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ORIGINAL ARTICLE

Spartina maritima (cordgrass) rhizosediment extracellular enzymatic activity and its role in organic matter decomposition processes and metal speciation

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Abstract

Seasonal monitoring was carried out to investigate the influence of extracellular enzymatic activity (EEA) on metal speciation and organic matter cycling in the rhizosediment of *Spartina maritima*. Heavy metal speciation was achieved by the Tessier scheme, and showed a similar pattern of variation of the organic-bound fraction, indicating a decomposition process in progress. Both humic acid and organic matter showed the same seasonal pattern. The basal respiration of the rhizosediments also presented a similar seasonal pattern, indicating a microbial degradation of organic matter. The high organic-bound fraction found in the summer gradually decreased towards the winter. This decrease was found to be related to the increase of activity of peroxidase, β-*N*-acetylglucosaminidase and protease. Also the activity of sulphatase was found to be related to the depletion on the exchangeable fraction, probably due to sulphide formation and consequent mobilization. The results show an interaction between several microbial activities, affecting metal speciation.

Problem

Salt marshes located in estuaries frequently receive large inputs of nutrients (Tobias *et al.* 2001), as well as particulate and dissolved organic matter. This high nutrient input makes salt marshes one of the most productive ecosystems of the planet. This high biomass production has as a consequence large necromass generation due to litter senescence (Caçador *et al.* 2009). In highly industrialized estuaries, along with this nutrient input there is also a large input of heavy metals, which will accumulate in salt marsh sediments (Doyle & Otte 1997). These high inputs make salt marshes key zones for the biogeochemistry of the estuary, but also for metal cycling (Weis & Weis 2004). The microbial decomposer communities of salt marsh sediments play an essential role in these cycling processes by decomposing the organic matter, as well as other large complex molecules that reach to the sediments, into more bioavailable forms (Ravit *et al.* 2003). As verified previously, salt marsh sediments are often very organic (Richert *et al.* 2000), providing large amounts of substrates for the proliferation of microbial decomposers. The generation of large amounts of below-ground necromass and the organic compounds exuded by living plants (Duarte *et al.* 2007) are the major contributors to the organic content of the rhizosediments. The large periods of submersion to which these sediments are subjected, lead to a low oxygenation of the sediments, with adverse effects on plant growth (Richert *et al.* 2000). Some salt marsh plants have the ability to pump oxygen from the atmosphere into the root zone, oxidizing the sediments and consequently promoting 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 on season (Wilczek *et al.* 2005; Yang *et al.* 2007). To process the organic matter, microbes produce extracellular enzymes which degrade these large complex molecules into smaller ones, which

are easily up taken by the microbes. These enzymes are divided into two major groups: oxidoreductases, which degrade large molecules by redox reactions, and hydrolases, which decompose complex molecules by breaking them down. These enzymes are very important in the ecosystem overall due to the large amount of necromass generated, maintaining an ecological balance. Several extracellular enzymes, such as proteases, phenol oxidases, peroxidases and *β-N*-acetylglucosaminidases, are very important in this process as they contribute to the breakdown of organic compounds (Kang *et al.* 2005; Oyekola & Pletschke 2006; Acosta-Martínez *et al.* 2007). During the early stages of decomposition (Brüchert & Pratt 1996 in Passier *et al.* 1999) it was observed that sulphur-linked reactions also play an important role in the decomposition process in salt marsh sediments, either by sulphidization reactions of the organic matter (Passier *et al.* 1999) or by leaching of labile organic sulphur present in tissues. Several studies have shown that plant litter occurs in three different stages, independently of the species (Valiela *et al.* 1985; Wilson *et al.* 1986; Benner *et al.* 1991). Initially, 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 bound to the more resistant fraction of the organic matter, remaining in the nearby sediment. Other factors such as oxygen in the rhizosphere, the redox state, temperature and the microbial community also influence this rate of decomposition (Pereira *et al.* 2007).

Heavy metal inputs reach salt marshes through tidal flooding and are retained in the sediments in various forms, depending on the bonds they establish with the sediment components (Tessier 1979). There is a large variability in this process, depending not only on the sediment characteristics but also on external factors (hydrodynamics, weather, seasonal variations and plant coverage), as seen in previous studies (Reboreda & Caçador 2007; Duarte *et al.* 2008). The literature provides evidence that microbial interactions with metals greatly influence their speciation (Gadd 2001, 2004; Tabak *et al.* 2005; Duarte *et al.* 2008). These transformations include metal precipitation reactions by metallic sulphides and redox reactions, causing changes on the metal species and its associations (Hullebusch *et al.* 2005).

Heavy metal speciation is very variable, depending both on salt marsh localization and physical–chemical characteristics, and on plant coverage (Reboreda & Caçador 2007, 2008). Although there was evidence of the influence of extracellular enzymes on metal speciation in the upper marsh colonized by *Halimione portulacoides* (Duarte *et al.* 2008), the sediment and hydrological conditions in the lower marsh mainly colonized by *S. maritima* are very different. In this paper the authors compare microbial speciation mechanisms in *S. maritima* with those previously verified in *H. portulacoides*, in order to understand if they are still valid in this environment.

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 *H. portulacoides* (Chenopodiacea) and *Sarcocornia fruticosa* (Chenopodiacea) and undergoes short submersion episodes during high tide. Between April 2006 and January 2007, four samplings were done: April (Spring), July (Summer), October (Autumn) and January (Winter). For each sampling, five sediment cores (50 cm depth) were taken in pure stands of *S. maritima*. The stands were located along the marsh always with a minimum distance of 10 m from each stand. All the collections were made during low tide. The cores were transported in refrigerated bags to the laboratory, where the sediment was sliced. According to previous studies (Reboreda & Caçador 2008) the depth between 5 and 10 cm proved to have high extracellular enzymatic activity (EEA) and it was used for analysis. These sediment samples surrounding the rhizosphere of *S. maritima* are referred hereafter as the rhizosediment.

Sediment parameters

Redox potential (Eh) and pH values were measured in the fresh, selected segment using a HANNA pH/mV (HI 9025). Calibration of redox potential measurements was done using a standard redox solution (Crison, Eh = 468 ± 5 mV at 25 °C). 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 for 2 h. Humic acids were extracted and quantified according to Adani *et al.* (2006) with some modifications. Dried and sieved sediment (5 g) was extracted by adding 25 ml of a solution containing 0.1 M NaOH and 0.1 M $Na_4P_2O_7$. The extraction was carried out in an end-to-end shaker for 24 h at 65 °C. After extraction the samples were centrifuged at 45,880 *g* for 20 min at 4 °C. The supernatant was totally recovered and distilled water was added to the solid residue, which was re-suspended and centrifuged again. This operation was

repeated until the supernatant was clear. The supernatant solutions were combined and acidified with 50% sulphuric acid to pH <1.5 to precipitate the humic acids. These were separated by centrifugation as described above, the supernatant completely evaporated at 60 °C until constant weight, and the humic acids weighed. Phenolic content in the sediment samples was determined according to Folin & Ciocalteau (1927) modified by Waterman & Molle (1994). Briefly, 5 g of fresh sediment was mixed with 50 ml distilled water and 10 ml of this slurry was centrifuged at 653 *g* for 2 min at 10 °C. The supernatant (0.5 ml) was treated with 2.5 ml of Folin-Ciocalteu's phenol reagent (0.2 N) and 2.5 ml alkali reagent, and left to stand for 2 h. After this period the absorbance was 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.

Respiratory activity and extracellular enzyme activity in the rhizosediment

Sediment basal respiration was determined by the NaOH-trap method (Isermeyer 1952). Briefly, 5 g of rhizosediment was placed in a tube and adjusted with distilled water to 55% of its water-holding capacity (WHC). This container was placed inside a jar containing 0.05 M NaOH, sealed air-tight and placed in the dark at room temperature for 2 days. After this period the carbon dioxide involved and trapped in the NaOH-trap was determined by titration with 0.05 M HCl. An excess of water was percolated to a portion of the sediment samples and the volume stored in the sample was used for calculation of the WHC.

All enzymatic determinations were carried out with colorimetric methods and the absorbances were read on a TECAN Absorbance Microplate Reader (SPECTRA Rainbow). The utilization of this plate reader system allowed three readings of the same replicate to be made. 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 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 was added to 2 ml of slurry and incubated at 30 °C with gentle agitation for 60 min (sulphatase) and 2 h (*β-N*-acetylglucosaminidase). After incubation, samples were centrifuged at 653 *g* for 15 min at 4 °C and 0.2 ml of 1 N NaOH was added to stop the reaction and reveal the *p*-nitrophenol (pNP) formed. Absorbance of the supernatant was read at 410 nm. The absorbance was compared with a calibration curve for pNP and the activity was expressed as *μ*g of pNP released per gram sediment dry weight per hour. Phenol oxidase and peroxidase were assayed using 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. Two ml was added to 2 ml of slurry (adding 0.1 ml of 0.3% H_2O_2 for peroxidase assay) and were incubated for 60 min for both enzymes. After incubation, samples were centrifuged at 653 *g* for 15 min at 4 °C. Absorbance of supernatant was read at 460 nm and the absorbance of phenol oxidase was subtracted from the absorbance of total peroxidase to obtain the real value for peroxidase activity alone. The absorbance was compared with a calibration curve for L-DOPA and the activity expressed as *μ*M L-DOPA oxidized per gram sediment dry weight per hour. Protease activity was assayed according to Ladd *et al.* (1976). Briefly, 1 g of fresh sediment was incubated with 5 ml of Tris (Tris hydroxymethyl-aminomethane) buffer (0.05 M, pH 8.1) and a 2% (w/v) casein solution, for 2 h at 50 °C. After incubation, the reaction was stopped with 1 ml trichloroacetic acid 17.5% (w/v) and centrifuged at 14,690 *g* for 15 min at 4 °C. For photometric analysis, 1 ml of supernatant was added to 1 ml of Folin-Ciocalteu's phenol reagent (0.2 N) and 2.5 ml alkali reagent, and left to stand for 90 min. The absorbance was measured at 700 nm and compared with the calibration curve for tyrosine. Activity was expressed as microgram tyrosine equivalents per gram sediment dry weight per hour.

Metal sequential extraction and elemental analysis

To determine chemical fractioning of Cu, Cd, Cr, Ni, Zn, Co and Pb on sediments, a sequential extraction procedure was used. For metal determinations all labware was soaked for 2 days in HCl (10%) and rinsed with distilled water to avoid contamination. The metal sequential extraction scheme adopted in this study was that described by Tessier (1979) and modified by Hullebusch *et al.* (2005). One gram of air-dried sediment was sequentially extracted by adding 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, comprising 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 of the procedure the sediment was centrifuged at 204 *g* for 10 min at 4 °C and the supernatant filtered using Whatman No. 42 filters (2.5 *μ*m pore diameter) and stored at 4 °C until analysis. This procedure

proved to be adequate for this kind of sediment, as described in Duarte *et al.* (2008). A total digestion was made with aqua regia in a Teflon reactor at 110 °C for 3 h to evaluate the efficiency of the sequential procedure. All efficiencies were between 95 and 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.

Statistics

Statistical analysis was performed using STATISTICA Software version 7.0 from StatSoft 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-parametric tests.

Results

Sediment characteristics

Organic matter content (LOI) and humic acid content in the rhizosediment of *S. maritima* (Table 1) showed a marked seasonal pattern ($P < 0.03$). The pattern between these two organic components was very similar, with a decrease from spring to summer followed by an increase to autumn. In winter an accentuated decrease was registered, compared with the values verified in the previous seasons. pH values were very constant, showing no significant seasonal pattern ($P > 0.05$). There was only a slight decrease in summer, this being the lowest value verified. There was no significant seasonal pattern for the redox state of the collected sediments. There was a great variability of Eh values in the collected replicates, as shown by the standard error. Although not significant ($P > 0.05$), a progressive increase in the phenolic content was seen towards the winter season.

Metal content and speciation

Observing the metal speciation results (Fig. 1) it is possible to see a large variation in the organic-bound fraction of metals, being more significant in Cu, Cr and Ni ($P < 0.03$ for Cu and $P < 0.05$ for Cr and Ni). The residual fractions of Co, Cu, Cr and Pb also showed a marked seasonal pattern ($P < 0.02$ for Co and Cr, and $P < 0.03$ Cu and Pb). Only Ni showed a marked seasonal pattern in all the fractions except for the residual fraction. All metals evaluated exhibited the same pattern of total and partial concentrations, increasing from spring to summer, corresponding to an increase in all fractions. In autumn and winter a progressive decrease in the concentrations of all fractions was observed similar to those verified in summer, except for Cr and Pb, where the concentrations remained above the summer values. The major fluctuations in the fractions were observed in the residual and organic-bound fractions for all the analyzed metals. All the concentrations found are in agreement with those previously in other studies. Cadmium is often the less abundant metal in salt marsh sediments (Caçador *et al.* 2000). Although it is found mostly in the organic-bound fraction and residual fraction, it was also possible to observe that about 10–15% of the metal is in the more labile fractions (exchangeable and carbonate-bound fractions), with a slight increase of these fractions in winter. Cobalt and Pb exhibit similar behaviour, with an increase of the residual fraction from spring to autumn and consequent depletion of the remaining fractions. In winter both metals showed an increase in the available and carbonate-bound fraction, although the total amount of metal did not increase. Cu and Cr were only found in considerable amounts in the organic and residual fractions, and in different proportions (Fig. 1). Both elements showed an increase of the organic-bound fraction in summer and autumn, with a depletion in spring and winter. Zinc fluctuations from spring to autumn are mostly due to depletions in the more labile fractions and increase of the residual fraction. An increase of the exchangeable and carbonate-bound fractions of zinc was found in winter, proportional to the values verified in spring. Observing Ni distribution in fractions it was possible to verify a strong seasonal pattern in the rhizosediment of *S. maritima* ($P < 0.03$ for the available and $P < 0.05$ for carbonate-bound and organic-bound fractions). During summer and autumn the carbonate-bound and available fractions prevailed, whilst in spring and winter these fractions almost disappeared, with a very significant increase in residual fractions.

Table 1. Sediment characteristics during the study period (mean value ± SE).

	spring	summer	autumn	winter
Eh (mV)	−12.09 ± 5.84	51.73 ± 14.50	10.65 ± 2.81	−9.43 ± 10.34
pH	7.21 ± 0.31	6.12 ± 0.73	6.60 ± 0.41	7.13 ± 0.56
LOI (%)	22.96 ± 0.58	19.64 ± 0.75	23.42 ± 0.48	18.07 ± 1.21
Humic acids (g HA·g^{-1} DW)	34.90 ± 6.57	19.50 ± 0.64	25.10 ± 2.68	8.70 ± 0.30
Phenolics (mEq GA·g^{-1} DW)	22.22 ± 2.37	292.46 ± 30.33	11.59 ± 0.19	1457.02 ± 82.02

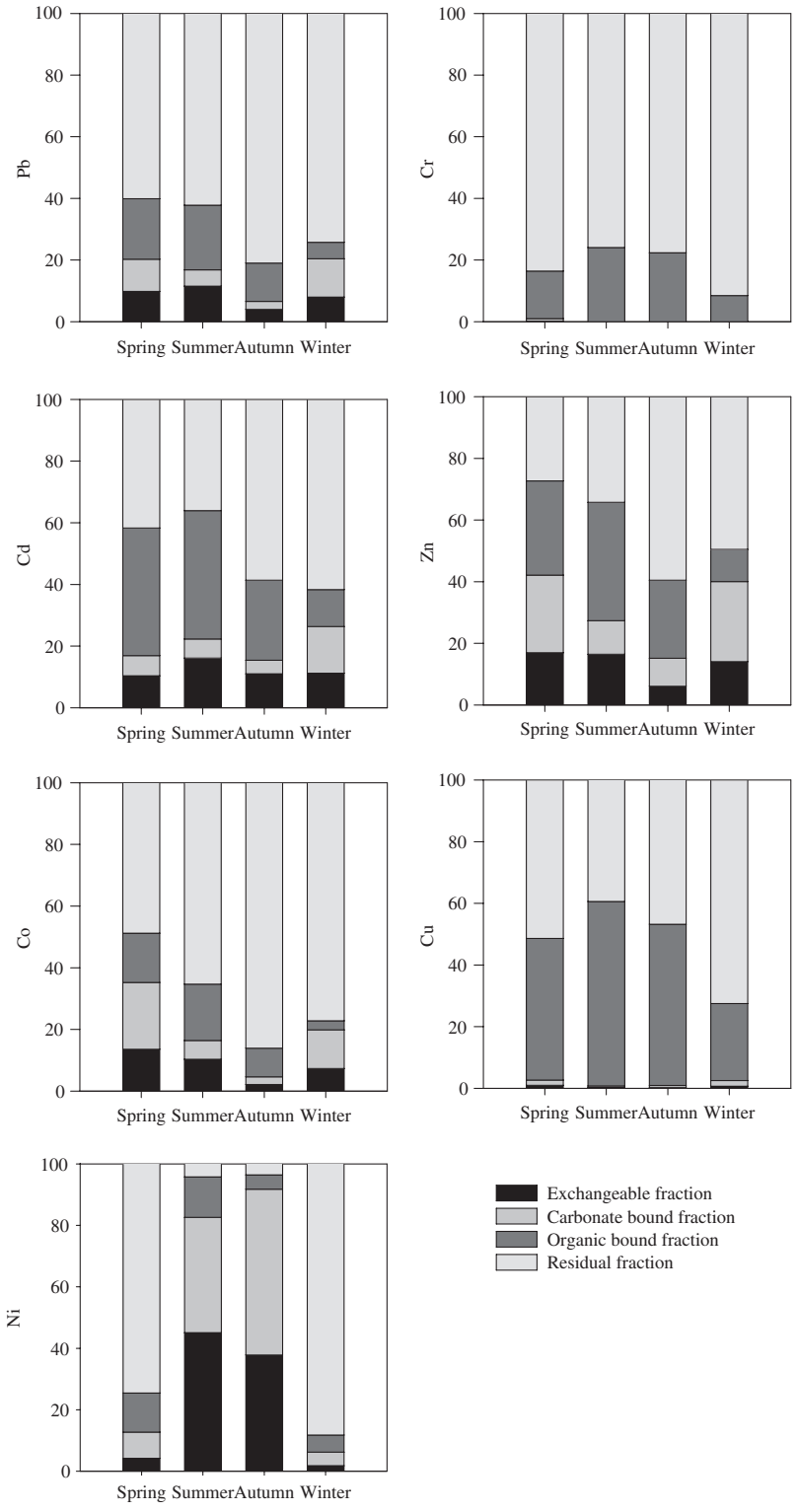

Fig. 1. Metal speciation in the rhizosediments of *S. maritima* throughout the study period.

Sediment basal respiration and EEA

Basal respiration in the rhizosediment of *S. maritima* (Fig. 2) did not show a significant seasonal pattern ($P > 0.05$), although a depletion from spring to summer and an increase to autumn could be observed. A decrease could be verified in winter. The EEA of several extracellular enzymes was also assessed (Fig. 3). The pattern of

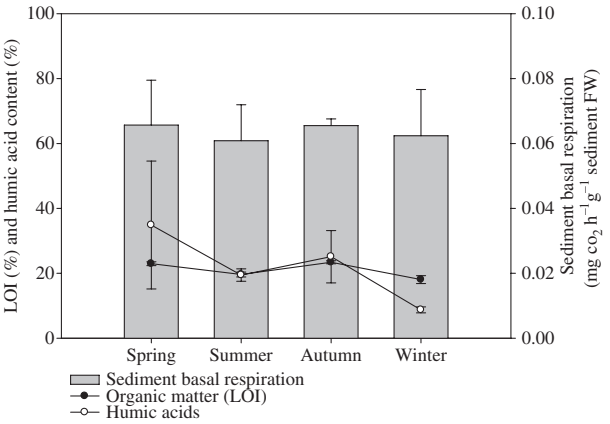

Fig. 2. Comparison between organic matter content (LOI), humic acids and sediment basal respiration.

seasonality observed in all the analyzed EEA was statistically significant only for peroxidase ($P < 0.02$). Peroxidase and phenol oxidase showed a contrasting behaviour. Whereas phenol oxidase was very active in spring and summer, peroxidase activity was only detected in autumn and winter. Protease activity was always detected in the studied periods, being highest in the warm seasons and slightly low in the colder seasons. The activity of *β-N*-acetylglucosaminidase was rather low from spring to autumn, followed by a great increase in winter. The activity for sulphatase was also assessed. In this case the enzyme did not show any detectable activity in spring, but during the rest of the year activity was very high and constant in the following seasons.

Discussion

Comparing the values of organic matter content, humic acid concentration and basal respiration it is possible to observe similar patterns in all these parameters. This indi-

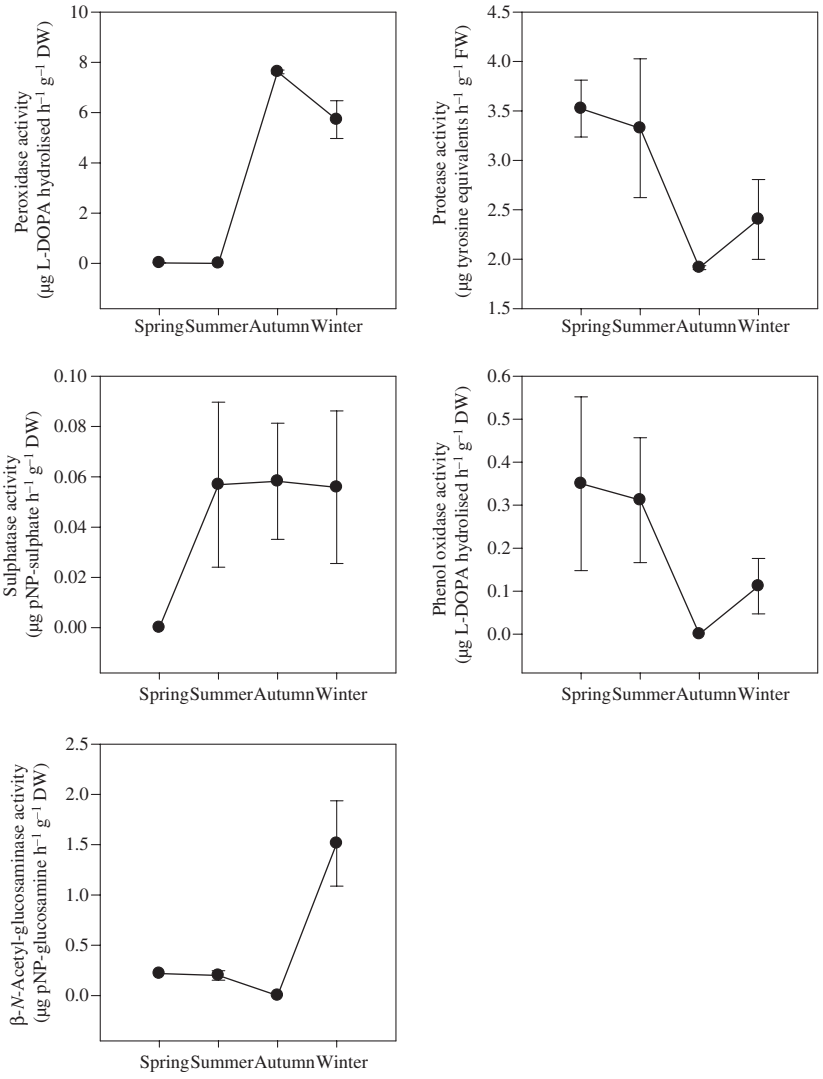

Fig. 3. Extracellular enzymatic activites assessed for the rhizosediment of *S. maritima* throughout the study period (SE bars represented).

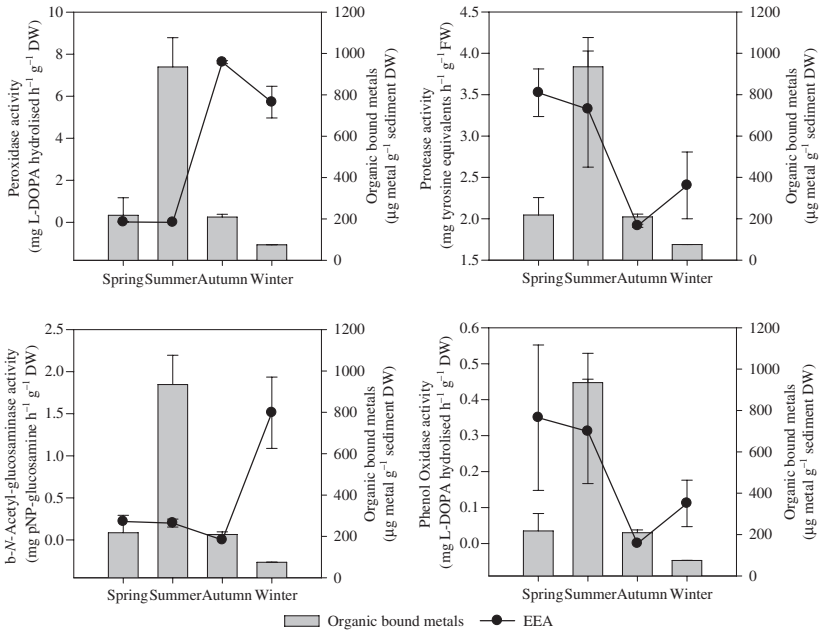

Fig. 4. Influence of extracellular enzymatic activities of protease, peroxidase, phenol oxidase and *β*-*N*-acetylglucosaminidase (line) on total organic-bound metals (bars).

cates that the microbial activity is dependent on the organic matter present in the sediment. This is important in particular for the respiratory activity, directly related to the degradation of organic substrates, which are the important ligands of heavy metals, affecting metal speciation, as has already been seen for the upper marsh sediments (Duarte *et al.* 2008). Considering the organic-bound fraction of all metals and organic matter and humic acid content in the sediment, the patterns exhibited by these parameters showed no similarity. This indicates that the peak of metals observed in summer was not due to an increase of organic matter free to establish bonds with metals, but to a beginning of the decomposition process. In this first step of decomposition the dominant process is the leakage of the more labile compounds, such as the low molecular weight (LMW) organic molecules. This is the case for the organic acids, as has been observed in previous studies that established chelator–metal complexes (Mucha *et al.* 2005; Duarte *et al.* 2007). With this leakage of LMW molecules the organic-bound fraction of metals in the rhizosediments increases, as can be seen from our results. As previously referred to, several enzymes are involved in the degradation and breakdown of organic matter. These enzymes have different patterns of activity throughout the seasons, leading to a differential degradation of the organic components of the sediment in different periods of the year. Taking these facts into consideration, the decomposition process will be discussed, in this case, from summer to winter. Carrying over data from enzymatic activities and from metal speciation analysis, it is possible to observe a common pattern (Figs 4 and 5). The values detected in spring are probably

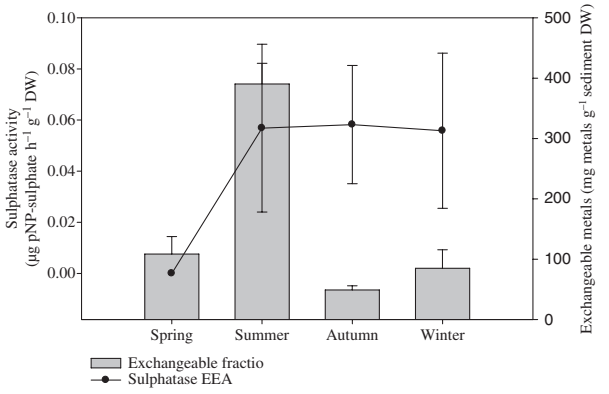

Fig. 5. Influence of extracellular enzymatic activities of sulphatase (line) on total exchangeable metals (bars).

the end of decomposition from a previous cycle, and so will not be considered for discussion proposes. Extracellular peroxidase is known to be produced mostly by lignin-olytic fungi to degrade plant litter (Johnsen & Jacobsen 2008) and catalyzes the degradation of ligninocellulosic litter in the presence of hydrogen peroxide. The degradation of lignin produces phenolic substances of LMW. The major activity of this enzyme was detected in autumn and winter, after the first step of decomposition, indicating the beginning of a second phase of decomposition. The observed decrease in the organic-bound fraction of metals in the seasons where peroxidase activity was high, indicated that probably the lignin-like compounds which were being degraded are molecules that were bound to the metals. With the decomposition of these molecules the metals are released from the organic-bound fraction.

Together with this depletion it was possible to observe an increase in the phenolic content of the rhizosediments, with the same pattern verified for peroxidase activity, supporting lignin degradation, as already pointed out. This accumulation of phenolic substances is also due to the low activity of phenol oxidase in winter. A great increase of activity of β-*N*-acetylglucosaminidase in winter was also assessed, coincident with the high depletion of organic-bound fraction of metals. Together with the high peroxidasic activity, this glucosamine polymer degradation contributes to the degradation of large polymers, typically found in teguments. The degradation renders proteins more accessible for protease degradation, as indicated by the small recovery of activity verified for protease in winter. All these three EEA contribute to a strong decrease of heavy metals in the organic-bound fraction, releasing them to the surrounding environment. Previous studies (Hullebusch *et al.* 2005) point out that high sulphatase activity, and consequent formation of sulphates by this enzyme into sulphides, can lead to its conversion into sulphides by sulphate-reducing bacteria (SRB). Sulphides can chemically reduce metals into a stable form for extended periods of time (Tabak *et al.* 2005), decreasing therefore the metal concentrations in the exchangeable fraction of this extraction scheme. This was also observed in this study. From summer to winter the organic matter degradation released heavy metals from the organic-bound fraction, and these processes would be expected to lead also to an increase of the heavy metals in the exchangeable fraction. This was not verified, probably due to the high activity of sulphatase and consequent sulphide generation and metal precipitation, as shown by the decrease of the exchangeable fractions. All these findings are in agreement with those described for the rhizosediment colonized by *H. portulacoides* (Duarte *et al.* 2008), pointing to a similar process independent of the plant coverage. Although the patterns of activity, absolute values of EEA and speciation are very different from those verified for *H. portulacoides* rhizosediment, the inherent mechanism seems to be the same. This supports the important role of microbial processes not only in the sediment biogeochemistry but also in metal speciation processes. This is a very important process for consideration within the entire ecosystem.

Conclusion

Organic matter is an important and effective sink of heavy metals in the sediments, providing a large source of strong ligands for these elements. All the processes of breakdown or modification that affect sediment organic matter will consequently affect the bonded metals, changing their speciation. It is very important to take these biochemical processes into consideration. Changing metal speciation means that metal availability and mobility are also altered, influencing their effect on the ecosystem community. These processes should also be taken in account when considering bioremediation processes. Stimulating or inhibiting some extracellular enzymes can lead, for example, to the leakage of metals, diminishing their concentration in the sediment.

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References

Acosta-Martínez V., Cruz L., Sotomayor-Ramírez D., Pérez-Alegria L. (2007) Enzyme activities as affected by soil properties and land use in a tropical watershed. *Applied Soil Ecology: A Section of Agriculture, Ecosystems & Environment*, **35**, 36–45.

Adani F., Ricca G., Tambone F., Genevini P. (2006) Isolation of the stable fraction (the core) of the humic acid. *Chemosphere*, **65**, 1300–1307.

Benner R., Fogel M., Sprague E. (1991) Diagenesis of belowground biomass of Spartina alterniflora in salt-marsh sediments. *Limnology and Oceanography*, **36**, 1358–1374.

Brüchert V., Pratt L. (1996) Contemporaneous early diagenetic formation of organic and inorganic sulfur in estuarine sediments from St. Andrew Bay, Florida, USA. *Geochimica et Cosmochimica Acta*, **60**, 2325–2332.

Caçador I., Vale C., Catarino F. (2000) Seasonal variation of Zn, Pb, Cu and Cd concentrations in the root-sediment system of *Spartina maritima* and *Halimione portulacoides* from Tagus estuary salt marshes. *Marine Environmental Research*, **49**, 279–290.

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.

Doyle M., Otte M. (1997) Organism-induced accumulation of Fe, Zn and AS in wetland soils. *Environmental Pollution (Barking, Essex: 1987)*, **96**, 1–11.

Duarte B., Delgado M., Caçador I. (2007) The role of citric acid in cadmium and nickel uptake and translocation, in *Halimione portulacoides*. *Chemosphere*, **69**, 836–840.

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.

Folin O., Ciocalteau V. (1927) On tyrosine and tryptophane determination in proteins. *Journal of Biological Chemistry*, **73**, 424–427.

Gadd G. (2001) Accumulation and transformation of metals by microorganisms. In: Rehm H.-J., Reed G., Puhler A.,

Stadler P. (Eds), *Biotechnology, a Multi-volume Comprehensive Treatise, Volume 10: Special Processes*. Wiley-VCH Verlag, Weinheim, Germany: 225–264.

Gadd G. (2004) Microbial influence on metal mobility and application for bioremediation. *Geoderma*, **122**, 109–119.

Hullebusch E., Utomo S., Zandvoort M., Lens P. (2005) Comparison of three sequential extraction procedures to describe metal fractioning in anaerobic granular sludges. *Talanta*, **65**, 549–558.

Isermeyer H. (1952) Eine einfache Method zur Bestimmung der Bodenatmug und der Kerbonate im Boden. In: Alef K., Nannipieri P. (Eds), *Methods in Applied Soil Microbiology and Biochemistry*. Academic Press, London: 26–38.

Johnsen A., Jacobsen O. (2008) A quick and sensitive method for the quantification of peroxidase activity of organic surface soil from forests. *Soil Biology and Biochemistry*, **40**, 814–821.

Kang H., Freeman C., Park S., Chun J. (2005) *N*-Acetylglucosaminidase activities in wetlands: a global survey. *Hydrobiologia*, **532**, 103–110.

Ladd J.N., Brisbane P.G., Butler J.H.A. (1976) Studies on soil fumigation. 3. Effects on enzyme-activities, bacterial numbers and extractable ninhydrin reactive compounds. *Soil Biology and Biochemistry*, **8**, 255–260.

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

Mucha A., Almeida C., Bordalo A., Vasconcelos M. (2005) Exudation of organic acids by a marsh plant and implications on trace metal availability in the rizosphere of estuarine sediments. *Estuarine, Coastal and Shelf Science*, **65**, 191–198.

Oyekola O., Pletschke B. (2006) ATP-sulphurylase: an enzymatic marker for sulphate reduction? *Soil Biology and Biochemistry*, **38**, 3511–3515.

Passier H., Böttcher M., De Lange G. (1999) Sulphur enrichment in organic matter of eastern Mediterranean sapropels: a study of sulphur isotope partitioning. *Aquatic Geochemistry*, **5**, 99–118.

Pereira P., Caçador I., Vale C., Caetano M., Costa A. (2007) Decomposition of belowground litter and metal dynamics in salt marshes (Tagus Estuary, Portugal). *Science of the Total Environment*, **380**, 93–101.

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**(2B), 465–474.

Reboreda R., Caçador I. (2007) Copper, zinc and lead speciation in salt marsh sediments colonised by *Halimione portulacoides* and *Spartina maritima*. *Chemosphere*, **69**, 1655–1661.

Reboreda R., Caçador I. (2008) Enzymatic activity in the rhizosphere of Spartina maritima: potential contribution for phytoremediation of metals. *Marine Environmental Research*, **65**, 77–84.

Richert M., Saarnio S., Juutinen S., Silvola J., Augustin J., Merbach W. (2000) Distribution of assimilated carbon in the system *Phragmites australis*-waterlogged peat soil after carbon-14 pulse labeling. *Biology and Fertility of Soils*, **32**, 1–7.

Tabak H., Lens P., Hullebush E., Dejonghe W. (2005) Developments in bioremediation of soils and sediments polluted with metals and radionuclides. – 1. Microbial processes and mechanisms affecting bioremediation of metal contamination and influencing metal toxicity and transport. *Reviews in Environmental Science and BioTechnology*, **4**, 115–156.

Tessier A. (1979) Sequential extraction procedure for the speciation of particulate trace metals. *Analytical Chemistry*, **51**, 844–851.

Tobias C.R., Macko S.A., Anderson I.C., Canuel E.A. (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., Teal J., Allen S., Van Etten R., Goehringer D., Volkmann S. (1985) Decomposition in salt marsh ecosystems: the phases and major factors affecting disappearance of above-ground organic matter. *Journal of Experimental Marine Biology and Ecology*, **89**, 29–54.

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: 39–57.

Waterman P., Molle S. (1994) *Analysis of Phenolic Plant Metabolites*. Blackwell, Oxford.

Weis J., Weis P. (2004) Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. *Environment International*, **30**, 685–700.

Wilczek S., Fischer H., Pusch M. (2005) Regulation and seasonal dynamics of extracellular enzymatic activities in sediments of a large lowland river. *Microbial Ecology*, **50**, 253–267.

Wilson J., Buchsbaum R., Valiela I., Swain T. (1986) Decomposition in salt marsh ecosystems: phenolic dynamics during decay of litter of Spartina alterniflora. *Marine Ecology Progress Series*, **29**, 177–187.

Yang R., Tang J., Chen X., Hu S. (2007) Effects of coexisting plant species on soil microbes and soil enzymes in metal lead contaminated soils. *Applied Soil Ecology*, **37**, 240–246.

Zawislanski P., Chau S., Mountford H., Wong H., Sears T. (2001) Accumulation of selenium and trace metals on plant litter in a tidal marsh. *Estuarine, Coastal and Shelf Science*, **52**, 589–603.