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Denitrification: an ecosystem service provided by salt marshes

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ABSTRACT: We hypothesized that denitrification rates, as an N removal process, would be enhanced in salt marsh rhizosediments as compared to sediments without vegetation (bare mudflats). Denitrification rates (measured by the ^{15}N-isotope pairing technique), potential nitrification, and nutrient fluxes were seasonally quantified in a *Spartina maritima* salt marsh and in adjacent bare mudflats. Potential nitrification rates were significantly higher in autumn and winter, but there were no significant differences between the bare mudflats and *S. maritima* vegetated sediment. Seasonally, denitrification rates in vegetated sediments under dark conditions were significantly higher in winter (676 ± 497 µmol N_2 m^{-2} h^{-1}, mean ± SD), whereas bare mudflats showed a maximum rate of 151 ± 24 µmol N_2 m^{-2} h^{-1} in summer. The high denitrification rates recorded in winter may be due to many abiotic and biotic factors, namely higher potential nitrification and nitrate availability in the water column, lower competition for nitrogen within the sediment, and less competition between plants, microphytobenthos, and nitrifiers, especially in dark conditions. Hence, during winter, there was a higher contribution of *S. maritima* marshes to N removal through denitrification, highlighting the role of the marshes in this ecosystem service. As a whole, considering the seasonal variations of the studied processes, it cannot be concluded whether or not annual denitrification was significantly different between the vegetated sediment and the bare mudflats.

KEY WORDS: Denitrification · Salt marshes · Ecosystem services · Eutrophication · *Spartina maritima* · ^{15}N-isotope pairing technique

INTRODUCTION

Salt marshes provide diverse ecosystem services that have been evaluated as being some of the most valuable services for humanity (Costanza et al. 1997, Wieski et al. 2010). These services and human benefits include disturbance regulation (e.g. shoreline erosion protection), waste treatment (e.g. nutrient removal and transformation, denitrification, and nutrient retention), recreation (e.g. bird watching), and productivity (e.g. primary and secondary production, including plant biomass production as a source of organic matter and nutrients, and fish production through fishing activities and aquaculture) (Boorman 2003, Gedan et al. 2009, Wieski et al. 2010).

Salt marshes are classified as sensitive habitats under the European Habitats Directive (Best et al. 2007). The reduction in salt marsh areas worldwide as a result of anthropogenic and natural disturbances, namely through habitat disruption and frag-

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mentation, pollution, climate change, storm events, and coastal development, is of major concern, and several studies on the ecology of estuaries have emphasized the negative consequences of its disappearance (e.g. Valiela et al. 2000, Boorman 2003, Best et al. 2007, Jin et al. 2007, Simas & Ferreira 2007, Gedan et al. 2009, Green et al. 2009).

Estuarine eutrophication occurs all over the world (Nixon 1995, Valiela et al. 2000, Lillebø et al. 2005, Dugdale et al. 2007) as a result of water column nutrient enrichment by landscape runoff, which in turn is frequently the result of anthropogenic activities (Vitousek et al. 1997, Hauxwell & Valiela 2004). In marine and estuarine systems, nitrogen (N) is frequently the limiting nutrient to primary production (Nixon 1981, Vitousek & Howarth 1991), and in the last few decades this nutrient has been recognized as being the major cause of eutrophication in coastal ecosystems (Howarth & Marino 2006). Thus, the increase in N loading in estuaries causes algal blooms and shifts in the primary producers (phytoplankton, macroalgae, and seagrasses) (Hauxwell & Valiela 2004, Howarth & Marino 2006). This emphasizes the importance of studying the N cycle.

Salt marshes are very important as N sinks through plant biomass production (i.e. the incorporation of N in standing biomass, detritus, litter, and sediments) (Edwards & Mills 2005, Caçador et al. 2007, Sousa et al. 2008) and denitrification (e.g. Teal & Howes 2000, Valiela & Cole 2002). These processes may contribute to counteract eutrophication in coastal areas (Seitzinger 1988). In fact, most of the land-derived nitrogen that loads to coastal environments, in non-human-impacted environments, could be denitrified in estuarine and shelf regions (Galloway 1998).

Even though anammox (anaerobic ammonium oxidation) contributes to N removal in aquatic ecosystems (Trimmer et al. 2003), denitrification seems to be the most significant process that produces N_2 in estuaries (Schlesinger 1997, Jaffe 2000). Accordingly, the organic enrichment of sediments seems to increase denitrification to a greater extent than anammox, since the latter process greatly depends on water depth (much less in estuaries than in marine waters) and mineralization rates (deeper water columns show lower organic enrichment) (Dalsgaard et al. 2005). For example, in the Thames estuary, anammox contributed to less than 10% of N_2 production (Trimmer et al. 2003), while Risgaard-Petersen et al. (2004b) reported that, compared to marine deep sea sediments (where anammox represented 30 to 70% of N_2 production), N_2 production by anammox represented 5 to 24% of total N_2 production in estuaries.

Denitrification in aquatic ecosystems depends on many physical, chemical, and biological factors. Nitrate concentration, availability of easily degradable organic carbon, oxygen availability, temperature, light, and water retention time are some of the physical-chemical factors influencing denitrification rates in these ecosystems (Thompson 1995, Cornwell et al. 1999, Piña-Ochoa & Álvarez-Cobelas 2006, Silvennoinen et al. 2008). Indirectly, sulfide concentration may also affect denitrification: for sulfide concentrations within a certain range, nitrification is suppressed, which consequently affects denitrification (Seitzinger 1988). In addition, biological factors such as plant roots, fauna (through bioturbation and bio-irrigation), and microbiological abundance and activity may affect denitrification. Plants can influence denitrification rates (Reddy et al. 1989, Howarth et al. 1996, Cornwell et al. 1999, Eriksson et al. 2003, Piña-Ochoa & Álvarez-Cobelas 2006, Koop-Jacobsen & Giblin 2009) due to O_2 diffusion through the aerenchyma (Cartaxana & Lloyd 1999, Maricle & Lee 2002) and the creation of oxic micro-zones surrounding the roots and rhizomes (the rhizosphere) at a certain depth in the sediment, which enhances coupled nitrification-denitrification. *Spartina maritima* also creates a more oxidized rhizosphere, which enhances sulfide oxidation and contributes to sulfide detoxification (Madureira et al. 1997). Moreover, in the presence of reduced sulfur forms (H_2S, S^{2-}, S), which act as electron donors, denitrification coupled to sulfur-oxidation occurs (Burgin & Hamilton 2007), thus contributing to nitrate removal in these sediments. It has been shown that bioturbation by benthic macrofauna significantly stimulates *in situ* sediment denitrification, which is associated with the sediment layer where infauna is more active (Gilbert et al. 1998). Denitrification rates can also be influenced by plants and microphytobenthos (MPB), which compete with microbial denitrifier communities for substrate (nitrate).

Nitrification (the microbial aerobic oxidation of NH_4^+ and NO_2^- to NO_3^-) is an important step in the nitrogen cycle, occurring in oxic surface sediments. Since many biotic and abiotic factors can influence potential nitrification (e.g. plant roots, fauna abundance, activity of nitrifying bacteria, temperature, oxygen penetration, and NH_4^+ concentration; Henriksen et al. 1981), it is important to quantify potential nitrification rates, which can be regarded as a proxy for the abundance of active nitrifiers (Risgaard-Petersen et al. 2004a). The product of this process (NO_3^-) is later denitrified (coupled nitrification-denitrification [D_n]) and can also diffuse from the sediment into the water column. Several studies have been performed

in order to quantify denitrification in different aquatic ecosystems, namely freshwater tidal marshes (Seitzinger 1988, Cornwell et al. 1999), estuaries, rivers, lakes, coastal waters (Steingruber et al. 2001, Piña-Ochoa & Álvarez-Cobelas 2006), and wetlands (Merrill & Cornwell 2000, Risgaard-Petersen 2003, Trimmer et al. 2003, Sundbäck et al. 2006). However, regarding denitrification in estuaries, there are only few studies concerning mudflats (e.g. Cabrita & Brotas 2000, Risgaard-Petersen 2003, Sundbäck et al. 2006) and even fewer concerning salt marshes (Valiela & Teal 1979, Koch et al. 1992, White & Howes 1994, Eriksson et al. 2003, Poulin et al. 2007). In these studies, different techniques were applied and therefore restrict comparisons between systems.

The present study aimed to evaluate the role of *Spartina maritima* salt marshes in the denitrification process, as a service provided by the ecosystem. In order to do so, we hypothesized that: (1) the rhizosphere environment may enhance nitrification and denitrification; and (2) in eutrophic systems, where nutrients are not limiting, marsh plants and bacteria do not compete for resources. Thus, we hypothesized that, compared to sediments without vegetation (bare mudflats), salt marsh rhizosediments enhance N_2 removal.

MATERIALS AND METHODS

Sampling site and procedure

Sampling took place in the Tagus estuary, located in the southern European Atlantic margin (Portugal) (38° 40′ 10″ N, 9° 00′ 13″ W). The Tagus estuary is one of Europe's largest estuaries (320 km^2), classified by the Convention of Wetlands as a Ramsar site. It is characterized by water temperatures ranging between 20 and 26°C in summer and 8 and 18°C in winter (Gameiro et al. 2007). Water column dissolved inorganic nitrogen (DIN) concentrations for the period 1999 to 2005 varied seasonally between 27 ± 19 µmol l^{-1} (mean ± SD) in summer and 84 ± 33 µmol l^{-1} in winter; the PO_4-P concentrations varied between 3.4 ± 1.1 µmol l^{-1} during winter and 4.5 ± 3.0 µmol l^{-1} in autumn (Gameiro et al. 2007).

Spartina maritima (Curt.) Fernald is an herbaceous perennial plant that colonizes estuarine intertidal mudflats and is distributed throughout the coasts of western, southern, and southeastern Europe, as well as in western Africa. It is one of the most common halophytes colonizing salt marshes in the Tagus estuary, which has 20 km^2 of salt marsh vegetation (Simas et al. 2001). *Spartina maritima* is the dominant species in the lower marsh, with an area covering 675 ha, which represents one-third of the total marsh area (Simas et al. 2001, Reboreda & Caçador 2007). It is described as a pioneer species, tolerating high salinity and long flooding conditions common in low marshes. In this system, the aboveground biomass of *S. maritima* is 0.60 ± 0.02 kg DW m^{-2}, while the belowground biomass is 3.60 ± 0.15 kg DW m^{-2} (Reboreda & Caçador 2007).

A seasonal study was performed from autumn 2007 to summer 2008. Sampling was carried out during spring tides at low tide. Ten sediment cores were collected in the *Spartina maritima* salt marsh (each core containing 1 or 2 shoots of *S. maritima*; the inter-core plant biomass was as similar as possible) using a Plexiglass core (∅ = 8 cm; 30 cm height). Each sediment core was 15 cm in depth. An additional 10 sediment cores (5 cm depth) were collected in order to characterize the vegetated sediment (n = 5) and to perform the potential nitrification experiment (n = 5). The same number and type of sediment cores were collected in the adjacent area without vegetation (henceforth called bare mudflats). Estuarine water was collected in containers and taken to the laboratory to be used in the incubation procedure. The *in situ* temperatures of the water and sediment were recorded, and all samples were immersed in estuarine water and taken to the laboratory within 1 h. The *in situ* temperature conditions were maintained in the laboratory using coolers.

Sediment characterization

The sediment was characterized for MPB, chlorophyll *a* (chl *a*) and sediment particle size. For chl *a* determination, the top 5 mm of the 5 sediment cores (5 cm depth) were collected, weighed, and stored at −80°C. Later on, this sediment was freeze-dried and weighed again. About 0.3 g of freeze-dried sediment was immersed in 5 ml of 90% acetone and stored at −20°C for 24 h. Then, the samples were stirred in the vortex, centrifuged for 10 min at 2800 × *g*, and the supernatant was analyzed in a UV-1603 spectrophotometer. The chl *a* values were obtained according to Lorenzen (1967). The chl *a* level was also estimated from the trichromatic equations of Jeffrey & Humphrey (1975), which do not include an acidification step.

Sediment particle size was determined by sequential sieving of the top 5 cm of the sediment cores and classified according to Folk (1954). Organic matter

was quantified as loss on ignition (% LOI) during 8 h at 500°C.

Potential nitrification measurements

Potential nitrification was measured through a slurry incubation experiment (adapted from Hansen et al. 1981) in *Spartina maritima* vegetated sediment (n = 5) and in sediment from the bare mudflat (n = 5). Homogenized surface sediment aliquots (0 to 5 mm depth; 2 ml) were incubated with 20 mM NH_4Cl and 4 mM KH_2PO_4 in 40 ml artificial seawater (ASW) adjusted to *in situ* salinity. Samples for the determination of nitrification rates were taken at timed intervals of 1 h, over 5 h. Samples were centrifuged (8 min at 1180 × *g*), and supernatant from the water sample was filtered and frozen for subsequent NO_x-N (NO_3-N + NO_2-N) analysis. The NO_x-N concentrations were expected to increase over time (5 h of incubation) in a linear manner, meaning that the added NH_4-N was immediately nitrified after the start of incubation. Potential nitrification was calculated from this increase in NO_x-N according to Hansen et al. (1981) and Rysgaard et al. (1994).

Incubation procedure: nutrient fluxes and O_2 consumption

In order to assess whether there was competition for resources between plants and bacteria (Risgaard-Petersen & Ottosen 2000) in non-N-limited conditions, nutrient fluxes in *Spartina maritima* vegetated sediment and in bare mudflats were quantified and compared. Incubations were performed in a batch mode assay in a tank/incubator with 10 cores each time (i.e. 5 cores with *S. maritima* and 5 cores of bare mudflat sediment, meaning that light and dark incubations were always performed on different cores). The cores were aerated overnight (with an air pump and a magnetic stirrer rotating a magnet inside each core, as described by Cabrita & Brotas (2000) and Dalsgaard et al. (2000) and under a natural seasonal light-dark cycle. On the following day, each core was sealed with Plexiglass stoppers and incubated as described by these authors. After measuring the nutrient fluxes, the cores were aerated overnight to re-establish the equilibrium between the sediment and water column, and denitrification rates were measured on the following day. Flux incubation time was calculated considering the reduction in O_2 concentration in the water column, which cannot decrease by more than 20% of the initial concentration (2 vegetated cores and 2 bare mudflat cores were sampled and measured after 1 h of incubation). Nutrients (NH_4-N, NO_x-N) and oxygen fluxes were calculated using a mass balance approach. Both dark and light incubations for nutrient fluxes were performed twice each season, using extra cores to increase the number of replicates, i.e. twice (5 light vegetated + 5 light bare mudflat) and twice (5 dark vegetated + 5 dark bare mudflat).

Nutrient and oxygen analyses

Dissolved oxygen was quantified by Winkler titration (Grasshoff et al. 1983). Inorganic nutrient concentrations were quantified in water samples that had previously been filtered through GF/C Whatman filter paper and immediately frozen. Following this, colorimetric analyses in a Tecator FIAstar 5000 Analyser were performed. The NO_3-N concentration was quantified according to Grasshoff (1976), NO_2-N according to Bendschneider & Robinson (1952), and NH_4-N using colorimetric methods in filtered samples according to Koroleff (1969/1970).

Incubation procedure: denitrification rate measurements

Denitrification measurements were performed on the same cores as nutrient fluxes, according to the isotope pairing technique (Nielsen 1992). Following this, $^{15}NO_3$ (from a $Na^{15}NO_3$ stock solution, 99%; Sigma Aldrich) was added to the estuarine water in the container with 10 sediment cores each time (5 light vegetated + 5 light bare mudflat and 5 dark vegetated + 5 dark bare mudflat), to a final concentration of at least 20% of the O_2 concentration and a final enrichment of at least 30 atom % in the nitrate pool (Dalsgaard et al. 2000). The diffusion time for $^{15}NO_3$ was about 15 min, and the time was calculated according to Dalsgaard et al. (2000). The sediment cores were closed with PVC lids and incubation started. The incubation time was calculated according to the O_2 fluxes performed the day before. At the end of incubation, the water samples were placed in Exetainer vials (Exetainer, Labco) for N_2 analyses (200 µl of $ZnCl_2$ [50% w/v] was added to stop any biological activity). The water samples were filtered and stored for NO_3 analyses. Immediately after, each core was carefully slurried in order to homogenize

the dissolved N_2 in the water column and in porewater, and new samples for N_2 analyses were collected. Thus, N_2 diffused into the water column during incubation and N_2 still in the porewater was sampled and quantified. Denitrification rates were calculated according to Nielsen (1992).

^{15}N IPT assumptions

The isotope pairing technique (IPT) has the following assumptions: (1) the added $^{15}NO_3$ does not affect the production of $^{14}N_2$; (2) the $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ produced is binomially distributed; and (3) $^{14}NO_3$ and $^{15}NO_3$ homogeneously mixes in the nitrate reduction zone in the sediment. In order to test these assumptions, a $^{15}NO_3$ concentration series experiment was performed following the method of Nielsen (1992). Seven different $^{15}NO_3$ concentrations were tested (20 to 160 µM, in order to include a wide range of NO_3 concentrations in the water column) and the denitrification rates were quantified.

Plant biomass and fauna characterization

After all incubations, *Spartina maritima* plants were carefully washed and rinsed with distilled water and then dried at 60°C for dry weight (DW) quantification per sample. Sediment from each core, with and without vegetation, was sieved through a 500 µm-sized mesh net and macrofauna were collected, identified, and the species abundance calculated.

The biological factors MPB, plant density, and fauna abundance were considered because they can affect oxygen production and consumption and, consequently, other chemical processes such as mineralization, denitrification, and other nutrient fluxes (Rysgaard et al. 1995, Hulth et al. 2005, Sundbäck et al. 2006).

Statistical analysis

Linear correlation was performed (Pearson's and Spearman's rank correlations) to test for correlations between $^{15}NO_3$ concentration in the water column denitrification of NO_3^- in bottom water (D_w) and D_n. Analyses were performed using SPSS 17.0 and the STATISTICA 9 software package. 2-way ANOVA was performed to test for differences in potential nitrification rates and in denitrification rates between dark/light conditions and *Spartina maritima* vegetated sediment/bare mudflat sediment. If needed, data were transformed to satisfy the ANOVA assumptions. Cochran's Q and Kolmogorov-Smirnov tests were used to analyze the homogeneity of variances and normality of data, respectively. 1-way ANOVA was performed to test for differences in D_t (total denitrification) between seasons. A principal components analysis (PCA) was performed (Zar 1998) using PRIMER ver. 5 software. Projections considered the principal components 1 and 2 for variable environmental vectors (water temperature, MPB, nitrate and ammonium concentrations, macrofauna abundance, salinity, potential nitrification rate; data in Table 1 and Fig. 1), and the study sites (*S. maritima* sediment and bare mudflat sediment from the 4 seasons). All concentration data were log(x + 1) transformed and all variables were normalized.

RESULTS

Sediment characterization, plant and macrofauna biomass

The temperature of the water and sediment showed a clear seasonal variation, with higher values in spring and summer and lower ones in autumn and winter (Tables 1 & 2). In both areas (*Spartina maritima* vegetated sediment and bare mudflats), the percentage of fine particles (silt and clay) was higher

Table 1. Water temperature and salinity in each season, and mean concentrations (± SE; min. n = 3) of oxygen and nutrients (NH_4-N, NO_x-N), of the incubation water, in the initial conditions. *In situ* temperature and salinity are from the Tagus estuary in 1999–2005 (Gameiro et al. 2007)

Season	Temperature (°C)		Salinity		O_2	NH_4-N	NO_x-N
	Incubation	*In situ*	Incubation	*In situ*	(µmol l^{-1})	(µmol l^{-1})	(µmol l^{-1})
Autumn	18	12–24	30	5–32	219 (± 8)	37.7 (± 0.2)	46.6 (± 1.4)
Winter	18	8–18	28	2–32	221 (± 3)	22.8 (± 3.2)	43.2 (± 4.2)
Spring	23	13–24	28	1–37	210 (± 10)	38.1 (± 8.7)	34.7 (± 1.7)
Summer	26	20–26	28	6–36	194 (± 5)	33.6 (± 7.6)	25.6 (± 2.0)

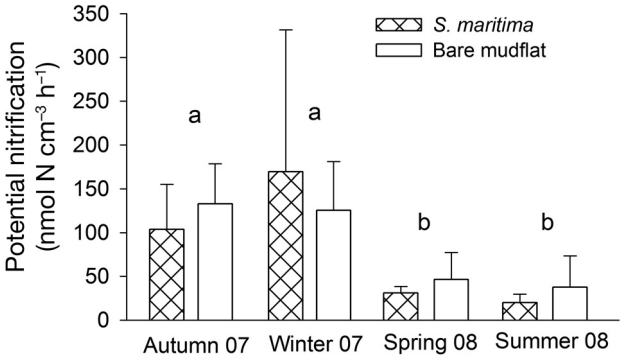

Fig. 1. *Spartina maritima.* Potential nitrification in vegetated sediment (cross-hatched bars) and bare mudflat sediment (white bars), during autumn, winter, spring, and summer (mean + SD, n = 5). Different lowercase letters (a, b) mean statistical significant differences ($p \leq 0.05$)

in the autumn/winter period. Both sediment and season influenced the percentage of organic matter (determined as the percentage of loss on ignition, % LOI) (there was interaction between both factors, 2-way ANOVA, $F = 77.69$, $p < 0.001$), with *S. maritima* vegetated sediment having a higher % LOI during summer and autumn, and bare mudflats showing a lower % LOI in summer and autumn (Table 2).

The MPB abundance (estimated as the concentration of chl *a*) depended on the sediment type and season (there was an interaction between both factors, 2-way ANOVA, $F = 4.55$, $p < 0.05$) and was comparatively higher in the *Spartina maritima* vegetated sediment. Even though *S. maritima* DW per core slightly increased along the growing season (ranging from 0.7 ± 0.2 to 2.2 ± 1.5 g DW), there was also an increase in the SD, thus reducing the meaning of the plant biomass increase (Table 2). In the cores from both sites, the most abundant infauna species were *Hydrobia ulvae, Scrobicularia plana, Hediste diversicolor,* and *Abra tenuis,* and their seasonal abundance was generally higher in spring. In *S. maritima* vegetated sediment, the mean macrofauna abundance was 1.1 to 2× higher than in the bare mudflats.

Potential nitrification rates

The slurry incubation salinities ranged between 28 and 30. Potential nitrification rates were significantly higher in winter and autumn compared to spring and summer (2-way ANOVA, $F = 11.99$, $p < 0.0001$) (Fig. 1). No statistically significant differences were found between bare mudflat and *Spartina maritima* vegetated sediments (2-way ANOVA, $F = 0.49$, $p > 0.05$).

O_2 and nutrient fluxes

Oxygen and nutrient concentrations at the beginning of the incubations varied seasonally (Table 1). In both areas (with vegetation and bare mudflats), net consumption of oxygen usually occurred under both dark and light conditions (Fig. 2A). Nevertheless, under dark conditions, when respiration is not compensated by primary production, the O_2 consumption was greater (2-way ANOVA, $F = 86.70$, $p < 0.0001$ for vegetated sediment, and $F = 5.50$, $p < 0.05$ for bare mudflat sediment). A seasonal variation was observed for both vegetated sediment, where the consumption was lower in autumn (2-way ANOVA, $F = 3.74$, $p < 0.05$), and in bare mudflat sediment (2-way ANOVA, $F = 3.92$, $p < 0.05$), where consumption was

Table 2. Sediment characterization and *Spartina maritima* biomass in each season. Granulometry: Fs: fine sand (values: 63–125 µm fraction + >125 µm fraction); Sc: silt and clay (≤63 µm). LOI: loss on ignition, MPB: microphytobenthos. MPB-chl *a* and *S. maritima* biomass: values are mean ± SD (MPB-chl *a*: n = 3 to 5, *S. maritima* biomass: n = 15)

Season	Mudflat type	Sediment					*S. maritima* biomass (g DW core^{-1})
		In situ temperature (ºC)	Granulometry Fs (%)	Sc (%)	LOI (%)	MPB-chl *a* (µg g^{-1})	
Autumn	*S. maritima*	14–15	13 + 64	14	17.5 ± 0.4	71.9 ± 16.2	2.2 ± 1.5
	Bare mudflat	15–17	9 + 65	19	10.8 ± 0.5	10.6 ± 2.6	
Winter	*S. maritima*	16–17	10 + 62	28	12.9 ± 0.5	46.4 ± 20.5	0.8 ± 0.5
	Bare mudflat	16–17	11 + 60	24	11.8 ± 0.5	7.9 ± 1.6	
Spring	*S. maritima*	24–25	18 + 64	10	17.2 ± 0.4	60.2 ± 15.8	0.7 ± 0.2
	Bare mudflat	23–25	9 + 70	13	14.3 ± 0.6	21.5 ± 1.4	
Summer	*S. maritima*	24–26	23 + 65	9	18.0 ± 0.3	41.9 ± 7.3	1.4 ± 1.1
	Bare mudflat	26–28	34 + 50	13	8.9 ± 0.7	12.7 ± 1.6	

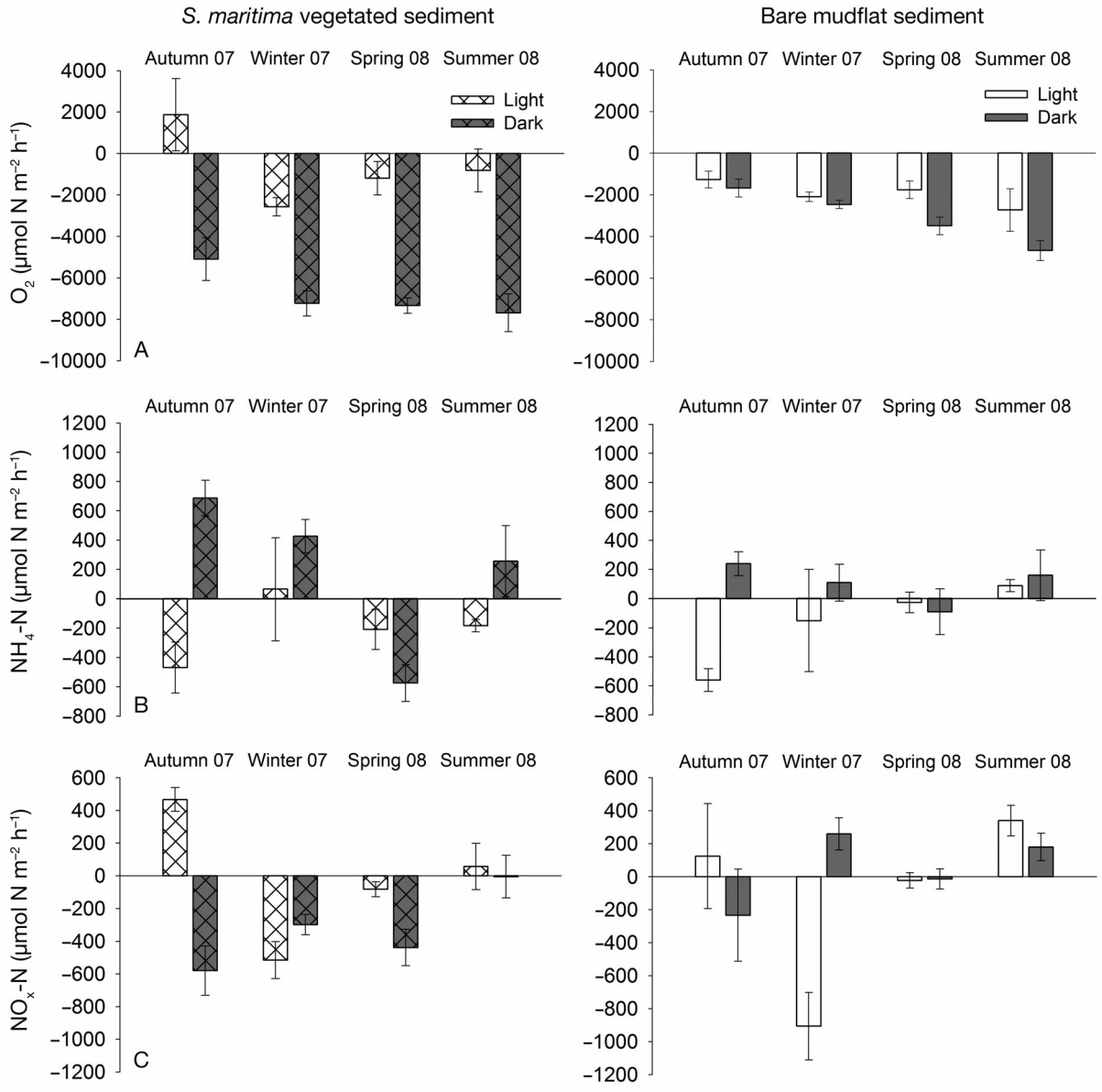

Fig. 2. *Spartina maritima.* Seasonal oxygen and nutrient fluxes (mean ± SE) in vegetated sediment and in bare mudflat sediment. Positive fluxes mean oxygen or nutrient efflux from the sediment, while negative fluxes represent uptake by the sediment. Light (white bars) and dark (grey bars) incubation results are shown. (A) O_2 fluxes; (B) NH_4-N fluxes; (C) NO_x-N fluxes

significantly lower in autumn than in summer. Regarding the nutrient fluxes, vegetated sediment under dark conditions showed an efflux of NH_4-N (except in spring) and NO_x–N consumption (except in summer) (Fig. 2B,C). During light conditions, NH_4-N was consumed (except in winter), and NO_x-N was consumed in winter and spring, with an efflux in autumn. Regarding the bare mudflat sediment, there was no clear trend concerning NH_4-N and NO_x-N fluxes throughout the year.

^{15}N-IPT assumptions

D_w was significantly correlated with the $^{15}NO_3$ concentration in the water column ($p < 0.05$, $r = 0.9816$), whereas D_n was constant at all $^{15}NO_3$ concentrations tested ($p > 0.05$, $r_S = 0.3907$) (Fig. 3). The results suggest that all of the assumptions of IPT were fulfilled (Nielsen 1992, Rysgaard et al. 1994, Steingruber et al. 2001, Eyre et al. 2002), which justifies the use of the IPT in this system.

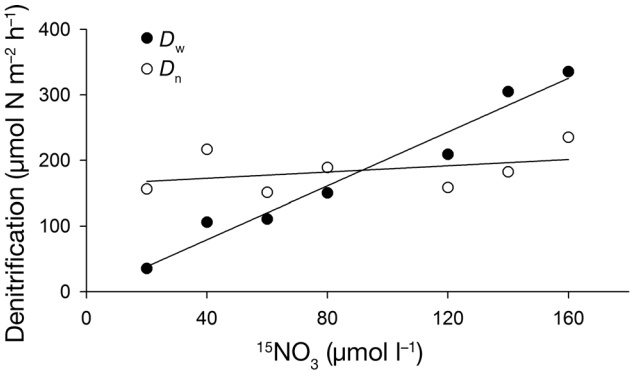

Fig. 3. Denitrification (D_n and D_w) along an increasing $^{15}NO_3$ concentration series. Denitrification in bottom water (D_w; ●) is correlated with the $^{15}NO_3$ in the water column ($p \leq 0.05$; $r = 0.9816$), while coupled nitrification-denitrification (D_n; ○) is similar with increasing $^{15}NO_3$ ($p > 0.05$; $r_S = 0.3907$)

Denitrification measurements

Within each season, denitrification rates were not significantly different between the areas (with vegetation and bare mudflats), except in winter under dark conditions, when the presence of *Spartina maritima* significantly enhanced the denitrification rates (697 ± 497 µmol N m^{-2} h^{-1}) (Fig. 4A). In autumn, the values ranged between 51 ± 22 and 69 ± 7 µmol N m^{-2} h^{-1}, and there were no significant differences in the total denitrification rates between the dark and light incubations for either area, or between areas under the same incubation conditions (Fig. 4A). In winter, the total denitrification rate was significantly higher in the presence of *S. maritima* (2-way ANOVA, $F = 24.06$, $p < 0.05$), and under dark conditions (2-way ANOVA, $F = 5.48$, $p < 0.05$), but there was no interaction be-

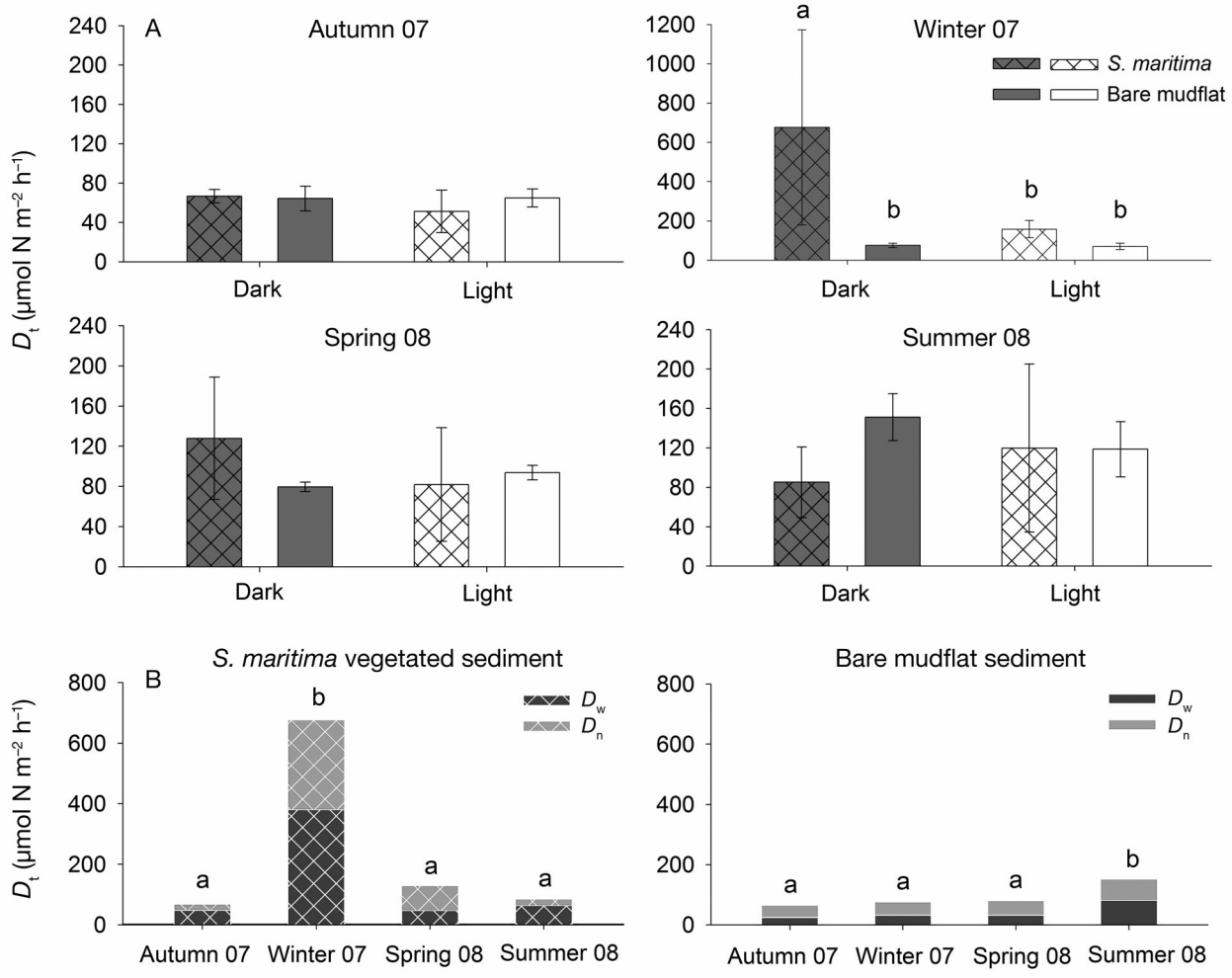

Fig. 4. (A) Total denitrification (D_t) (mean ± SD; n = 3 in autumn; n = 5 in the other seasons) in different seasons, comparing dark vs. light conditions, and *Spartina maritima* vegetated sediment vs. bare mudflat sediment. (B) D_t (mean) in *S. maritima* vegetated sediment and bare mudflat sediment in dark conditions. D_w and D_n absolute values are shown (µmol N m^{-2} h^{-1}; mean; n = 3 in autumn; n = 5 in the other seasons). Different lowercase letters (a, b) indicate statistically significant differences ($p \leq 0.05$)

tween these 2 variables (Fig. 4A). In spring, the values ranged between 80 ± 5 and 128 ± 61 µmol N m^{-2} h^{-1}, and there were no significant differences in the total denitrification rates between the areas under the same incubation conditions, or between dark and light incubations within each area. In addition, there were no differences in the summer incubations between either type of sediment (vegetated vs. bare mudflat) or light vs. dark conditions; values ranged between 85 ± 36 and 151 ± 24 µmol N m^{-2} h^{-1} (Fig. 4A). Fig. 4B shows that, for the dark conditions, denitrification rates in the bare mudflat areas were significantly higher in summer, whereas in colonized areas the denitrification rates were significantly higher in winter. Between seasons and within each season, the contributions of D_n and D_w to the denitrification rates between the areas (with vegetation and bare mudflat) did not show a clear trend (Fig. 4B, see Table 3).

The PCA (Fig. 5) showed that the principal components 1 and 2 explained 77% of the variance. Fig. 5 shows that principal component 1, which explained 47% of the variance, clearly separated autumn and winter from spring and summer seasons on the right side of the axis (positive values). The right side of the axis is characterized by higher concentrations of nitrate, lower salinity, higher potential nitrification, and higher denitrification under dark conditions; whereas the left side of the axis (negative values) is characterized by higher temperature, presence of fauna, and higher concentrations of ammonium. Principal component 2, which explained 30% of the variance, clearly separates winter from autumn, whereas spring and summer are not differentiated. In summary, winter was clearly different from the other seasons and characterized by comparatively higher potential nitrification and higher denitrification rates under dark conditions.

Table 3. Seasonal or annual denitrification (µmol N m^{-2} h^{-1}) in salt marshes: unvegetated and vegetated sediment ability to remove N in salt marshes through denitrification. All these studies were performed using ^{15}N, and values shown include both light and dark incubations (single values correspond to the mean, while ranges correspond to minimum and maximum values recorded). Values for D_t are shown in **bold**

		Vegetated sediment			Unvegetated sediment			Reference
		Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	
Tagus estuary, Portugal	D_t	**51–67**	**158–676**	**82–128**	**85–120**	**64–65**	**71–76**	**80–94**	**119–151**	Present study
	D_w	19–48	57–381	34–46	37–63	24–27	23–32	32–43	41–81	
	D_n	19–32	102–295	48–81	22–83	38–40	44–48	47–51	70–78	
Tagus estuary, Portugal	D_t	–	–	–	–	**0–100 (R)**	**20–250 (R)**	–	**0–50 (R)**	Cabrita & Brotas (2000)[a]
						0–35 (P)	**45–150 (P)**		**0–55 (P)**	
	D_w	–	–	–	–	10–30 (R)	10–24(R)	–	0–10 (R)	
						0–28 (P)	0–38(P)	–	0–13 (P)	
	D_n	–	–	–	–	0–65 (R)	5–200(R)	–	0–12 (R)	
						0–10 (P)	0–55 (P)		0–40 (P)	
Venice lagoon, Italy	D_t	**125–250**	–	**21–125**	**7–21**	**143–286**	–	**72–143**	**14–43**	Erickson et al. (2003)[b]
Colne Point, England	D_t			**8.3**		–	–	–	–	Aziz & Nedwell (1986)[c]
Great Sippewissett marsh, USA	D_t			**75**		–	–	–	–	White & Howes (1994)[d]
Pointe-au-Père, Canada	D_t	**11–25**	**6–15**	–	**18–42**	–	–	–	–	Poulin et al. (2007)[e]
Kertinge Nor, Denmark	D_t	–	–	–	–	**3–5**	**35–75**	**20–30**	**7.5–12.5**	Rysgaard et al. (1995)
	D_w	–	–	–	–	1	10–16	10–15	3	
	D_n	–	–	–	–	2–5	20–60	7.5–15	7.5–12.5	
Estuarine fjord, Denmark	D_t	–	–	–	–	–	**29**	**8–17**	**5**	Christensen et al. (2000)

[a]R: Rosário salt marsh; P: Pancas salt marsh. [b]Data in bare mudflat column corresponds to creek sediment; study performed with *Limonium serotinum*. [c]Study performed with *Puccinelia/Halimione*. [d]Cape Cod, MA, USA; study performed with *Spartina alterniflora*. [e]St. Lawrence estuary, Canada; study performed with *Spartina alterniflora*

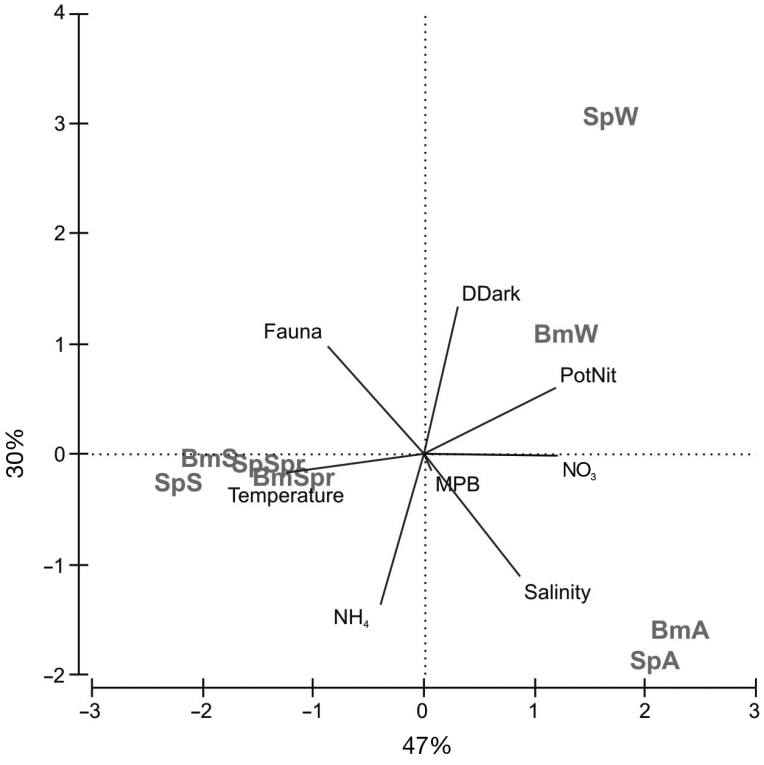

Fig. 5. PCA results, showing principal components 1 and 2 for environmental variable vectors (nitrate [NO_3], ammonium [NH_4], temperature, salinity, denitrification in dark conditions [DDark], microphytobenthos [MPB], fauna, potential nitrification [PotNit], and the seasons; A: autumn; W: winter; Spr: spring; and S: summer). Sp: *Spartina maritima* vegetated sediment; Bm: bare mudflat sediment

DISCUSSION

The role of denitrification as a N removal process has been demonstrated across several ecosystem types (from agricultural landscapes to aquatic ecosystems, namely marine, estuarine, and freshwater systems) (e.g. Cornwell et al. 1999, David et al. 2006, Seitzinger et al. 2006). In addition, other pathways contribute to this N removal in aquatic ecosystems (salt marshes included), such as dissimilatory nitrate reduction to ammonium (DNRA), or autotrophic pathways, such as anammox (anaerobic ammonium oxidation) or chemoautotrophic denitrification via sulfur or iron oxidation; the magnitude of which depends on the ecosystem type and its inherent characteristics (Eriksson et al. 2003, Risgaard-Petersen et al. 2004b, Dalsgaard et al. 2005, Burgin & Hamilton 2007, Poulin et al. 2007).

The biogeochemistry of the salt marshes' sediment (and consequently, the nitrogen removal capacity) depends on a complex interaction of biotic and abiotic factors. In the present work, denitrification rate and nutrient flux measurements showed some variability within each season and within sites, as also reported in other works (e.g. Cabrita & Brotas 2000, Eriksson et al. 2003, Poulin et al. 2007). Accordingly, differences within and among sites, result from the natural variability within each system, given the number of parameters/variables that may interact and influence all of these processes. These variables can include the abundance of biota (from microorganisms to macrofauna, and from MPB to macrophytes), which may have an interacting effect on the aforementioned biogeochemical processes (e.g. Lillebø et al. 1999, Eriksson et al. 2003, Gilbert et al. 2003, Risgaard-Petersen 2003, Hou et al. 2007). Despite the inherent environmental variability, it is important to understand the contribution of salt marshes to the removal of excess nitrogen through denitrification in coastal areas. Even though denitrification is an important reactive nitrogen sink (David et al. 2006), there are still uncertainties concerning the fate of all land-derived nitrogen (Galloway et al. 2004). Thus, even in heavily altered regions, rivers (important sources of nitrogen to coastal systems) represent small sources of reactive nitrogen to the open ocean (Galloway et al. 2008).

Potential nitrification can be regarded as a proxy for abundance of active nitrifiers (Risgaard-Petersen et al. 2004a). In the Tagus estuary, potential nitrification rates were significantly higher in autumn and winter, and there were no statistically significant differences between bare mudflat and *Spartina maritima* vegetated sediment. Two main reasons could explain these seasonal differences: (1) in this warm-temperate system, the water temperature in autumn/winter ranges between 15 and 17°C, which corresponds to the higher end of the temperatures that temperate estuaries generally experience during winter (e.g. Lillebø et al. 2005); (2) during the warmer growing season, plants and MPB may outcompete nitrifiers (Cabrita & Brotas 2000).

Total denitrification ranged between 64 ± 13 and 151 ± 24 µmol N m^{-2} h^{-1} in the bare mudflat sediment, and between 51 ± 22 and 676 ± 497 µmol N m^{-2} h^{-1} in *Spartina maritima* vegetated sediment. As a whole, denitrification was not higher in the marsh, considering the seasons and dark/light conditions. However, under dark winter conditions, the rates

were significantly higher in the vegetated sediment, meaning that at least during winter, areas colonized by *S. maritima* enhance the removal of nitrogen through denitrification. As shown in the PCA, this seems to be related to the higher potential nitrification, as well as to the higher availability of inorganic nitrogen (namely nitrate) in the water column. These results are in agreement with those of other studies (Koch et al. 1992, Eriksson et al. 2003, Piña-Ochoa & Álvarez-Cobelas 2006, Koop-Jacobsen & Giblin 2009). The greater availability of nitrate, derived from freshwater inputs during winter (Lillebø et al. 2005), reduces the competition for nitrogen within the sediment and may contribute to higher denitrification levels (Rysgaard et al. 1995, Ogilvie et al. 1997, Cabrita & Brotas 2000). Accordingly, on a daily basis (day and night fluxes), winter was the season with the highest net consumption of NO_x–N. Moreover, there was less competition between plants and MPB and nitrifiers during winter, especially under dark conditions, due to a lower NH_4–N uptake (Rysgaard et al. 1993, 1995, Risgaard-Petersen et al. 1994, Lillebø et al. 2002). In addition, O_2 diffusion through the plant aerenchyma, namely in *S. maritima* (Cartaxana & Lloyd 1999), and the creation of oxic microzones at the rhizosphere may increase D_n (Koop-Jacobsen & Giblin 2010), which may explain our results. Conversely, bare mudflat sediment showed higher denitrification rates in summer under dark conditions. Considering the previous work carried out in the Tagus estuary (Cabrita & Brotas 2000), it was expected that denitrification rates in bare mudflat were higher during winter, as occurred in the vegetated sediment. The higher abundance of active nitrifiers recorded in winter than in summer (given the potential nitrification rates) could ultimately lead to higher denitrification rates. However, denitrification rates in winter were lower than in summer. Accordingly, it should be taken into account that denitrification rates obtained in the present study in winter (bare mudflats) are within the ranges obtained by Cabrita & Brotas (2000) in the same estuary and season. Nevertheless, further studies should be performed in order to better understand this biogeochemical process. Differences in the summer denitrification rates between bare mudflat and *S. maritima* vegetated sediment may have been due to the less abundant MPB community in the bare mudflat (<50% chl *a* content in the surface sediment), meaning that there was only minor competition between MPB and nitrifiers, in addition to the absence of plants, which also compete for nitrogen sources.

Table 3 summarizes the results of the literature review of seasonal and annual denitrification rates in the salt marshes measured using a ^{15}N tracer. The Tagus estuary denitrification rates in bare mudflat sediment were within the same range as in the Venice lagoon (Eriksson et al. 2003), as well as within the range previously recorded in the Tagus estuary (Cabrita & Brotas 2000), but they were higher than those recorded in Denmark (Rysgaard et al. 1995, Christensen et al. 2000). On the other hand, the denitrification rates in the Tagus areas colonized by *Spartina maritima* were comparatively higher than in other vegetated sediments, namely *Limonium serotinum* in the Venice lagoon (Eriksson et al. 2003). The comparison with other vegetated sediments, namely *S. alterniflora* (Valiela & Teal 1979, White & Howes 1994) and *Puccinelia*/*Halimione* (Aziz & Nedwell 1986 in White & Howes 1994) becomes limited due to differences in the methodologies used, even though they were all were based on tracing ^{15}N (for more detailed information about the methodologies used see the review by Cornwell et al. 1999). Nevertheless, differences between comparable results may be due to: (1) species-specific interactions, i.e. different salt marsh species may create specific rhizosphere effects, depending on their life cycles, physiology, root systems, and nutritional status, and thus influence the microbial community and competition with nitrifiers; (2) bioturbation and bioirrigation, i.e. benthic fauna diversity and abundance will also change the redox state of the sediment and thus influence nutrient fluxes and the microbial community; (3) the MPB, which may also change the top sediment redox state and compete with the microbial community for nutrients; (4) geographical environmental characteristics, namely temperature range and seasonal availability of nitrate. More specifically, differences in denitrification rates between ecosystems may be due to several physical, chemical, and biological factors, such as temperature, light, NO_3 concentrations, oxygen availability, benthic microalgae, benthic fauna, and the presence/absence of plants (e.g. Kaplan et al. 1979, Valiela & Teal 1979, Risgaard-Petersen et al. 1994, Cornwell et al. 1999, Herbert 1999, Eriksson et al. 2003, Pinã-Ochoa & Alvarez-Cobelas 2006, Poulin et al. 2007, Koop-Jacobsen & Giblin 2009).

Seasonally, the relative contribution of D_n and D_w to total denitrification in the bare mudflats was rather similar. In sediments colonized by *Spartina maritima*, the relative contribution of D_n and D_w to total denitrification was more variable, although no clear seasonal variation was found. As shown in other studies, one could expect an increased contribution of D_w in

winter due to an increase in NO_3 availability in the water column (e.g. Rysgaard et al. 1995, Ogilvie et al. 1997). In the Tagus estuary, Cabrita & Brotas (2000) only observed a relatively small seasonal increase in D_w in one of their bare mudflat study sites. As described earlier, many variables can control the processes behind D_n, which renders D_n rates highly variable. Nitrification process is generally limited by low oxygen and ammonium concentrations (Henriksen & Kemp 1988). In turn, oxygen penetration depends on plant and microbial activity and infauna bioturbation (Cartaxana & Lloyd 1999, Maricle & Lee 2002, Nizzoli et al. 2007, Volkenborn et al. 2007), whilst ammonium availability depends on the balance between ammonification and processes using NH_4^+ (e.g. uptake by primary producers and nitrification). Therefore, both nitrification and D_n may be affected by these variables.

On the whole, denitrification is influenced by multiple interacting variables (Seitzinger 1988, Thompson 1995, Cornwell et al. 1999, Piña-Ochoa & Álvarez-Cobelas 2006, Silvennoinen et al. 2008), which may result in an inherent variability under identical experimental conditions. The present study showed that there is a significantly higher contribution of *Spartina maritima* marshes to N removal during winter. However, on an annual basis, and considering the seasonal variations observed, it cannot be concluded whether or not denitrification in the vegetated sediment is significantly different from the bare mudflats.

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