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Enzymatic Activity in the Rhizosphere of *Spartina maritima*: Potential Contribution for Phytoremediation of metals

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1 Abstract

2	Extracellular Enzymatic Activity (EEA) of five enzymes (peroxidase, phenol
3	oxidase, β -glucosidase, β -N-acetylglucosaminidase and acid phosphatase) was analysed
4	in sediments colonised by Spartina maritima in two salt marshes (Rosário and Pancas)
5	of the Tagus estuary (Portugal) with different characteristics such as sediment
6	parameters and metal contaminant levels. Our aim was a better understanding of the
7	influence of the halophyte on microbial activity in the rhizosphere under different site
8	conditions, and its potential consequences for metal cycling and phytoremediation in
9	salt marshes. Acid phosphatase and β -N-acetylglucosaminidase presented significantly
10	higher EEA in Rosário than in Pancas, whereas the opposite occurred for peroxidase.
11	This was mainly attributed to differences in organic matter between the two sites. A
12	positive correlation between root biomass and EEA of hydrolases (β -glucosidase, β -N-
13	acetylglucosaminidase and acid phosphatase) was found, indicating a possible influence
14	of the halophyte in sediment microbial function. This would potentially affect metal
15	cycling in the rhizosphere through microbial reactions.
16	
17	Keywords: enzyme activity, halophytes, metals, phytoremediation, salt marsh, Spartina
18	<u>maritima</u> .
19	

20 Abbreviations: EEA (extracellular enzyme activity), β-N-acetyl-glucosaminidase
21 (chitobiase)

1. Introduction

2

1

Salt marshes usually receive high inputs of contaminants coming from nearby
urban areas, river transport and tidal inundation. They are considered to act as sinks for
contaminants, namely heavy metals (review by Williams et al., 1994; Doyle and Otte,
1997). Metals accumulate mainly in the sediments, especially in vegetated areas of the
marsh (Caçador et al., 1996).

8 It has been shown that halophytes have a dominant role in the metal cycling in salt marshes (Otte, 1991; Caçador et al., 2000). The roots take up metals, which are 9 10 mainly accumulated in root tissues and only a small fraction is translocated to the above 11 ground parts of the plants (Alberts, 1990). When plants die, litter decomposition leads to 12 the mobilisation of metals and their return to sediment, at a rate that is dependent on the 13 type of tissue but also on plant species (Windham et al., 2003). Apart from direct uptake 14 of metals, wetland plants contribute to metal removal through other important processes 15 such as substrate stabilization, rhizosphere oxidation and supply of organic matter for microorganisms (Kosolapov et al., 2004). 16

17 Microbial activity in sediments is involved in several processes useful for 18 bioremediation of metals, like metal precipitation by sulphate reduction and redox 19 transformations of metals among others (Kosolapov et al., 2004). Wetland plants 20 interact with microorganisms of the rhizosphere by supplying organic matter that is 21 required for such reactions and thus providing an appropriate environment for metal 22 cycling (Jacob and Otte, 2003). For instance, stimulated sulphate reducing bacteria 23 activity caused by <u>S. maritima</u> colonisation was thought to be one of the reasons for 24 variations in metal sulphides in salt marshes of Ría de Ortigueira, Spain (Otero and 25 Macías, 2002). In fact, some phytoremediation strategies involve inoculation of plants 26 with rhizosphere microbiota and arbuscular mycorrhiza (Khan, 2005).

1	Enzymes activities are a useful index of microbial activity (Sinsabaugh, 1994;
2	Ravit et al., 2003). Enzymes can exist extracellularly in sediments, attached to clay
3	minerals or complexed with humic colloids (Burns, 1982). Extracellular enzymes
4	excreted by fungi and bacteria effectively metabolize humic compounds reducing their
5	molecular weight, consequently increasing the solubility and mobility of these potential
6	carriers of heavy metals (Gramss et al., 1999). The enzyme activity assay gives the
7	maximum potential activity of enzymes occurring in sediment rather than the actual
8	enzyme activity (Alef and Nannipieri, 1995).
9	In this paper we compared the EEA (Extracellular Enzymatic Activity) of five
10	enzymes in sediments colonised by Spartina maritima in two salt marshes of the Tagus
11	estuary (Portugal) with marked differences in their characteristics such as sediment
12	parameters and metal contaminant levels (Caçador et al., 1999). Our aim was to
13	compare the possible influence of the plant on the microbial function of the rhizosphere
14	under different site conditions. This would help to elucidate how halophyte-
15	microorganism interactions may contribute for the potential use of halophytes in
16	phytoremediaton of metal contaminated environments.
17	
18	2. Materials and Methods
19	
20	2.1 Site description
21	Between February-March 2005 five sediment cores were collected in areas
22	Colonised by <u>Spartina maritima</u> in each of two salt marshes of the Tagus estuary:
23	Rosário (38° 40'N, 9° 01'W) and Pancas (38° 48'N, 8° 55'W). Rosário is a mature salt
24	marsh (Valiela et al., 2000) with muddy sediments located in the southern part of the
25	estuary, in the vicinity of urbanised and industrial areas. Pancas is a young salt marsh

1	(Valiela et al., 2000) with muddy-sand sediments located in the northeast part of the
2	estuary, within the Tagus Nature Reserve. Distance between these sites is about 18 km.
3	The studied salt marshes contain extensive intertidal mudflat areas colonised by
4	halophyte plant communities dominated by Spartina maritima (Curtis) Fernald,
5	Halimione portulacoides (L.) Aellen, Sarcocornia fruticosa (L.) A. J. Scott and
6	Sarcocornia perennis (Mill.) A. J. Scott. These sites experience two tidal flushing a day
7	(for a more detailed description of the estuary see Caçador et al. (1996)). Rosário
8	presents higher levels of metal contamination than Pancas (Table 1, after Caçador and
9	Vale, 2001).
10	9
11	2.2 Sampling
12	Collection of samples was made at low tide. Sediment was removed using a
13	stainless steel corer with 6.8 cm inner diameter and 50 cm depth. In the field, cores were
14	sliced to separate the following segments: 0-5 cm, 10-15 cm and 40-45 cm. These
15	segments were expected to be representative of surface sediment, sediment with high
16	root biomass and deep sediment with little or inexistent root influence, respectively.
17	Redox potential (Eh) and pH values of fresh sediment were determined in situ using a
18	HANNA pH/mV meter (HI 9025). The samples were placed in plastic bags and
19	transported to the laboratory refrigerated. Those samples for determination of enzymatic
20	activity were frozen at -20°C until enzymatic analysis was carried out (Kuperman and
21	Carreiro, 1997). Root biomass was determined in fresh sediment separating root
22	material from sediment and then drying to constant weight at 70°C (Gross et al., 1991).
23	2.3 Sediment parameters

1	Sediment (~20 g) was dried 48 h at 60°C and relative moisture calculated
2	(percentage of fresh weight). In order to determine organic matter content (LOI, loss on
3	ignition) 2g of dry sediment was burned at 600°C for 2 hours.

4 2.4 Enzyme activities in sediment

5 6	Enzymatic activity of 5 enzymes (two oxidoreductases and three hydrolases)
7	was analysed: phenol oxidase, peroxidase, β -glucosidase, acid phosphatase and β -N-
8	acetylglucosaminidase (chitobiase). These enzymes are involved in metabolic processes
9	such as C cycling (β -glucosidase, catalyzes the final step of cellulose degradation), N
10	and C cycling (β -N-acetylglucosaminidase, hydrolyzes the glucosamine), P cycling
11	(acid phosphatase, hydrolyzes organic-P compounds) and decomposition of plant litter
12	(phenol oxidase, catalyzes polyphenol oxidation; and peroxidase, catalyzes the
13	oxidation of various compounds such as phenols and amines). We used the method
14	described in Ravit et al. (2003) with a modification in the incubation temperature and
15	without dilution of the supernatant. Sediment samples were prepared for analysis adding
16	75 ml of sodium acetate buffer (pH 5) to 5 g fresh sediment. The sample was mixed for
17	1 min to obtain the sediment slurry. The substrates used for β -glucosidase, acid
18	phosphatase and β -N-acetylglucosaminidase (chitobiase) were p-Nitrophenyl- β -D-
19	glucopyranoside, p-Nitrophenyl phosphate and p-Nitrophenyl-N-acetyl-β-D-
20	glucosaminide, respectively. All of them released p-nitrophenol (pNP) when hydrolized.
21	Two millilitres of substrate (5 mM) was added to 2 ml of the slurry in a test tube and
22	incubated at 30°C in gentle agitation for 60 min (β-glucosidase), 30 min (acid
23	phosphatase) and 2 hours (chitobiase). After incubation the samples were centrifuged at
24	2000 rev min ⁻¹ for 15 min and 0.2 ml of 1 N NaOH was added immediately to stop the
25	reaction and cause colour change. Determination of phenol oxidase and peroxidase
26	activities was carried out using L-DOPA (L-3,4-dihydroxyphenylalanine) as substrate

1	(5 mM) following the same procedure: 2 ml slurry + 2 ml substrate (adding 0.1 ml of
2	0.3% H ₂ O ₂ for peroxidase). Incubation time was 60 min for both enzymes, followed by
3	centrifugation. Absorbance of supernatant was read on a TECAN Absorbance
4	Microplate Reader (SPECTRA Rainbow) at 410 nm for pNP enzymes and at 460 nm
5	for L-DOPA enzymes. Absorbance for phenol oxidase was substracted from absorbance
6	of peroxidase total (which assay gives the activity of peroxidase + phenol oxidase) in
7	order to calculate absorbance for peroxidase alone. Controls of sediment slurry and
8	substrate were made, and their absorbance subtracted from the absorbance read for each
9	sample. Enzymatic activity was expressed as <u>μ moles product g dry wt^{-1} h^{-1}.</u>
10	2.5 Statistic analyses
11	
12	Significant differences in soil characteristics between sites were tested using
13	one-way ANOVA. Significant differences in extracellular enzymatic activities between
14	the two sites at the different depths were compared using one-way ANOVA. The same
15	analysis was used to test differences with depth at each site separately. This was
16	followed by a Tukey- HSD test when appropriate. Pearson's coefficient was calculated
17	to test correlation between activities of different enzymes and correlation between
18	enzymes activities and soil characteristics.
19	
20	3. Results and discussion
21	
22	Sediment characteristics (Eh, pH, LOI, relative moisture) and root biomass of S.
23	maritima in Rosário and Pancas are shown in Table 2. Sediment redox potential (Eh)
24	was significantly higher (p<0.05) in Pancas, except on the surface, where Rosário
25	showed significantly higher Eh. Organic matter (LOI) and moisture were significantly
26	higher (p<0.05) in Rosário at every depth studied in areas colonised by <u>S. maritima</u> ,

whereas pH was significantly higher in Rosário only at the root depth (10-15 cm). Root
 biomass of <u>S. maritima</u> was significantly higher in Rosário than in Pancas (p<0.05).

3

4 The extracellular enzymatic activity (EEA) of peroxidase was much higher (up 5 to 10 fold) than the EEA of the other four enzymes at every depth studied (Figure 1). The dominance of peroxidase over hydrolytic enzymes may indicate the presence of 6 7 refractory organic matter in the salt marsh (Sinsabaugh et al., 2003). Gramss et al. 8 (1999) demonstrated that extracellular enzymes released by fungi and bacteria, as well 9 as commercial enzymes including peroxidase and β -glucosidase, were able to 10 effectively metabolize soil humic extracts (to which heavy metal ions and xenobiotics 11 are potentially attached), reducing their molecular weight and consequently increasing 12 their solubility and motility. Therefore, high extracellular activity of peroxidase in 13 sediments colonised by S. maritima might influence the mobilization of metals in the 14 studied salt marshes. In addition to supplying plant litter for decomposition, the halophyte may contribute to high EEA of peroxidase by the release of peroxidases from 15 16 plant roots (Salt et al., 1998). However, it should be taken into account that the EEA 17 assay gives the maximum potential activity of enzymes occurring in sediment rather 18 than the actual enzyme activity (Alef and Nannipieri, 1995).

19

When comparing EEA's in sediments colonised by <u>S. maritima</u> in Rosário and Pancas, significant differences were observed for peroxidase, acid phosphatase and chitobiase (p<0.05) (Figure 1). Peroxidase presented higher EEA in Pancas than in Rosário, whereas acid phosphatase and chitobiase presented higher EEA in Rosário than in Pancas. Correlation coefficients between EEA's and sediment characteristics revealed that acid phosphatase and chitobiase activities were positively correlated with organic matter content and moisture (Table 3). In addition, chitobiase activity was positively

1	correlated with pH. A negative correlation occurred between EEA of peroxidase and
2	organic matter content. These results suggest that differences in the organic matter
3	content between the two sites could partly explain the observed pattern in extracellular
4	activities of the enzymes in areas colonised by S. maritima. Higher organic matter
5	content and moisture in sediment from Rosário seemed to be related to higher EEA's of
6	acid phosphatase and chitobiase (also influenced by higher pH) in this salt marsh,
7	whereas peroxidase activity was higher in Pancas, where organic matter content of the
8	sediment was lower. Results from Caçador et al. (2004) showed that litter
9	decomposition in Pancas was faster than in Corroios, another salt marsh with
10	characteristics similar to Rosário (located about 12 Km to the southwest from this)
11	(Caçador et al., 1999). This may be related to higher activities of peroxidase in Pancas,
12	as it is involved in the degradation of complex organic compounds. There was no
13	correlation between EEA's and redox potential of the sediment. This was surprising,
14	especially for phenol oxidase, that needs O_2 for its activity. An oxygen limitation on
15	phenol oxidase is associated with inhibition of hydrolytic enzymes in peatlands through
16	accumulation of phenolic compounds (Freeman et al, 2001; Freeman et al. 2004). The
17	low EEA observed for phenol oxidase (in the same order of magnitude as the
18	hydrolases) is likely to be caused by O ₂ limitation in the sediment, since it is expected to
19	be scarce in salt marsh sediments. Although no reductive conditions were found in the
20	sediments studied (positive Eh) it should be taken into account that it does not indicate
21	abundance of O ₂ , as other oxidant compounds could be present. Accumulation of
22	phenolic compounds due to restricted phenol oxidase activity would have important
23	consequences for the cycling of heavy metals in the salt marsh, since phenols are known
24	to bind metals (Ambrosi et al., 2003; Mota et al., 2005).

1	Hydrolytic enzymes activities (β-glucosidase, acid phosphatase and chitobiase)
2	were positively correlated with root biomass of S. maritima (Table 3). Microbial
3	activity is stimulated around plant roots through exudation of organic anions and sugars
4	(Ryan et al., 2001). Karjalainen et al. (2001) found that Lobelia dortmanna L., a
5	submersed plant species, enhanced microbial activity and biomass in sediments through
6	organic carbon release. Thus, it would be reasonable that higher root biomass would
7	result in higher exudation of organic compounds, enhancing microbial activity around
8	plant roots. Burke et al. (2002) found a positive interaction between a group of
9	<u>Protobacteria</u> and root biomass of <u>Spartina patens</u> and <u>Phragmites australis</u> in salt
10	marsh sediments. The release of soluble sugars by roots supply the substrates needed by
11	β -glucosidase and chitobiase (β -N-acetylglucosaminidase), probably inducing the
12	excretion of higher amounts of these enzymes by microorganisms (Burns, 1982). In
13	spite of this, β -glucosidase activity was not significantly higher in sediment from
14	Rosário, where <i>S. maritima</i> root biomass was significantly higher (Figure 1; Table 3).
15	This might be caused by an inhibition of this enzyme in the presence of higher metal
16	concentrations in Rosário (Table 1) (Geiger et al., 1998). Exudation of phosphatases
17	from plant roots is one of the strategies used by plants to cope with P deficiency (Ryan
18	et al., 2001). This might be a strategy of S. maritima as well, given the correlation found
19	between acid phosphatase activity and root biomass of the plant (Table 3). Ravit (2005)
20	concluded that, in salt marsh environments of the USA, the sediment microbial
21	community structure and function is determined by the interaction of hydrological
22	conditions and the effect of plant roots. From the observed results we deduce that S.
23	maritima roots could influence the extracellular enzymatic activity of the hydrolytic
24	enzymes analysed and, accordingly, it may affect the microbial function in the
25	rhizosphere of the plant in Tagus estuary salt marshes. Given the importance of

1	microbially mediated reactions in metal cycling around plant roots in wetlands (Jacob
2	and Otte, 2003), and the proposed use of halophytes like S. maritima for
3	phytostabilization of metals in metal contaminated salt marshes (Reboreda and Caçador,
4	2007; Reboreda et al., In press), the halophyte-microorganism interactions arise as an
5	additional concern for phytoremediation studies.
6	
7	Variations in EEA with depth were only significant (p<0.05) for β -glucosidase
8	and acid phosphatase from Rosário. Still, enzymatic activities were in general lower in
9	the horizon 40-45 cm, most likely as a consequence of low organic matter content and
10	oxygen availability (Table 2). In Pancas, the lack of significant differences in EEA with
11	depth (p>0.05) was surprising considering the 2-orders of magnitude change in root
12	biomass and organic matter (Table 2). Other sediment parameters such as grain size
13	(muddy-sand in Pancas; Reboreda et al., in press) may have a stronger effect on
14	enzymes distribution (Kandeler et al., 2000).
15	In Rosário, EEA's of β -glucosidase and chitobiase were positively correlated
16	with EEA of phenol oxidase (Table 4). This might suggest a similar behaviour of these
17	enzymes, in organic matter rich/oxygen limited salt marshes, as that found by Freeman
18	et al. (2001) and Freeman et al. (2004) in peatlands, which was described above. EEA

- 19 of β -glucosidase also showed correlation with peroxidase activity. There were no
- 20 significant correlations between EEA's of the studied enzymes in Pancas.
- 21
- 22 **4. Conclusions**

Sediments colonised by *S. maritima* show differences in some extracellular
 enzymes activities between salt marshes in the Tagus estuary, which appears to be
 related to differences in sediment organic matter and root biomass. The results support

the idea that *S. maritima* could influence to some extent the microbial function in the
 rhizosphere, which in turn may potentially influence microbially mediated metal cycling
 in the sediment. In addition, site characteristics seem to play a relevant role in
 enzymatic activity as well, given the differences observed between the two sites.

5 Consequently, we suggest that halophyte-microorganism interactions are worthy of

6 further study for its usefulness in phytoremediation strategies.

7

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1 2 **Figure caption**

- 3
- Figure 1. Extracelullar enzymatic activities (EEA) (μ moles h⁻¹ g dry weight⁻¹) in three 4
- 5 depths of sediment at Rosário and Pancas sites colonised by Spartina maritima
- Accepter 6 (mean±SD). *Significant differences (p<0.05) between sites.



1 **Table 1.** Concentrations (mean \pm standard deviation) of Zn, Pb, Cu and Cd (μ g g⁻¹ dry 2 weight) in surface sediments (0-5 cm) ; intermediate rooted sediment layers (10-15 cm)

3 and deeper layers (40-45 cm) in areas colonised by Spartina maritima, from Pancas and

4 Rosário salt marshes (after Caçador & Vale, 2001)

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<u>Cu µg g⁻¹ dw</u> <u>Pb</u>μg g⁻¹dw Zn µg g⁻¹ dw $Cd \mu g g^{-1} dw$ Depth (cm) Sites (0-5)95<u>+</u>17 42<u>+</u>4 22+3 1.1<u>+</u>0.2 Pancas (10-15) 177<u>+</u>18 53<u>+</u>4 33<u>+</u>6 1.3<u>+</u>0.1 (40-45)93<u>+</u>5 43<u>+</u>9 14.7<u>+</u>3 0.4 <u>+</u> 0.1 1.2 <u>+</u>0.2 395 <u>+</u>16 51+2 (0-5) 402<u>+</u> 27 Rosário 78<u>+</u> 15 116<u>9+</u>74 (10-15) 577<u>+</u>21 1.7 <u>+</u>0.2 98<u>+</u> 10 55.3<u>+</u>11 (40-45) 12.7<u>+</u> 3 0.4 <u>+</u>0.1 8

1 Table 2. Eh (mV), pH, organic matter (Loss on Ignition, %), moisture (%) and root

- biomass (g m⁻²) in sediments from Rosário and Pancas colonised by S. maritima at three 2
- 3 sediment horizons (mean ± SD).
- 4

	Depth	Eh (mV)	pH (14°C)	LOI	Moisture (%)	Biomass (g m ⁻²)
	0-5 cm	84.0±24.7*	5.9±0.5	10.3±0.6*	52.3±0.7*	264.6±26.3*
Pancas	10-15 cm	80.9±23.2*	5.9±0.4*	8.0±0.1*	50.7±1.7*	681.0±9.6*
	40-45 cm	76.3±11.9*	5.5±1.3	0.6±0.7*	16.4±0.6*	9.6±3.0*
	0-5 cm	107.4±23.0*	5.5±0.1	19.0±5.9*	64.1±5.0*	798.8±50.2*
Rosário	10-15 cm	29.0±7.0*	6.7±0.2*	14.5±2.0*	60.0±2.8*	1305.0±124.3*
	40-45 cm	20.0±6.8*	5.8±0.1	11.8±2.1*	49.7±8.5*	31.8±6.0*

5 *Significant differences between sites (one-way ANOVA, p<0.05). MANUS

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- 1 Table 3. Correlation coefficients (Pearson's) between sediment characteristics and
- 2 extracellular enzymatic activities of phenol oxidase, peroxidase, β-glucosidase, acid
- 3 phosphatase and chitobiase in salt marsh sediments colonised by Spartina maritima.

				1	
	Eh (mV)	pН	LOI (%)	Moisture (%)	Biomass (g m ⁻²)
Phenol ox.	0.48	-0.01	-0.38	-0.17	-0.13
Peroxidase	0.46	0.00	-0.54*	-0.31	-0.11
β-glucosidase	0.32	0.46	0.26	0.31	0.86**
Phosphatase	0.47	-0.05	0.70**	0.51*	0.58*
Chitobiase	-0.17	0.57*	0.69*	0.59*	0.88**

* Correlation is significant at the 0.05 level. 4

MANUS 5 ** Correlation is significant at the 0.01 level.

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- Table 4. Correlation between the activities of phenol oxidase, peroxidase, β -1
- 2 glucosidase, acid phosphatase and chitobiase in sediments of Rosário and Pancas
- 3 colonised by S. maritima (Pearson's Correlation).
- 4

		Enzyme	Phenol ox.	Peroxidase	β-gluc	Phosphatase	Chitobiase
		Phenol ox.					
	io	Peroxidase	0.454				
	osáı	β-glucosidase	0.591*	0.650*			\mathcal{O}
	Ŗ	Phosphatase	0.211	0.198	0.398		
		Chitobiase	0.707**	0.473	0.796**	0.530	
		Phenol ox.					
	as	Peroxidase	0.497				
	anc	β-glucosidase	0.089	-0.230			
	Р	Phosphatase	0.025	0.192	-0.046	6	
-		Chitobiase	-0.079	0.328	0.204	0.033	
5	* Co	rrelation is signific	cant at the 0.05	ilevel.		$\langle \rangle$	
7	*** C	orrelation is signif	icant at the 0.0	n level.			
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