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

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- 7 **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 *maritima*.

19
20 Abbreviations: EEA (extracellular enzyme activity), β -N-acetyl-glucosaminidase
21 (chitobiase)

1 1. Introduction

2
3 Salt marshes usually receive high inputs of contaminants coming from nearby
4 urban areas, river transport and tidal inundation. They are considered to act as sinks for
5 contaminants, namely heavy metals (review by Williams et al., 1994; Doyle and Otte,
6 1997). Metals accumulate mainly in the sediments, especially in vegetated areas of the
7 marsh (Caçador et al., 1996).

8 It has been shown that halophytes have a dominant role in the metal cycling in
9 salt marshes (Otte, 1991; Caçador et al., 2000). The roots take up metals, which are
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
16 microorganisms (Kosolapov et al., 2004).

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 *S. maritima* 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 phytoremediation of metal contaminated environments.

18 2. Materials and Methods

20 2.1 Site description

21 Between February-March 2005 five sediment cores were collected in areas
22 colonised by *Spartina maritima* 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

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

24

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 hydrolyzed.
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% H₂O₂ 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 subtracted 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 $\mu\text{moles product g dry wt}^{-1} \text{ h}^{-1}$.

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 *S. maritima*,

1 whereas pH was significantly higher in Rosário only at the root depth (10-15 cm). Root
2 biomass of *S. maritima* 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).
6 The dominance of peroxidase over hydrolytic enzymes may indicate the presence of
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
15 halophyte may contribute to high EEA of peroxidase by the release of peroxidases from
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

20 When comparing EEA's in sediments colonised by *S. maritima* in Rosário and
21 Pancas, significant differences were observed for peroxidase, acid phosphatase and
22 chitinase ($p < 0.05$) (Figure 1). Peroxidase presented higher EEA in Pancas than in
23 Rosário, whereas acid phosphatase and chitinase presented higher EEA in Rosário than
24 in Pancas. Correlation coefficients between EEA's and sediment characteristics revealed
25 that acid phosphatase and chitinase activities were positively correlated with organic
26 matter content and moisture (Table 3). In addition, chitinase 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 chitinase (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 O₂ 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 O₂ 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 O₂, 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).

25

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 *Protobacteria* and root biomass of *Spartina patens* and *Phragmites australis* 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 *S. maritima* 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.

22 **4. Conclusions**

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

1 the idea that *S. maritima* could influence to some extent the microbial function in the
2 rhizosphere, which in turn may potentially influence microbially mediated metal cycling
3 in the sediment. In addition, site characteristics seem to play a relevant role in
4 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

4 **Figure 1.** Extracellular enzymatic activities (EEA) ($\mu\text{moles h}^{-1} \text{g dry weight}^{-1}$) in three
5 depths of sediment at Rosário and Pancas sites colonised by *Spartina maritima*
6 (mean \pm SD). *Significant differences ($p<0.05$) between sites.

7

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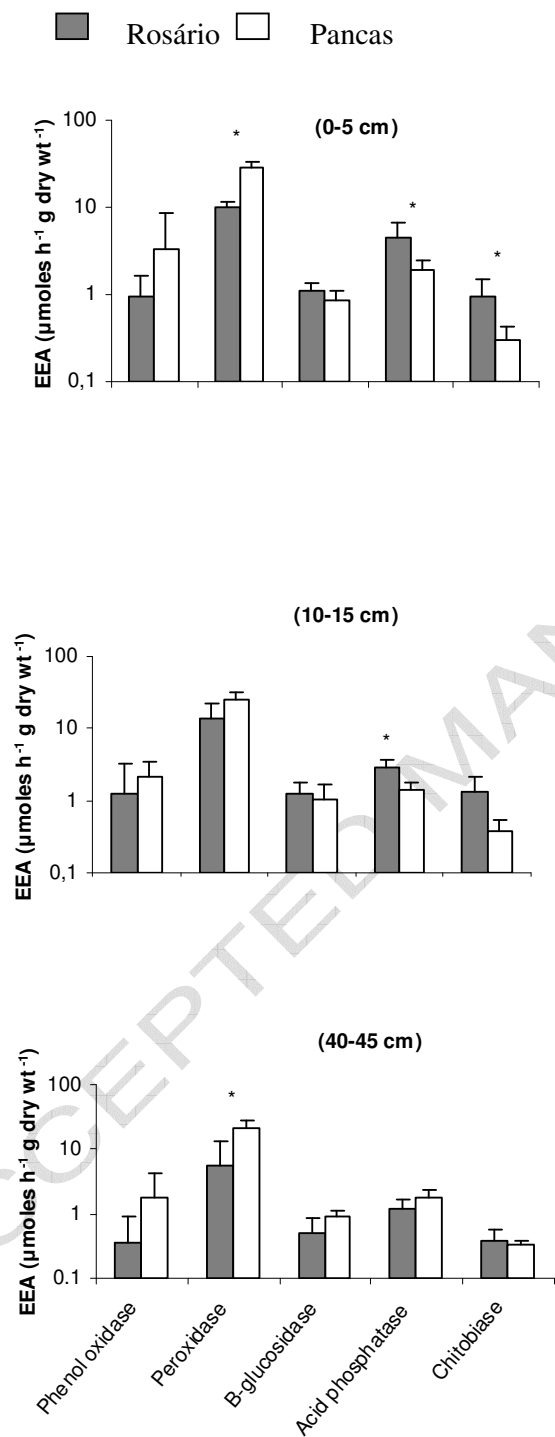
1 **Figure 1**

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1 **Table 1.** Concentrations (mean \pm standard deviation) of Zn, Pb, Cu and Cd ($\mu\text{g g}^{-1}$ 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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Sites	Depth (cm)	Zn $\mu\text{g g}^{-1}$ dw	Pb $\mu\text{g g}^{-1}$ dw	Cu $\mu\text{g g}^{-1}$ dw	Cd $\mu\text{g g}^{-1}$ dw
Pancas	(0-5)	95 \pm 17	42 \pm 4	22 \pm 3	1.1 \pm 0.2
	(10-15)	177 \pm 18	53 \pm 4	33 \pm 6	1.3 \pm 0.1
	(40-45)	93 \pm 5	43 \pm 9	14.7 \pm 3	0.4 \pm 0.1
Rosário	(0-5)	402 \pm 27	395 \pm 16	51 \pm 2	1.2 \pm 0.2
	(10-15)	1169 \pm 74	577 \pm 21	78 \pm 15	1.7 \pm 0.2
	(40-45)	98 \pm 10	55.3 \pm 11	12.7 \pm 3	0.4 \pm 0.1

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1 **Table 2.** Eh (mV), pH, organic matter (Loss on Ignition, %), moisture (%) and root
 2 biomass (g m^{-2}) in sediments from Rosário and Pancas colonised by *S. maritima* at three
 3 sediment horizons (mean \pm SD).

4

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

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

6

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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*.

	Eh (mV)	pH	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**

- 4 * Correlation is significant at the 0.05 level.
 5 ** Correlation is significant at the 0.01 level.

1 **Table 4.** Correlation between the activities of phenol oxidase, peroxidase, β -
 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
Rosário	Phenol ox.					
	Peroxidase	0.454				
	β -glucosidase	0.591*	0.650*			
	Phosphatase	0.211	0.198	0.398		
	Chitobiase	0.707**	0.473	0.796**	0.530	
Pancas	Phenol ox.					
	Peroxidase	0.497				
	β -glucosidase	0.089	-0.230			
	Phosphatase	0.025	0.192	-0.046		
	Chitobiase	-0.079	0.328	0.204	0.033	

5 * Correlation is significant at the 0.05 level.

6 ** Correlation is significant at the 0.01 level.

7