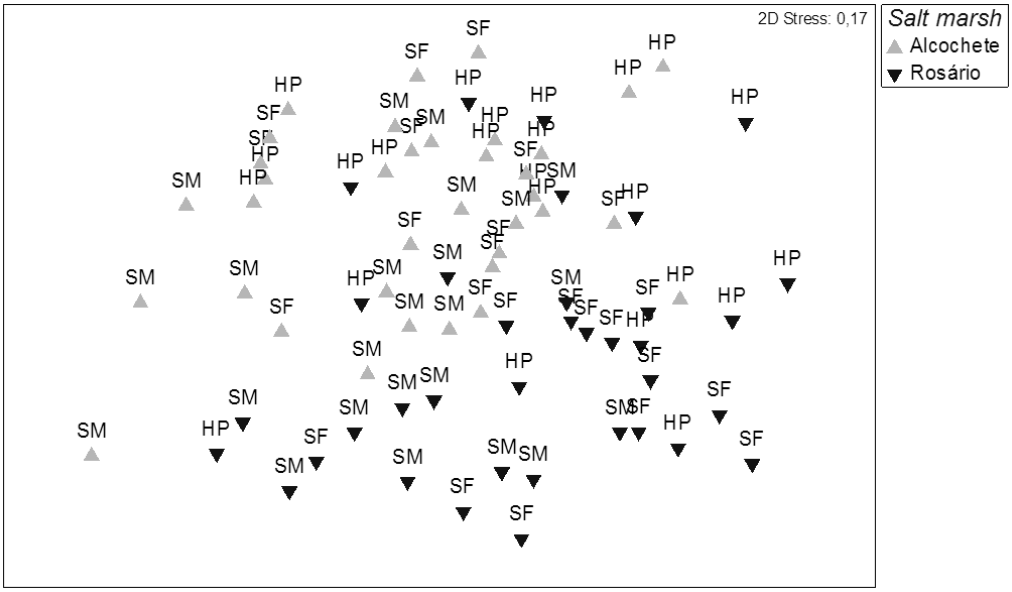

Figure 1. Multi-Dimensional Scalling of all the analysed rhizosediments according to the salt marsh and colonizing halophyte specie (HP – *Halimione portulacoides*; SF – *Sarcocornia fruticosa*; SM – *Spartina maritma*).

The pH values varied between 6.70 and 7.99 in Alcochete salt marsh rhizosediments, and 6.72 and 7.86 in Rosário salt marsh. In both salt marshes pH was significantly higher in winter ($p < 0.01$). Regarding rhizosediment pH no significant differences between Alcochete and Rosário were found. In Alcochete salt marsh *S. maritima* rhizosediments had the highest pH values among all plant species in every season, except in summer when *S. fruticosa* had the highest pH value, although it was very similar to *S. maritima* rhizosediments pH. During summer and spring *H. portulacoides* rhizosediments had the lowest pH, whilst in winter and autumn pH values of *H. portulacoides* and *S. fruticosa* were very similar. In Rosário salt marsh *S. maritima* rhizosediments had the highest pH in every season except in winter when *H. portulacoides* had the highest pH values and *S. maritima* had the lowest. During spring *H. portulacoides* rhizosediments had the lowest pH, while *S. fruticosa* rhizosediments had the lowest pH during autumn and summer (Figures 2 and 3).

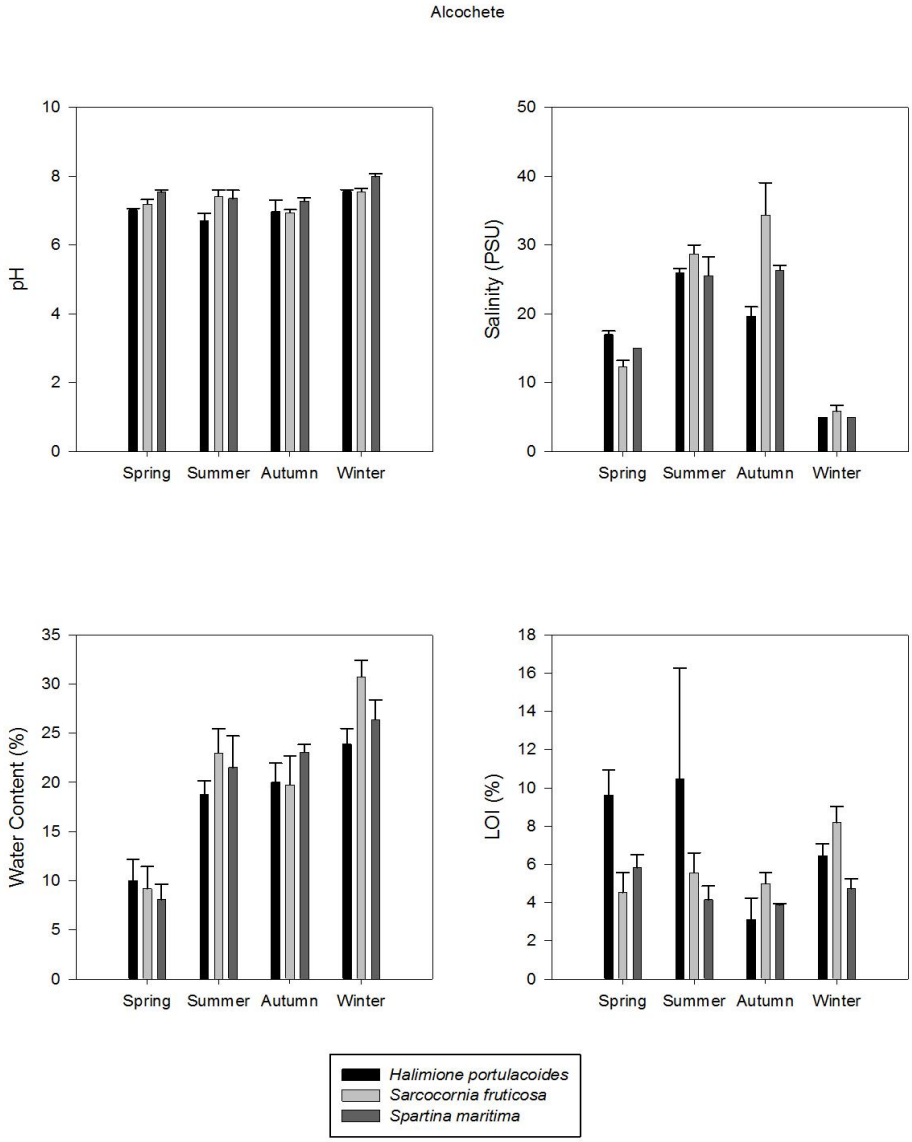

Figure 2. Alcochete rhizosediment physic-chemical characteristics. Error bars represent standard error

Alcochete salt marsh rhizosediments had significantly lower water content than Rosário salt marsh ($p < 0.01$), although *H. portulacoides* rhizosediments during autumn and winter had higher water content in Alcochete than in Rosário. Among seasons, the rhizosediments collected during spring had significantly lower water contents than rhizosediments collected during the rest of the year ($p < 0.05$) for both Rosário and Alcochete salt marshes. Using SIMPER analysis it was found that water content was the factor that contributed the most for the dissimilarity between spring and the other seasons. In Alcochete salt marsh during summer and winter *S. fruticosa* rhizosediment had the highest water content and *H. portulacoides* rhizosediment had the lowest water content. During spring, the water content of *H. portulacoides* rhizosediment was the highest, in contrast to *S. maritima* rhizosediment, which had the lowest water content. During autumn, the water content of *H. portulacoides* and *S. fruticosa* rhizosediments were similar while in *S. maritima* rhizosediment the water content was the highest (Figures 2 and 3).

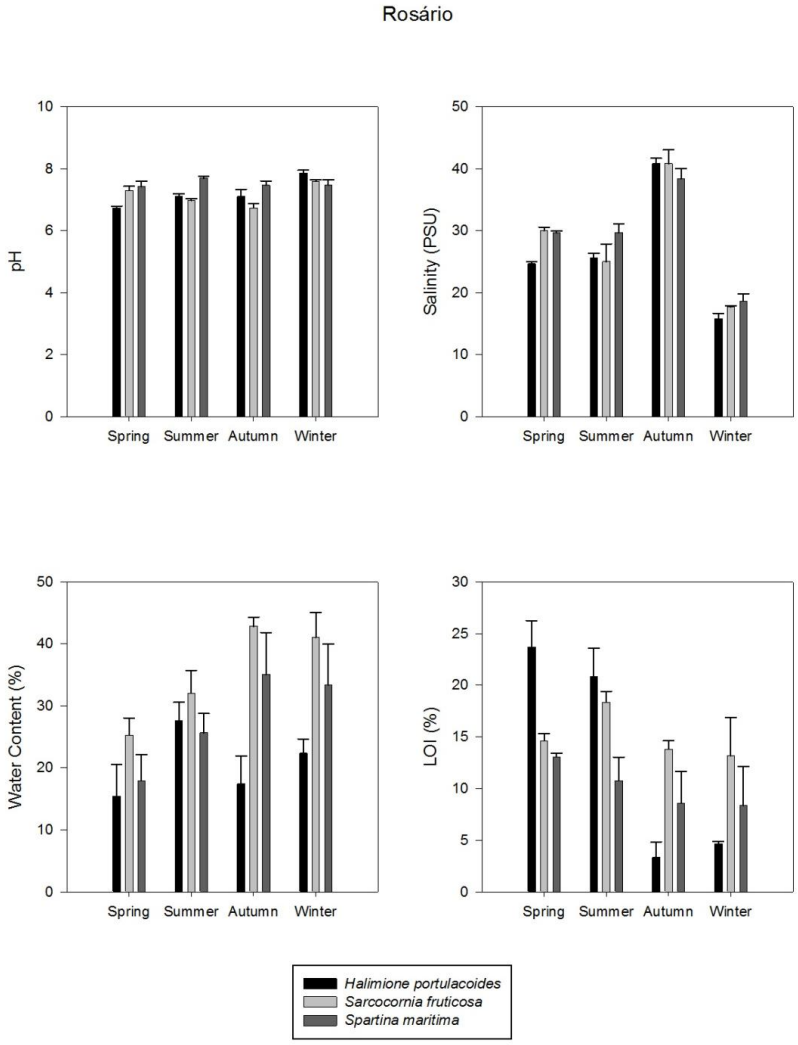

Figure 3. Rosário rhizosediment physic-chemical characteristics. Error bars represent standard error.

Regarding salinity, no significant differences were found among plant species, but rhizosediments salinity was significantly higher in Rosário than in Alcochete ($p < 0.01$). During winter, rhizosediment salinity was significantly lower than the rest of the seasons ($p < 0.05$) and for rhizosediments collected during autumn salinity was significantly higher than winter and spring ($p < 0.01$) although there were no significant differences between autumn and summer (Figures 2 and 3). According to SIMPER alysis, salinity was the factor that most contributed to the dissimilarities between winter and summer as well as to the differences between winter and autumn. Salinity was also the second most important factor contributing to the dissimilarities between spring and winter.

Rhizosediment dehydrogenase activity

DHA was significantly higher in *S. maritima* rhizosediments than in the rhizosediments of the remaining analyzed species independently of the marsh site ($p < 0.05$). This was confirmed by the SIMPER analysis, that showed that DHA was the factor that most contributed to the dissimilarities between *S. maritima* and *H. portulacoides* as well as to the dissimilarities between *S. maritima* and *S. fruticosa*. In Alcochete salt marsh *H. portulacoides* had the lower DHA in all seasons except for spring when *S. fruticosa* rhizosediment had the lower activity. On the other hand DHA was higher during winter in *S. maritima* rhizosediments, but the same was not observed in *H. portulacoides* and *S. fruticosa* rhizosediments which had very low DHA all year long, never reaching 2 µg TPF g^{-1} FW h^{-1}. However, in Rosário salt marsh all plant rhizosediments had a higher DHA during winter, with *H. portulacoides* and *S. fruticosa* rhizosediments showing very similar DHA during all seasons, except in summer when *S. fruticosa* had the lowest DHA (Figure 4). There was found a positive correlation between rhizosediment DHA and pH ($R^2=0.463$, $p < 0.05$).

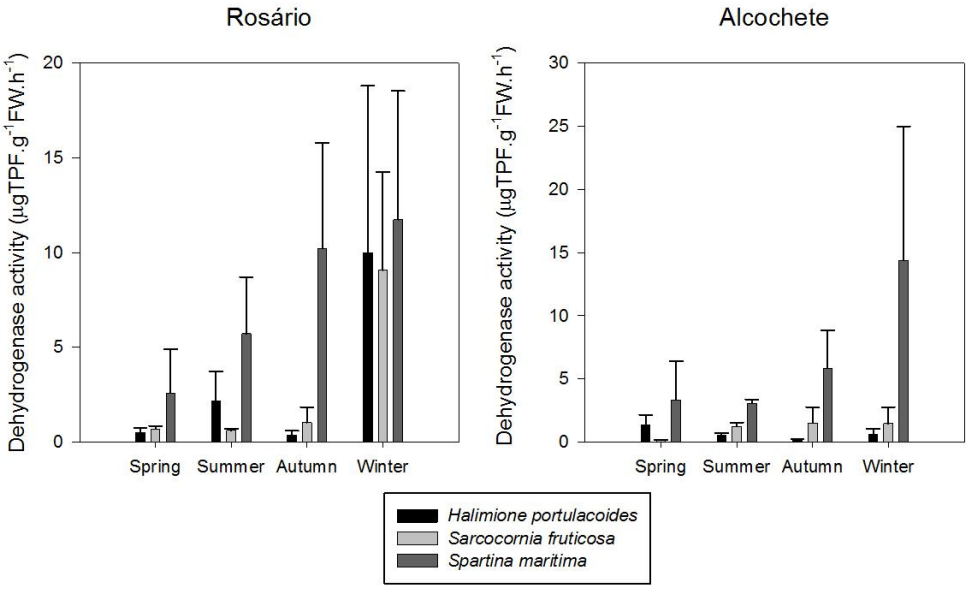

Figure 4. Dehydrogenase activity in Rosário and Alcochete salt marshes. Error bars represent standard error.

Influence of biogeochemical factors in dehydrogenase activity

CAP analysis using sediment physical-chemical characteristics showed distinct groups formed by the samples collected during winter, autumn and the warmer seasons (spring and summer) separately. Winter group was mostly influenced by pH and salinity (Figure 5). SIMPER test shows pH as the factor contributing the most for similarities among winter samples and salinity as the main factor contributing to the similarities among autumn samples. This analysis also showed that spring and summer were the most similar seasons.

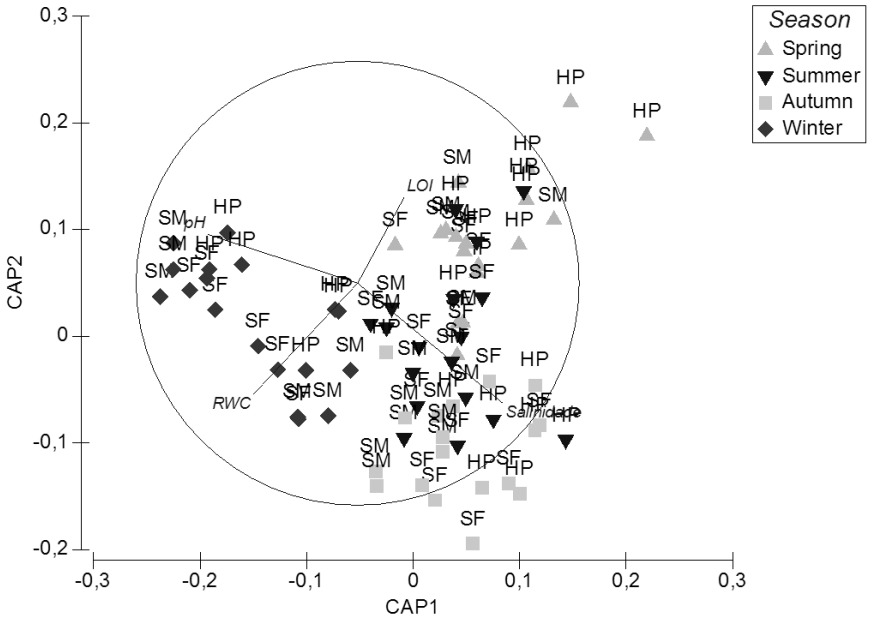

Figure 5. CAP analysis using rhizosediment physical chemical characteristics.

Discussion

There are evident differences that distinguish the analyzed samples, both spatially (salt marsh), temporally (seasonality) and ecologically (plant coverage).

Several physic-chemical characteristics of the sediments revealed to have a straight influence on DHA spatial-temporal variability. For instance, pH revealed to have an important correlation with the DHA, which is in accordance with Trevors (1984). Trevors (1984) also observed that there was very little DHA below pH 6.6 and above pH 9.5, which is in the range of the sampled sediments (minimum pH 6.7 and maximum pH 7.99). Harrison and Loveless (1971) propose that pH levels lower than 6.6 limit microbial growth due to the elevated energy requirements for pumping H^+ to the exterior in order to maintain intracellular pH. At elevated pH (8.7) Maurer *et al*. (2005) found that genes coding for proteins responsible for the proton import, such as ATPase were up-regulated, while genes coding for enzymes responsible for proton export, such as dehydrogenases were down-regulated, maintaining this way the intracellular pH. Rhizosediment DHA depends on the status of the microbial communities. It would be expected that factors such as community size or synthesis and activity of dehydrogenase in the cell would affect overall dehydrogenase activity. Consequently, it is possible that although dehydrogenase synthesis may be down regulated in higher pH environments, a large microbial community may reverse the tendency to decrease DHA in the rhizosediment if the microbial community has an optimum growth at higher pH. In fact Trevors (1984) found that soil samples adjusted to a more acidic pH presented resilience and were able to return to a pH 7.7 which points out to an optimum pH for microbial community growth around this value. However it seems clear that there is more than one factor influencing DHA and because of that there isn't a clear linear relation between pH and DHA explaining the relatively low R^2 value (0.463).

High salinities have been shown to inhibit growth of most microorganisms, yet some microorganisms successfully adapted to high concentrations of NaCl or even depend on high concentrations of NaCl to survive (Lanyi, 1974). In the particular case of microbial respiration several marine bacteria genera have been shown to have a specialized protein (NADH:ubiquinone oxidorreductase) that is responsible for the transfer of electrons form NADH or deaminoNADH to ubiquinone being this electron transfer stimulated by high concentrations of Na^+ (Krebs *et al.*, 1999; Skulachev, 1989, Tokuda and Kogure, 1989; Steuber, 2001). However, it seems that the rhizosediment microbial community is not very well adapted to high salinities or at least to the abrupt seasonal fluctuations that can occur at this level. The peak in DHA occurred during

winter when sediment present significantly lower salinities due to higher freshwater inputs. In accordance to the present study, Carrasco *et al.* (2006) and Caravaca *et al.* (2005a) found a negative correlation between salinity and DHA in salt marsh sediments.

Water content seems to be another important factor controlling DHA activity, since both these parameters present similar seasonal variations, with for e.g. lower values in spring. Marzadori *et al*. (1996) showed that water content influenced DHA and Brzezińska *et al*. (1998) proposed that water content influences DHA indirectly throughout changes in oxidation-reduction status.

Spartina maritima rhizosediments had the highest DHA which can probably attributed to sediment pH. Besides this, *S. maritima* is a specie that mainly colonizes the lower salt marsh, undergoing higher submersion periods which may be another factor to take into account. Brzezińska *et al*. (1998) showed that flooding affected DHA as well as dehydrogenase Q_{10} (increase of DHA due to an increase in temperature of 10 ºC). Also, Doi and Ranamukhaarachchi (2008) pointed out that air-dried sediment may lead to microbial cell death due to loss of sediment structure, decreasing the possibility of recovery during rewetting. Another important fact to consider is the plant coverage by itself. Salt marsh plants follow zonation patterns determined by factors such as sediment salinity and Eh. Larger sediment nitrogen content may promote the synthesis of nitrogen-containing osmotic regulator promoting the stabilization of a plant species in detriment of another (Caçador *et al*., 2007). Caravaca *et al* (2005b) found that halophytic species determined rhizosediment microbial activities, which may be due to differences in microbial community. Garbeva *et al* (2008) showed that plant species affected the structure of microbial community and thus plant species colonizing the rhizosediment may also be a factor influencing dehydrogenase activity.

Conclusion

Dehydrogenase activity showed a strong spatial-temporal variability. Salt marsh rhizosediments are biogeochemically different among the different colonizing plant species, mostly due to differences in its physical chemical characteristics. There were significant in DHA differences across rhizosediments from different plant species, due to differences in pH, water content and submersion periods. Concomitantly also the vegetation is known to be distributed along a physic-chemical gradient within the marsh. This way, it is not strange to find that also the plant coverage and its rhizosphere micro-environment play an important role influencing DHA.

References

Alef, K. and Nannipieri, P. (eds) 1995. Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London.

Alef, K., 1998. Dehydrogenase activity. In: Alef, K. and Nannipieri, P. (Eds.), Methods in Applied Sediment Microbiology and Biochemistry. Academic Press, London.

Brzezińska, M., Stepniewska, Z. and Stepniewski, W. 1998. Sediment oxygen status and dehydrogenase activity. *Soil Biology and Biochemistry*, 30(13):1783-1790.

Caçador, I., Tibério, S., Cabral, H.N. 2007. Species zonation in Corroios salt marsh in the Tagus estuary (Portugal) and its dynamics in the past fifty years. *Hydrobiologia,* 587:205-211.

Caetano, M., Bernárdez, P., Santos-Echeandia J., Prego, R., Vale C., 2011. Tidally driven N, P, Fe and Mn exchanges in salt marsh sediments of Tagus estuary (SW Europe). *Environmental Monitoring Assessment*.

Caravaca, F., Alguacil, M.M., Torres, P. and Roldán, A., 2005a. Microbial activities and arbuscular mycorrhizal fungi colonization in the rhizosphere of the salt marsh plant inula crithmoides l. Along a spatial salinity gradient. Wetlands, 25(2):350–355.

Caravaca, F., Alguacil, M.M., Torres, P. and Roldán, A., 2005b. Plant type mediates rhizospheric microbial activities and sediment aggregation in a semiarid Mediterranean salt marsh. Geoderma, 124:375-382.

Carrasco, L., Caravaca, F., Álvarez-Rogel, J. and Roldán, A., 2006. Microbial processes in the rhizosphere sediment of a heavy metals-contaminated Mediterranean salt marsh: A facilitating role of AM fungi. *Chemosphere*, 64:104–111.

Chapman, S.J., Campbell, C.D., and Artz, R.R.E. 2007. Assessing CLPPs Using MicroResp™. A Comparison with Biolog and multi-SIR. *Journal of Soils Sediments,* 7(6):406–410.

Clarke, K. and Gorley, R., 2006, PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth.

Daily, G.C., Matson, P.A., Vitousek, P.M., 1997. Ecosystem services supplied by soil. *In*: Daily, G.C. (Ed.), Nature's Services: Societal Dependence on Natural Ecosystems. Island Press, Washington, DC, pp. 113–132.

Doi, R. and Ranamukhaarachchi, S.L., 2008. Soil dehydrogenase in a land degradation-rehabilitation gradient: observations from a savana site with a

wet/dry seasonal cycle. *International Journal of Tropical Biology*, 57(1-2):223-234.

Garbeva, P, van Elsas, J.D. and van Veen, J.A. 2008. Rhizosphere microbial community and its response to plant species and soil history. Plant Soil, 302:19–32

Gardi, C., Montanarella, L., Arrouays, D., Bispo, A., Lemanceau, P., Jolivet, C., Mulder, C., Ranjard, L., Römbke, J., Rutgers, M. and Menta, C. 2009. Soil biodiversity monitoring in Europe: ongoing activities and challenges. *European Journal of Soil Science*, 60:807–819.

Harrison, D.E.F. and Loveless, J.E. 1971. The effect of growth conditions on respiratory activity and growth efficiency in facultative anaerobes grown in chemostat culture. *Journal of General Microbiology*, 68:35-43.

Krebs, W., Steuber, J., Gemperli, A.C. and Diroth, P. 1999. Na^+ translocation by the NADH:ubiquinone oxidoreductases (complex I) from *Klebsiella pneumonia. Molecular Microbiology,* 33(3):590-598.

Lanyi, J.K. 1974. Salt-dependent properties of proteins from extremely halophilic bacteria. *Bacteriological Reviews,* 38(3):272-290.

Lillebø, A.I., Flindt, M.R., Pardal, M.A. and Marques, J.C., 2006. The effect of Zostera noltii, Spartina maritima and Scirpus maritimus on sediment pore-water profiles in a temperate intertidal estuary. *Hydrobiologia* 555, 175–183.

Ludemann, H., Arth, I., Wiesack, W., 2000. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy sediment cores. Applied Environmental Microbiology 66, 754–762.

Marzadori, C., Ciavatta, C., Montecchio D. and Gessa C. 1996. Effects of lead pollution on different soil enzyme activities. *Biology and Fertility of Soils,* 22:53-58.

Maurer, L.M., Yohannes, E., Bondurant, S.S., Radmacher, M. and Slonczewski, J.L. 2005. pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. *Journal of Bacteriology*, 187(1):304-319.

Murata, T., Kanao-Koshikawa, M. And Takamatsu, T. 2005. Effects of Pb, Cu, Sb, Inand Ag contamination on the proliferation of soil bacterial colonies, soil dehydrogenase activity, and phospholipid fatty acid profiles of soil microbial communities. *Water, Air and Soil Pollution*, 164:103-118.

Nannipieri, P. and Badalucco, L. 2003. Biological processes. In*: Processes in the Soil–Plant System: Modelling Concepts and Applications* (eds D.K. Bembi and R. Nieder). The Haworth Press, Binghamton, NY, in press.

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G. and Renella, G., 2003. Microbial diversity and soil functions. *European journal of soil science*, 54:655–670.

Pandey, S. and Singh, D.K. 2006. Soil dehydrogenase, phosphomoesterase and arginine deaminase activities in an insecticide treated groundnut (*Arachis hypogaea* L.) field. *Chemosphere*, 63:869-880.

Pascaud, A., Soulas, ML., Amellal, S. and Soulas, G., 2012. An integrated analytical approach for assessing the biological status of the soil microbial community. *European Journal of Soil Biology*, 49:98-106.

Skulachev, V.P. 1989. The Sodium Cycle: A new type of bacterial energetic. *Journal of Bioenergetics and Biomembranes.* 21(6):635-647.

Steuber, J. 2001. Na^+ translocation by NADH:quinone oxidoreductases: an extension to the complex-I family of primary redox pumps. *Biochimica et Biophysica Acta,* 1505:45-56.

Thalmann A (1968) Zur Methodik der Bestimmung der Dehydrogenaseaktivität im Boden mittels Triphenyltetrazoliumchlorid (TTC). Landwirtsch Forsch 21:249-258.

Tokuda, H. and Kogure, K. 1989. Generalized distribution and common properties of Na^+-dependent NADH:quinone oxidoreductases in Gram-negative marine bacteria. *Journal of General Microbiology*, 135:703-709.

Trevors, J.T., 1984. Effect of substrate concentration, inorganic nitrogen, O_2 concentration, temperature and pH on dehydrogenase activity in soil. *Plant and Soil*, 77:285-293.

Xie, W., Zhou, J., Wang, H., Chen, X., Lu, Z., Yu, J. and Chen, X. 2009. Short-term effects of copper, cadmium and cypermethrin on dehydrogenase activity and microbial functional diversity in soils after long-term mineral or organic fertilization. *Agriculture, Ecosystems and Environment*, 129:450-456.

Chapter 3

Biogeochemical drivers of phosphatase activity in salt marsh sediments

Abstract

Although nitrogen has become a major concern for wetlands scientists dealing with eutrophication problems, phosphorous represent another key element as well as its biogeochemical cycling. Microbial communities are a central component in trophic dynamics and biogeochemical processes on coastal systems, since most of the processes in sediments are microbial-mediated throughout the enzymatic action. In the present work, the authors investigate the biogeochemical sediment drivers that control these enzymatic activities and how the different enzyme forms are controlled and its influence on the enzyme-mediated phosphorous cycling processes in salt marshes. Bare sediments differed significantly from vegetated sediments in respect to several characteristics such as pH, total and acid phosphatase activities, water content, humic acids and organic matter contents, however, no significant differences were found among vegetated sediments. Acid phosphatase was the most active pH-isoform and its activity was driven mostly by pH and humic acid content, although inorganic phosphorus was also found to have a clear relation with phosphatase activity.

Keywords: Phosphorus; biogeochemical cycling; extracellular phosphatase activity; eutrophication

Introduction

Salt marshes are highly productive areas located in the interface between freshwater and marine systems often subjected to high nutrient loadings due to anthropogenic activity that can lead to systems eutrophication (Tobias et al., 2001). Although Nitrogen (N) is considered to be a major concern when dealing with eutrophication, phosphorus (P) is also considered to be one of the key limiting nutrients to primary productivity and therefore it is one of the nutrients responsible for eutrophication (Correl, 1998; Paerl 2009). Phosphorous is delivered to aquatic systems in several forms such as longer chain poly-phosphates, pyrophosphates, organic phosphate esters and phosphodiesters, and organic phosphonates. This element may also be delivered both in the dissolved and particulated form being deposited in the salt marsh sediments (Correl, 1998). However, P is only biologically available when it is in the inorganic form, as orthophosphate.

Salt marsh plants release oxygen from the root system into the sediments promoting reactions with reduced species and favouring microbial growth (Caetano et al., 2011; Lillebo et al. 2006). Sediment microbial communities are an essential component in trophic dynamics and biogeochemical processes in coastal ecosystems. The microbial community synthesizes extracellular enzymes that mineralize organic phosphorus into inorganic and more easily metabolized ones. One large and important group of these enzymes are the phosphomonoesterases and their pH-isoforms: alkaline, acid and neutral phosphatases, being therefore active in several kinds of sediments (Alef et al., 1998).

In the present work the authors focus on extracellular enzymatic activities and the sediment biogeochemical variables that drive their activity and consequently their influence in phosphorous biogeochemistry. Due to the biogeochemical gradient observed between salt marsh areas, four salt marsh plants rhizosediments (*Halimione portulacoides, Sarcocornia fruticosa, Sarcocornia perennis and Spartina maritima*) and the bare sediments were evaluated and their biogeochemical environment were compared in order to understand the role of the biogeochemical features while controllers of phosphatase extracellular activity.

Material And Methods

Site description and Sampling

Rosário (38°40'N, 9°01'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 *Halimione portulacoides* (Chenopodiaceae), *and Sarcocornia fruticosa* (Chenopodiaceae) and undergoes short submersion episodes during high tide. The middle marsh is colonized mainly by *Sarcocornia perennis* (Chenopodiaceae), which can also be found although in smaller extents in the lower marsh where *Spartina maritima* (Poaceae) is dominant. Three transects were assessed perpendicular to the margin. In each transect five sediment cores were sampled (four in pure stands of each species and one in the bare bare sediments). All the samplings were made during low tide. According to previous studies (Reboreda and Caçador, 2008), the sediment layers between 5 and 10 cm proved to have high extracellular enzymatic activity (EEA). For this reason all analysis were carried out in the sediment samples collected at between these depths, and are referred hereafter as rhizosediment.

Sediment physical-chemical characteristics

Sediment pH was mesasured using a HANNA pH/mV (HI 9025) electrode directly in the sediment. 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°C during 2h. Sediment water content was determined by drying sediment samples at 60°C until constant weight. Total and Inorganic C and N were determined in air-dried and burned sediment samples, using a CHNS/O analyser (Fisons Instruments Model EA 1108). Organic C and N were determined as the difference between total fraction and inorganic fraction. For phosphorous determinations all lab wares were soaked for two days in HCl (10%) and rinsed with distilled water to avoid contaminations. Inorganic and total phosphorous was extracted according to Ruban et al. (2001) and Ladakis et al. (2006). Briefly, two sub-samples of 200 mg of rhizosediment were used for phosphorous determinations per sample. Sediments were passed through a 2 mm mesh to remove plant and shell detritus. For total phosphorous (TP) analysis one subsample was burned at 450°C for 3h and extracted with 20 ml of HCl 3.5 M, overnight (about 16 hours). After this extraction period the slurry was centrifuged at 200 x g for 15 min at 4°C. The supernatant was frozen in amber glass vials until analysis. A second subsample was used for inorganic

phosphorous (IP) extraction and was extracted with 20 ml of HCl 1M overnight, after which it was centrifuged as described above for TP. The supernatant was also frozen in amber glass vials until analysis. Phosphorous concentration was measured by the molybdenum-blue colorimetric analysis with a Tecator FIAstar™ 5000 Analyser. Organic phosphorous (OP) was determined as the difference between TP and IP. All phosphorous concentrations were expressed as mg $P\text{-}PO_4^{3-}$ per gram sediment dry weight (DW).

Phosphatase activity

All enzymatic determinations were carried out with colorimetric methods and the absorbance were read on a TECAN Absorbance Microplate Reader (SPECTRA Rainbow). Phosphatase activity was assayed according to Ravit et al., (2003) with a modification in the incubation temperature and without dilution of the supernatant. The buffers used were acetate buffer 50 mM (pH 5.0), TRIS buffer 100 mM (pH 8.7) and citrate buffer 50 mM (pH 7.1), respectively for acid, alkaline and neutral phosphatase assays. Briefly, 75 ml of buffer was added to 5 g of fresh sediment, and mixed for 1 min in order to obtain the sediment slurry. Two ml of p-nitrophenyl-phosphate 5 mM were added to 2 ml of slurry and incubated at 30°C with gentle agitation for 30 min. After incubation, samples were centrifuged at 6.530 x g for 15 min, at 4°C and 0.2 ml of 0.1 N NaOH was added in order to stop the reaction and reveal the p-nitrophenol (pNP) formed. The absorbance of the supernatant was read at 410 nm and compared with the calibration standards for pNP. The phosphatase activity was expressed as μg of pNP released per gram sediment dry weight per hour and normalized for organic carbon content.

Statistical Analysis

Statistical analysis was performed using Statistica Software version 10 from Statasoft Inc. The lack of normality and homogeneity of the data package lead to the application Kruskal-Whallis non-parametrical tests for significance analysis.

Results

Sediment physic-chemical characteristics

Bare sediments presented the lowest water, humic acid and organic matter contents, while *S. perennis, H. portulacoides* and *S. fruticosa* rhizosediments showed respectively the highest value of water, humic acids and organic matter contents. In which concerns these parameters, bare sediments differed significantly ($p < 0.01$) from vegetated sediments. In which concerns the rhizosediment pH it varied between 6 and 7 while in the bare sediments was higher (8.18). Again, bare sediments differed significantly ($p < 0.01$) from vegetated rhizosediments, however there were no significant differences among plant rhizosediments. (Table 1).

Table 1. Sediment physical-chemical characteristics (average ± standard deviation).

	Water Content (%)	pH	LOI (%)	Humic Acids ($g.g^{-1}DW$)
Sarcocornia fruticosa	60.412 ± 4.599	6.09 ± 1.43	20.905 ± 4.044	0.104 ± 0.026
Halimione portulacoides	63.123 ± 1.474	6.87 ± 0.12	19.007 ± 0.420	0.118 ± 0.053
Sarcocornia perennis	64.566 ± 6.892	6.03 ± 1.21	19.655 ± 5.559	0.104 ± 0.016
Spartina maritima	57.785 ± 2.943	6.98 ± 0.12	14.487 ± 1.673	0.091 ± 0.006
Mudflat	37.851 ± 9.732	8.18 ± 0.23	6.685 ± 1.896	0.044 ± 0.012

Regarding sediment elemental composition, *S. fruticosa* rhizosediments had the lowest values of total and organic carbon and nitrogen. These were also the sediments with lowest inorganic carbon content. However the mean inorganic nitrogen content in *S. fruticosa* sediments was similar to the contents found in the rest of the sampling sites. *S. perennis* sediments had the highest values of total and organic carbon and nitrogen contents. In which concerns inorganic nitrogen concentrations, it varied between 0.017 % in *Spartina maritima* sediments and 0.021 % in bare sediments. Total C/N ratios varied between 12.51 in *S. maritima* rhizosediments and 13.98 in *S. fruticosa,* being the lowest organic C/N ratio observed also in *S. maritima* rhizosediments (12.98) and the highest was in *S. fruticosa* (15.73). On the other hand, the inorganic C/N ratios varied between 0.312 and 1.66 in *S. fruticosa* and *H. portulacoides* rhizosediments respectively (Table 2). In which concerns carbon and nitrogen contents (all forms and ratios) the statistical analysis didn't show any

significant differences among sediment samples while comparing bare sediments and vegetated rhizosediment nor when comparing between plant rhizosediments.

Table 2. Sediment elemental composition (average ± standard deviation).

Carbon	Total			Organic			Inorganic		
Sarcocornia fruticosa	2.178	±	0.215	2.173	±	0.217	0.006	±	0.002
Halimione portulacoides	6.367	±	0.752	6.338	±	0.774	0.028	±	0.022
Sarcocornia perennis	6.828	±	1.514	6.807	±	1.525	0.021	±	0.012
Spartina maritima	5.442	±	1.264	5.428	±	1.267	0.014	±	0.016
Mudflat	5.019	±	0.959	5.006	±	0.947	0.013	±	0.012
Nitrogen	**Total**			**Organic**			**Inorganic**		
Sarcocornia fruticosa	0.156	±	0.018	0.139	±	0.019	0.018	±	0.001
Halimione portulacoides	0.499	±	0.070	0.478	±	0.067	0.020	±	0.006
Sarcocornia perennis	0.520	±	0.113	0.503	±	0.112	0.018	±	0.002
Spartina maritima	0.432	±	0.040	0.415	±	0.040	0.017	±	0.001
Mudflat	0.399	±	0.054	0.378	±	0.051	0.021	±	0.009
C/N	**Total**			**Organic**			**Inorganic**		
Sarcocornia fruticosa	13.977	±	0.269	15.727	±	0.601	0.312	±	0.109
Halimione portulacoides	12.794	±	0.482	13.278	±	0.627	1.661	±	1.696
Sarcocornia perennis	13.115	±	0.052	13.538	±	0.074	1.242	±	0.879
Spartina maritima	12.505	±	1.711	12.984	±	1.741	0.772	±	0.873
Mudflat	12.524	±	0.764	13.211	±	0.993	0.636	±	0.416

Sediment phosphorus content and phosphatase activity

Bare sediments had the lowest total, inorganic and organic phosphorus contents of the analyzed sediments. Among the vegetated rhizosediments, *H. portulacoides* had the lowest amount of total and inorganic phosphorus while *S. fruticosa* sediments had the highest amounts of both this phosphorous forms. While comparing vegetated rhizosediments, the organic phosphorus content was found to be highest in *S. fruticosa* and *H. portulacoides* sediments while the lowest vales were observed in *S. perennis* sediments (Figure 1).

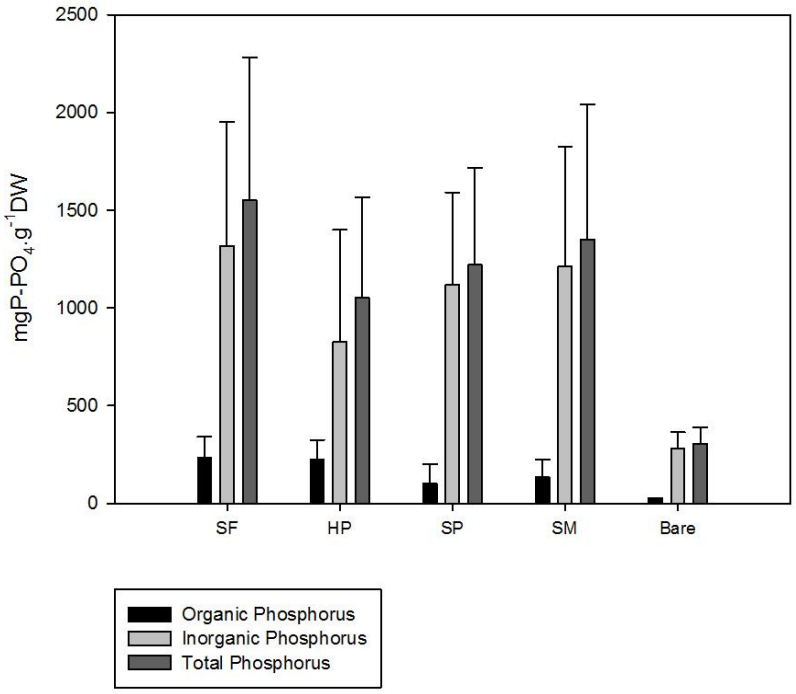

Figure 1. Average total, inorganic and organic phosphorus content in all the surveyed sites (error bars stand for standard deviation) SF- *S. fruticosa* rhizosediments, HP- *H. portulacoides* rhizosediments, SP- *S. perennis* rhizosediments, SM- *S. maritima* rhizosediments, Bare - Unvegetated sediments

Although bare sediments had the lowest total, acid, alkaline and neutral phosphatase activities, these differences were only found to be significant for total and acid phosphatase ($p < 0.05$), being acid phosphatase the enzyme with higher activity followed by alkaline phosphatase and neutral phosphatase. Considering the vegetated sites, *S. fruticosa* rhizosediments had the highest total, acid and alkaline phosphatase activity, but had the lowest neutral phosphatase activity. Furthermore, in *S. fruticosa* rhizosediments, acid phosphatase was found to have higher activity than alkaline phosphatase. Among vegetated sediments, *H. portulacoides* sediments had the lowest total, acid and alkaline phosphatase activity, but had the highest neutral phosphatase activity. Acid phosphatase evaluated in *H. portulacoides* sediments was the most active enzyme while alkaline phosphatase was the less active enzyme. *S. perennis* and *S. maritima* sediments had similar total, acid and alkaline phosphatase activities, although neutral phosphatase activity was higher in *S. maritima* sediments than in *S. fruticosa* sediments. In both plant rhizosediments the acid phosphatase was the most active enzyme and neutral phosphatase was the less active enzyme. Regarding enzyme activities, statistical analysis did not show any significant differences across vegetated sediments. Acid phosphatase was the most active enzyme in all the analyzed samples (Figure 2).

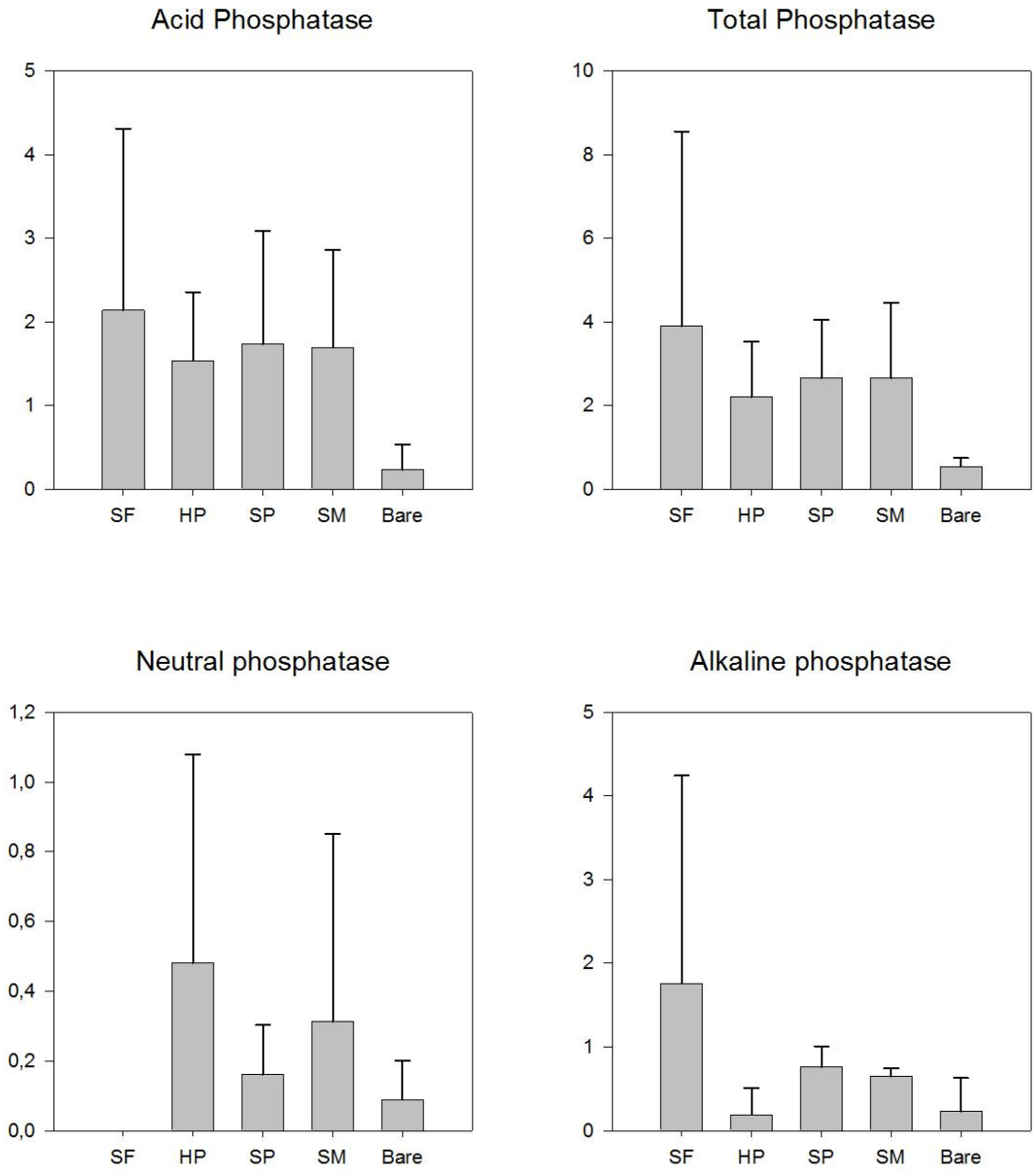

Figure 2. Total, acid, alkaline and neutral phosphatase activities (µg pNP-phosphate.h-1.g-1DW), error bars stand for standard deviation. SF- S. fruticosa rhizosediments, HP- H. portulacoides rhizosediments, SP- S. perennis rhizosediments, SM- S. maritima rhizosediments, Bare - Unvegetated sediments

Integrating all the data in a principal component analysis (Figure 3), it was possible to observe three different groups of samples. A first group containing the bare sediments samples associated to the high pH and inorganic N values and to low organic and water contents. A second group grouping out the sediments colonized by *S. fruticosa* mostly associated to high alkaline phosphatase activity and high organic carbon to organic nitrogen ratios. Finnaly also a third group could be distinguished contained the rhizosediments from *H. portulacoides, S. perennis* and *S. maritima* pure

stands, associated to high amounts of organic carbon and nitrogen as well as high neutral phosphatase activity.

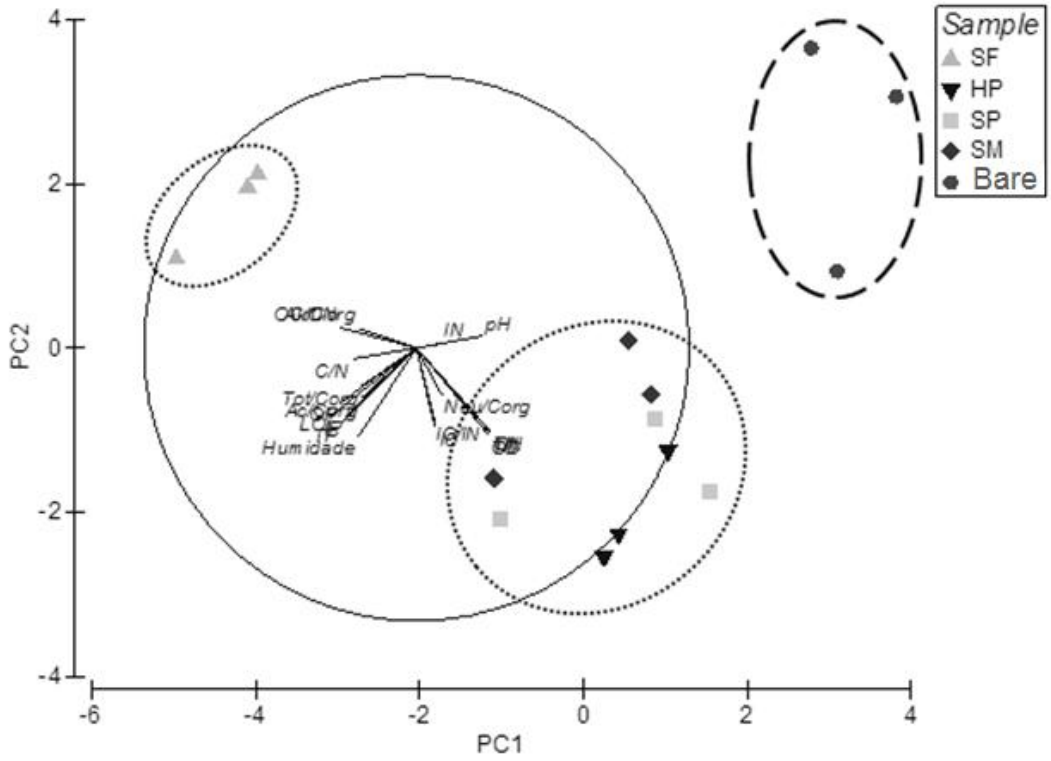

Figure 3. PCA ordination of all tested samples. SF- Sarcocornia fruticosa rhizosediments, HP-Halimione portulacoides rhizosediments, SP- Sarcocornia perennis rhizosediments, SM- Spartina maritima rhizosediments, MF- Bare sediments. Within the dotted lines are plant colonized rhizosediments while mudflat sediments are surrounded by the dashed line.

Discussion

Observing the data from Table 1 it was possible to observe that the rhizosediments from the upper marsh retained more water than the ones from the lower marsh, although these last undergo higher submersion periods. Along with this, there is also a higher content in organic matter in the rhizosediments from the upper marsh. This is very common in marsh sediments since soil organic matter has a greater water-holding capacity than mineral soil (Franzluebbers, 2002), Concomitantly in highly mineral sediments (like bare and *S. maritima* rhizosediments) there is lower water retention. Although the comparisons among rhizosediments from the four different plant species did not show significant differences, the PCA ordination separated *S. fruticosa* from the rest of plant rhizosediments and bare sediments mostly due to differences in total and organic carbon and nitrogen contents since these were lower in *S. fruticosa* rhizosediments than in the rest of the analysed sediments. *S. fruticosa* rhizosediments had the lowest amount of total and organic carbon and nitrogen, as well as the highest amount of organic matter and phosphorus, mostly due to tidal N and C fluxes. *S. fruticosa* is mostly found in higher salt marsh being this way subjected to shorter flooding periods than the rest of the analyzed plants, and consequently receiving lower amounts of dissolved and particulated tidal inputs. Also in previous works (Caetano et al., 2011) it was found that salt marsh plants have different sink/sources roles in elemental cycling affecting the import/export fluxes differentially (Duarte et al., 2008; Caçador et al., 2009). Plants have a clear effect on the sediments (Reboreda and Caçador, 2007), possible to observe in the higher phosphatase activities and phosphorus contents found in the analyzed rhizosediments. Inorganic phosphorus content seems to be higher in rhizosediments with higher phosphatase activity, although this is not so evident for neutral phosphatase, pointing out to a direct effect of phosphatase activity in inorganic phosphorous production, as it was described for other ecosystems (Alef et al., 1998). Another evidence of the halophyte effect on the phosphorous biogeochemistry and retention can be observed while comparing the bare and vegetated sediments in terms of both inorganic and organic phosphorus contents. These were found to be higher in plant rhizosediments than in bare sediments, probably due to tidal fluxes and to halophyte rhizosphere retention (Reboreda and Caçador, 2007). Since plant aboveground biomass intrinsic network can act as a sediment trap it is likely to have a retention effect on the incoming tidal particulate phosphorus. Also, *S. fruticosa* had higher amounts of total, organic and inorganic phosphorus than the rest of the rhizosediments probably due to shorter

flooding periods (Chambers, 1992). Although *S. maritima* is a specie colonizing mostly the lower pioneer marsh it also had high values of total and inorganic phosphorus. Previous works (Silva et al., 2009) showed that there are considerable differences in the sedimentation rates along the marsh due to different plant colonisations. Lower marsh areas colonized by *S. maritima* are subjected to longer submersion periods and thus to higher sedimentation rates (Silva et al., 2009). Wolaver and Spurrier (1988) found that phosphate was removed from the flooding water and resided at low and high marsh. Palomo and Neil (2009) found that *Sarcocornia perennis* rhizosediments act as a sink of phosphorus. Concomitantly also in this work, plant rhizosediments seem to be acting like a sink of phosphorus, having 4.23 times more phosphorous than the bare sediments. Allison (2006) observed that an increase in humic acid content could inhibit enzyme activity, including acid phosphatase. Enzymes may be inhibited due to the formation of complexes with humic acids leading to changes in protein conformation or even to a blocked active site (Allison, 2006). In fact, in the present work rhizosediments, excluding bare sediments, higher humic acid contents in sediments correspond to lower acid phosphatase activities. For alkaline phosphatase the pattern is not so clear, although *H. portulacoides* rhizosediments presented the lowest alkaline phosphatase activity among vegetated rhizosediments and simultaneously had the higher humic acid content.

Acid phosphatase activity was higher than neutral and alkaline phosphatase activities in plant rhizosphere, probably due to the pH of rhizosediments that was found to be always bellow 7 in the analyzed rhizosediments. In bare sediments where the pH is 8.18, there was a smaller difference between acid and alkaline phosphatase activity. Sediment pH seems to be an important driver of acid phosphatase activity as it could be observed in *S. fruticosa* rhizosediments with low pH values and high acid phosphatase activity. Neither alkaline nor neutral phosphatase activities seemed to be influenced by pH fluctuations. Bare sediments had lower phosphatase activities than vegetated sediments. Previous studies (Duarte et al, 2007; Gaume et al., 2001), have shown that root biomass can influence enzymatic activities directly, throughout exudation of phosphatases or low molecular weight organic molecules, increasing or enhancing enzymatic activity or indirectly throughout sediment physical-chemistry modification (Reboreda and Caçador, 2007). Overall the data showed significant differences when comparing bare sediments with vegetated rhizosediments, as shown by the PCA ordination, showing that plants have a significant influence in sediment characteristics and extracellular enzyme activity. In accordance with our results Cleary

et al (2012) showed that there were differences between vegetated and unvegetated habitats. Unvegetated habitats had higher pH, lower rates of enzymatic activity.

Conclusions

Phosphorous biogeochemical cycling in salt marsh sediments present an evident gradient driven mostly by physic-chemical drivers modulating enzymatic activity. Phosphatase activity is higher in vegetated sediments than in unvegetated sediments. Also the pH and humic acid content seem to be the most important factors influencing phosphatase activity. Thus, acid phosphatase was found to give the highest contribution for total phosphatase activity among the three pH-isoforms present in salt marsh sediments, favoured by acid pH in colonized sediments. On the other hand also appear to have an important role controlling phosphatase activity since high contents in humic acids seem to inhibit phosphatase. There is also a clear relation of phosphatase activity and inorganic phosphorous, reinforcing the role of phosphatase in phosphorous cycling, but also pointing out to a possible priming effect from inorganic phosphorous controlling enzymatic activity as a possible feedback mechanism.

References

Alef, K., Nannipieri, P., Trazar-Cepeda, C., 1998. Phosphatase activity. *In*: Alef, K. and Nannipieri, P. (Eds.), Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London.

Allison, S.D., 2006. Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes. *Biogeochemistry*, 81:361-373.

Caçador, I., Caetano, M., Duarte, B., Vale, C., 2009. Stock and losses of trace metals from salt marsh plants. *Marine Environmental Research*, 67:75-82.

Caetano, M., Bernárdez, P., Santos-Echeandia J., Prego, R., Vale C., 2011. Tidally driven N, P, Fe and Mn exchanges in salt marsh sediments of Tagus estuary (SW Europe). *Environ Monit Assess*. DOI 10.1007/s10661-011-2439-2.

Chambers, R.M., Harvey, J.W., Odum,W.E., 1992. Ammonium and Phosphate Dynamics in a Virginia Salt marsh. *Estuaries*,15(3):349-359.

Cleary, D.F.R., Oliveira, V., Gomes, N.C.M., Pereira, A., Henriques, I., Marques, B., Almeida, A., Cunha,A., Correia, A., Lillebø A.I., 2012. Impact of sampling depth and plant species on local environmental conditions, microbiological parameters and bacterial composition in a mercury contaminated salt marsh. Marine *Pollution Bulletin*, 64:263–271.

Correl, D., 1998. The Role of Phosphorus in the Eutrophication of Receiving Waters: A Review. *Journal of Environmental Quality* 27, 261-266.

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, 1056-1073.

Duarte, B., Reboreda,R., 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.

Franzluebbers, A.J., 2002. Water infiltration and soil structure related to organic matter and its stratification with depth. *Soil & Tillage Research* 66:197–205.

Gaume, A., Mächler, F., De León, C., Narro, L. and Frossard, E., 2001. Low-P tolerance by maize (Zea mays L.) genotypes: Significance of root growth, and organic acids and acid phosphatase root exudation. *Plant and Soil* 228, 253-264.

Ladakis, M., Dassenakis, M. and Pantazidou, A., 2006. Nitrogen and Phosphorous in Coastal Sediments covered by Cyanobacterial Mats. *Journal of Soils and Sediments* 6, 46-54.

Lillebø, A.I., Flindt, M.R., Pardal, M.A. and Marques, J.C., 2006. The effect of Zostera noltii, Spartina maritima and Scirpus maritimus on sediment pore-water profiles in a temperate intertidal estuary. *Hydrobiologia* 555, 175–183.

Paerl, H. 2009. Controlling Eutrophication along the Freshwater–Marine Continuum: Dual Nutrient (N and P) Reductions are Essential. *Estuaries and Coasts* 32, 593–601.

Palomo, L. And Neil, F.X., 2009. Primary production and nutrient budgets *of Sarcocornia perennis* ssp. alpini (Lag.) Castroviejo in the salt marsh of the Palmones River estuary (Southern Spain). *Aquatic Botany*, 91:130-136.

Ravit, B., Ehrenfeld, J.G., Haggblom, M.M., 2003. A comparison of sediment microbial communities associated with Phragmites australis and Spartina alterniflora in two brackish wetlands of New Jersey. *Estuaries* 26, 465–474.

Reboreda, R. and Caçador, I., 2007. Halophyte vegetation influences in salt marsh retention capacity for heavy metals. *Environmental Pollution* 146, 147-154.

Reboreda, R. and Caçador, I., 2008. Enzymatic activity in the rhizosphere of Spartina marítima: Potential contribution for phytoremediation of metals. *Marine Environmental Research* 65, 77-84.

Ruban, V., López-Sánchez, J., Pardo, P., Rauret, G., Muntau, H. and Quevauviller, P. 2001. Development of a harmonized phosphorous extraction procedure and certification of a sediment reference material. *Journal of Environmental Monitoring* 3, 121-125.

Tobias C., Macko S., Anderson I. and Canuel E., 2001. Tracking the fate of a high concentration groundwater nitrate plume through a fringing marsh: a combined groundwater tracer and in situ isotope enrichment study. *Limnology and Oceanography* 46, 1977–1989.

Valiela, I., Cole, M., McClelland, J., Cebrian, J., Joye, S.B., 2000. Role of salt marshes as part of coastal landscapes. In: Weinstein, M.P., Kreeger, D.A. (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, London.

Wolaver, T.G. and Spurrier, J.D., 1988. The exchange of phosphorus between a euhaline vegetated marsh and the adjacent tidal creek. *Estuarine, Coastal and Shelf Science* 26:203-214.

Chapter 4

Final Remarks

It is important to understand the factors underlying microbial activity in salt marsh sediments in order to understand the role of these ecosystems in the natural remediation of eutrophication. In this work it becomes clear that sediment pH and salinity are crucial to sediment function as they influence salt marsh plant zonation and consequently with reflections at the microbial level.

Salt marsh microbial communities play a central role in phosphorus biogeochemical cycle, mainly due to acid phosphatase activity. Dehydrogenase and phosphatase activities are both influenced by pH and plant coverage. Rhizosediment phosphatase activity is the main contributor to acid phosphatase, probably the better-adapted isoform to rhizosediment pH.

This way, when considering salt marshes as key players to achieve ecosystem natural remediation, it is important to consider plant coverage as a main factor controlling salt marsh efficiency as a phosphorus sink. It is also important to take into account, that in eutrophic systems if the nutrient load is reduced, the sediments may eventually become a source of phosphorus to the coastal waters. The evident evident strong spatial-temporal variability of microbial activities, showed by the dehydrogenase activities, has implications on the natural remediation processes, also subjected to the same variability. Thus, it is important to take not only the seasonal variability into account but also the vegetation coverage when considering the importance and efficiency of these remediation processes.