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The role of plant type and salinity in the selection for the denitrifying community structure in the rhizosphere of wetland vegetation

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Summary. Coastal wetlands, as transient links from terrestrial to marine environments, are important for nitrogen removal by denitrification. Denitrification strongly depends on both the presence of emergent plants and the denitrifier communities selected by different plant species. In this study, the effects of vegetation and habitat heterogeneity on the community of denitrifying bacteria were investigated in nine coastal wetlands in two preserved areas of Spain. Sampling locations were selected to cover a range of salinity (0.81 to 31.3 mS/cm) and nitrate concentrations (0.1 to 303 μM NO_3^-), allowing the evaluation of environmental variables that select for denitrifier communities in the rhizosphere of *Phragmites* sp., *Ruppia* sp., and *Paspalum* sp. Potential nitrate reduction rates were found to be dependent on the sampling time and plant species and related to the denitrifier community structure, which was assessed by terminal restriction fragment length polymorphism analysis of the functional genes *nirS*, *nirK* and *nosZ*. The results showed that denitrifier community structure was also governed by plant species and salinity, with significant influences of other variables, such as sampling time and location. *Ruppia* sp. and *Phragmites* sp. selected for certain communities, whereas this was not the case for *Paspalum* sp. The plant species effect was strongest on *nirK*-type denitrifiers, whereas water carbon content was a significant factor defining the structure of the *nosZ*-harboring community. The differences recognized using the three functional gene markers indicated that different drivers act on denitrifying populations capable of complete denitrification, compared to the overall denitrifier community. This finding may have implications for emissions of the greenhouse gas nitrous oxide. [**Int Microbiol** 2012; 15(2):89-99]

Keywords: bacterial communities · denitrification · eutrophication gradient · salinity gradient · rhizosphere ecology · wetlands · coastal lagoons

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Introduction

Coastal wetlands are transient links between terrestrial and marine environments and act as biodiversity reservoirs for characteristic flora and fauna. These natural wetlands provide a wide range of ecosystem services important to

humankind, but are being degraded and lost more rapidly than other ecosystems. At the same time, the demand for the retention and removal of nitrogen through the microbial process of denitrification in these systems has increased and is being increasingly exploited in constructed wetlands. Denitrification is an anaerobic respiration pathway through which nitrate is reduced to N_2 in four consecutive steps, each catalyzed by different enzymes. Genetic analysis of cultured denitrifiers has highlighted the wide taxonomic diversity of this functional group [19,38]. This property restricts the use of functional genes encoding the different reductases in the denitrification pathway as molecular markers [34]. Of those that are available, the most common targets are the genes *nirS* and *nirK*, coding for the copper-containing (NirK) and cytochrome cd_1 type (NirS) nitrite reductases, respectively, along with *nosZ*, coding for the nitrous oxide reductase. These genes have been used to study denitrifying communities in various environments [9,16,27], including constructed wetlands with high nitrogen loadings located downstream from wastewater treatment plants [20,32,33].

The specific setting of natural coastal wetland environments and the fact that they are highly influenced by changing external conditions enable the co-existence of divergent habitats in close proximity [36]. Moreover, salinity gradients are easily established. In a recent biogeographical study of denitrifiers, salinity was a major driver of denitrifying communities in aquatic environments at the global scale [18] and salinity, as well as eutrophication gradients, also had a significant impact on the vegetation colonizing coastal wetlands [23].

The variation in vegetation is likely to directly impact the denitrifying community structure in wetlands, since plants induce and stimulate the growth of specific bacterial groups and harbor well-defined bacterial communities around the root surface as compared to the bulk soil or sediment [6,8,17,35]. Most of the work on denitrifiers in the rhizosphere has been done in arable soils, since their activity results in a net nitrogen loss and thereby negatively affects crop production [3,15,29]. Experimental data on how denitrifying bacterial communities are altered by emergent vegetation in aquatic systems is limited to riparian soils and constructed wetlands [1,32].

In this study, we examined whether habitat heterogeneity and the different types of vegetation in coastal wetlands select for specific denitrifier communities. We therefore investigated the community structure of denitrifying bacteria in the rhizosphere of three widely distributed helophytes, *Phragmites australis*, *Ruppia* spp. and *Paspalum distichum*,

at two time points in nine natural wetlands located in two preserved areas along the Mediterranean and Atlantic coastlines of the Iberian Peninsula. The sampling locations covered a range of different salinity and nutrient concentrations in order to evaluate whether and how environmental variables select for certain denitrifier communities. Since denitrifiers express either the NirS or NirK nitrite reductases, which might be subject to different community assembly rules [18], community composition was studied by terminal restriction fragment length polymorphism (T-RFLP) of *nirS* and *nirK*. The *nosZ* gene, found among two thirds of the sequenced genomes of denitrifiers [19], was also targeted because environmental drivers may act differently on denitrifiers with complete denitrifying capacity, as compared to those that lack the last step with nitrous oxide as the end product of denitrification.

Materials and methods

Site description and sampling. Samples were obtained from wetlands located in two environmentally protected areas in Spain, the Empordà wetlands and Baix Ter (42º02′ N, 3º11′ E, Girona) on the Mediterranean coast, and the Doñana National Park (36º49′ N, 6º22′ W, Huelva) on the Atlantic coast. The Empordà wetlands (Aiguamolls de l'Empordà) include a group of Mediterranean coastal lagoons and salt marshes with variable depths (average depth of 0.60 m and maximum depth close to 2 m). They show a typical Mediterranean hydrological regime, which is greatly affected by the proximity of the sea [30]. The hydrology of this area depends mainly on the sudden and irregular intrusions during sea storms and the intense rainfall and entry of fresh water from the rivers Ter, Darò, Fluvià and Muga. The marshes are confined for long periods of time and tend towards desiccation [2,22]. In Doñana, some flooded areas are under tidal influence as well as the influence of the Guadalquivir River. Salt marshes, which follow a seasonal flooding pattern, become dry during the summer but there are also many lagoons where water remains throughout the year (Fig. 1) [31].

Within each area, different lagoons were sampled in January and May 2007 coinciding with the non-growing and growing seasons of plants, respectively, except for Túries and Algaida, which were sampled only in May. Bassa Ànser, Basses d'en Coll, and Laguna Dulce represent oligohaline environments (conductivity values < 2.5 mS/cm); Santa Olalla, Ter Vell and Lucio del Cangrejo, mesohaline environments (2.5–8.0 mS/cm); and Túries, Fra Ramon and Algaida, euhaline lagoons (>15.0 mS/cm).

The sediment was sampled in triplicate by collecting three randomly distributed samples in a square meter surface using a 7-cm diameter Plexiglas tube mounted in a manual core sampler in monospecific stands of the dominant plant species. Only the upper 4 cm of the sediment was used in the analysis. Visible roots were removed from sediment samples using sterile forceps. The sediments were homogenized, transported in a portable ice-box, and frozen at –20 ºC within 4 h after sampling.

Roots of the main plant species in each lagoon were collected for nucleic acid analysis and activity measurements according to Trias et al. [35]. Sampled plant species included the ditch grasses *Ruppia cirrhosa* (collected from Túries and Fra Ramon) and *Ruppia maritima* (Algaida), the emergent
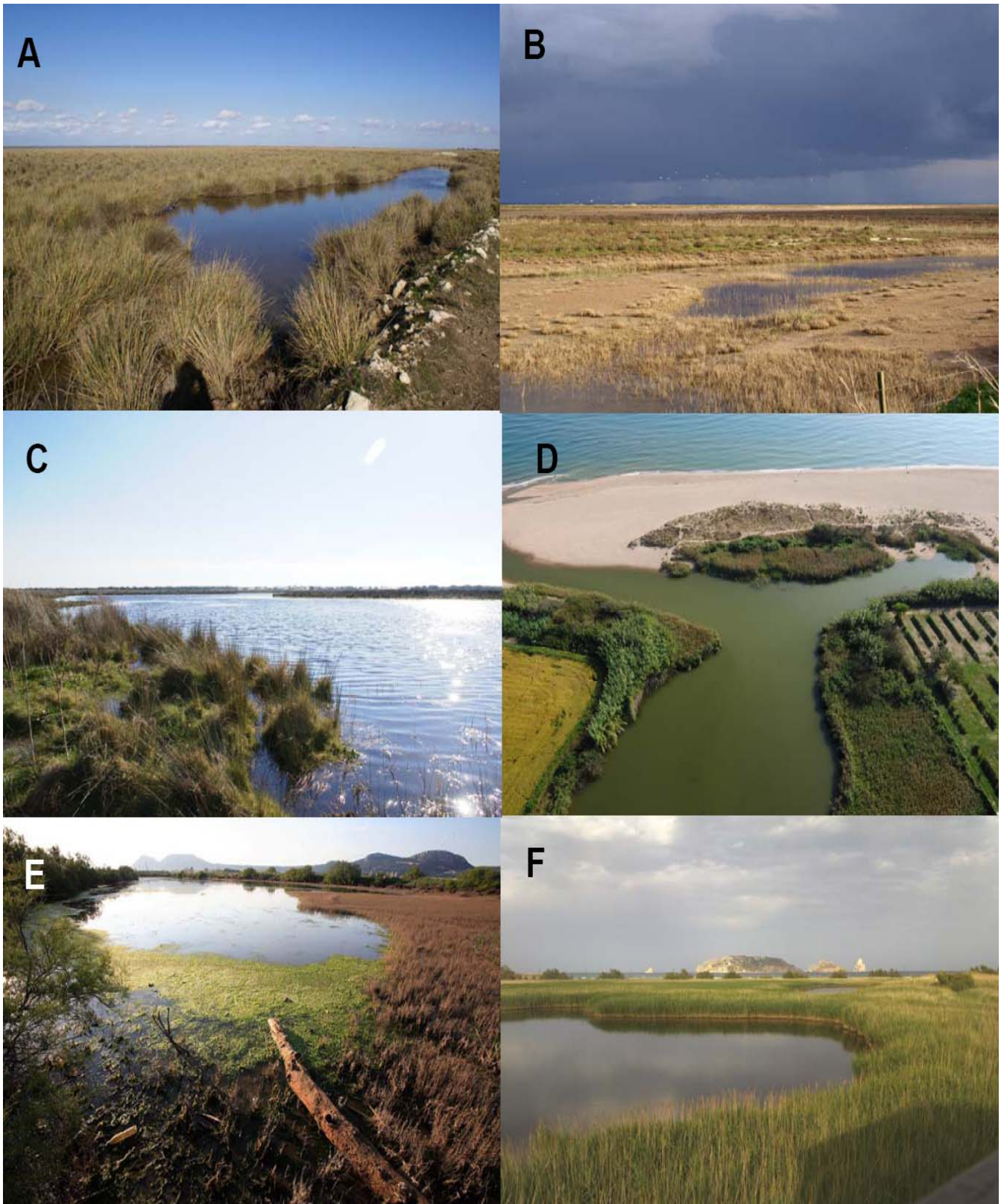
Fig. 1. Several of the wetlands studied in this work. (**A**) Algaida, Doñana. (**B**) Lucio del Cangrejo, Doñana. (**C**) Santa Olalla, Doñana. (**D**) Basses d'en Coll, Empordà. (**E**) Fra Ramon, Empordà. (**F**) Ter Vell, Empordà (see cover and p A2). Photographs by: (A-C) Rocío López-Flores. (D) Ricard Corbí (archives of the Cátedra de Ecosistemas Litorales Mediterráneos, University of Girona). (E) David Estany. (F) Rocío López-Flores.

macrophyte *Phragmites australis* (Basses d'en Coll, Ter Vell and Lucio del Cangrejo), and the knot grass *Paspalum distichum* (Laguna Dulce and Santa Olalla). Samples were distributed in aliquots of approximately 2 g (fresh weight) and kept at –20 ºC for further analysis.

Chemical determinations. Ammonium ($N–NH_4^+$), nitrite ($N–NO_2^-$) and nitrate ($N–NO_3^-$) concentrations were analyzed from filtered water samples (Whatman GF/F glass-fiber filter) using standardized methods for seawater analyses [12,13]. Total nitrogen (TN) was analyzed from unfiltered water samples as previously described [13]. Total and dissolved organic carbon (TOC and DOC) in water samples were measured from acidified unfiltered and filtered samples using a TOC analyzer (TOC 5000 Shimadzu, Shimadzu Scientific Instruments).

The TC and TN contents in the bulk sediment were analyzed by combustion of the dried samples (60 ºC for 3 h) at 975 ºC in a Perkin Elmer AE SeriesII equipped with a TCD detector. The results were evaluated using the K factor method with cystine ($C_6H_{12}N_2O_4S_2$) as the standard. Duplicates were performed for all chemical determinations.

Potential nitrate reduction activity. Potential nitrate reduction was measured using 10 g of clean, cut roots placed in 250-ml flasks with 49 ml of sterile isotonic solution (Ringer ¼ solution; Scharlau 06–073, Barcelona, Spain), but otherwise as previously described [32]. The samples were incubated at 25 ºC with continuous agitation (150 rpm) for 8 h, during which aliquots of the liquid phase were taken for nitrite and nitrate determination every 30 to 45 min for the first 2 h, and every hour for the next 6 h. Nitrate and nitrite were analyzed by ion chromatography (IC) using an IC-Pak Anion HC column (4.6 × 150 mm) and cartridge (Waters Corporation, Barcelona, Spain) in a Waters HPLC modular system according to the manufacturer's instructions. Initial and final ammonium concentrations in each flask were determined according to Ruiz-Rueda et al. [32]. Rates were calculated using linear-decay kinetics from the first 4 h of incubation and standardized in terms of dry weight (DW).

DNA extraction and PCR amplification. Nucleic acids were extracted from 1 g (wet weight) of roots. Nitrous oxide reductase genes (*nosZ*) were PCR-amplified as previously described [32]. Touchdown PCR was performed in a minicycler (MJ Research) according to the PCR conditions described by Enwall et al. [10]. The nitrite reductase genes *nirS* and *nirK* were amplified with primer pairs Cd3aF-R3Cd and F1aCu-R3Cu [34]; the expected PCR product sizes were 425, 470 and 453 bp for *nirK*, *nirS* and *nosZ*, respectively. In all cases, the forward primers were fluorescently labeled at the 5´end using 5-hexachlorofluorescein (HEX).

T-RFLP analyses of *nosZ*, *nirK* and *nirS* genes. Amplified gene fragments were independently digested with three endonucleases. BstUI, HhaI and Sau96I were used to digest the *nosZ* gene fragments, BstUI, HaeIII and HhaI for *nirS*, and DpnI, HpyCH4IVand Sau96I for *nirK*. Briefly, 12 µl of PCR products were digested in a final volume of 16 µl including 10 U of the endonuclease and the buffer provided by the manufacturer. The reactions were incubated for 2 h at 37 ºC, except in the case of the BstUI digestion, at 60 ºC. Digestions with endonuclease HhaI also contained bovine serum albumin at a final concentration of 0.1 mg/ml.

The digested DNA (1.4 µl) was separated and the fluorescently labeled TR fragments were detected using an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Ten µl of formamide and 0.04 µl of GS ROX-500 size standard (Applied Biosystems) were added to every digestion prior to denaturation at 95 ºC for 5 min. The data were analyzed using the software Peak Scanner (Applied Biosystems). Upper and lower thresholds for fragment sizes were set at 500 and 50 bp, respectively; peaks with fluorescence values below 50 were discarded. Relative peak areas were calculated by dividing each raw peak area by the total area in the sample digestion to normalize for variations in DNA concentration in T-RFLP analyses. Peaks with relative areas lower than 0.5 % of the total area were excluded.

Statistical analyses. Pearson's correlation coefficients were determined for pairwise comparisons of physicochemical variables at $P < 0.05$. Differences in the potential nitrate+nitrite reduction rates were analyzed for the effects of sampling time within every lagoon by non-parametric Welch tests. All of the analyses were performed using the SPSS software v16.0 software (SPSS Inc. Chicago, IL, USA). The effects of physicochemical variables, plant species and geographic location on the T-RFLP profiles obtained for the three genes were analyzed separately by nonmetric multidimensional scaling (NMS). The slow and thorough autopilot mode specifications were used throughout this study (maximum iterations = 400; starting number of axes = 4; instability criterion = 0.00001; number of real runs = 250; number of randomized runs = 250). Data were square-root transformed and distance measures between samples were calculated using the Bray-Curtis coefficient. Joint plots were used to explore relationships between environmental variables and the ordination. Radiating vectors were depicted as the hypotenuse of a right triangle whose sides were the r^2 values between the variable and the NMS axes. Spatial (based on sampled regions) and temporal differences in the diversity of the denitrifying community within every sampled lagoon were explored using multiple response permutation procedures (MRPP). Rank-transformed Bray-Curtis distance matrices were used in all cases to better assess the results obtained with NMS [24]. Additionally, the relationships between individual environmental variables and the structure of *nirS*, *nirK* and *nosZ* communities were determined by analyzing the correlation between Bray-Curtis derived dissimilarity matrices using the Mantel test with 999 Monte Carlo simulations. NMS, MRPP and Mantel tests were performed using the PC-ORD software (version 5.28 MjM software, Oregon, USA).

Results

Characterization of wetlands. The lagoons in the two geographic areas, Empordà and Doñana, differed according to salinity (from oligo- to euhaline), eutrophication and the dominance of specific plant species (Table 1). Water conductivity ranged from 0.8 (Laguna Dulce) to 31.3 mS/cm (Túries), which were the most extreme values recorded. In general, small variations in water conductivity were determined between January and May, except for Túries where an almost two-fold increase was detected in May. In the Empordà region, on both sampling occasions, the highest nitrate and ammonia concentrations occurred in freshwater or transitional ecosystems (Bassa Ànser, Basses d'en Coll and Ter Vell). There, negative correlations were obtained between

Table 1. Chemical properties of the overlying water and sediment of the wetlansds studied, in Doñana and Empordà. Data from sediments correspond to mean values and standard deviations of two replicate samples obtained where rhizosphere samples were collected

Wetland (abbreviation)	Water								Sediment	
	pH	Cond (mS/cm)	Temp (ºC)	N-NO$_3^-$ (µg/l)	N-NH$_4^+$ (µg/l)	TN (mg/l)	DOC (mg/l)	TOC (mg/l)	TC (%)	TN (%)
Empordà										
Bassa Ànser (BA)	8.8	1.4	10.9	2638.0	56.1	3.46	4.87	5.47	ND	ND
Basses d'en Coll (BC)	8.8	1.7	12.4	1972.0	52.9	3.30	5.45	5.60	1.18 ± 0.74	0.09 ± 0.04
Ter Vell (TV)	ND	4.5	12.5	16.9	82.4	1.00	6.32	6.27	0.95 ± 0.38	0.12 ± 0.04
Túries (TU)	10.1	17.6	8.1	1.9	6.7	1.64	15.21	15.31	ND	ND
Fra Ramon (FR)	ND	25.0	13.5	10.6	5.4	1.60	17.11	17.31	3.61 ± 0.12	< 0.05
Doñana										
Laguna Dulce (LD)	7.7	1.0	8.5	1.87	25.6	2.12	30.37	30.04	0.39 ± 0.40	0.11 ± 0.07
Santa Olalla (SO)	8.4	3.6	9.7	48.8	469.8	5.27	51.65	54.25	0.07 ± 0.03	< 0.05
Lucio del Cangrejo (LC)	7.2	7.2	7.5	189.2	< 1.4	1.09	8.31	9.30	2.75 ± 0.58	0.13 ± 0.06
Algaida (AL)	7.8	28.3	8.2	1310.1	104.6	2.38	8.31	8.24	ND	ND
Empordà										
Bassa Ànser (BA)	8.3	1.2	21.0	4251.6	128.6	4.83	28.52	20.64	ND	ND
Basses d'en Coll (BC)	8.8	2.3	19.0	1274.1	172.9	2.02	25.57	23.44	1.17 ± 0.62	0.07 ± 0.04
Ter Vell (TV)	ND	5.4	24.7	12.8	117.1	0.69	60.03	67.40	1.41 ± 1.32	0.35 ± 0.03
Túries (TU)	8.8	31.3	21.1	7.6	28.9	2.06	18.84	19.54	2.97 ± 0.59	< 0.05
Fra Ramon (FR)	9.1	23.1	29.9	< 0.28	4.4	2.42	47.10	71.52	7.80 ± 0.25	0.54 ± 0.23
Doñana										
Laguna Dulce (LD)	7.5	0.8	17.9	< 0.28	50.0	2.85	35.53	35.48	3.30 ± 1.42	0.45 ± 0.22
Santa Olalla (SO)	8.2	2.8	19.4	35.1	129.0	3.97	44.46	50.71	7.63 ± 1.06	0.59 ± 0.09
Lucio del Cangrejo (LC)	6.9	4.8	21.7	< 0.28	96.2	1.97	11.36	15.35	3.01 ± 0.32	0.10 ± 0.10
Algaida (AL)	9.5	24.7	24.4	< 0.28	5.4	2.50	22.22	19.17	5.15 ± 0.21	0.28 ± 0.03

January 2007 / *May 2007* (row-group labels in left margin)

ND, not determined.

conductivity and nitrate ($r = –0.896$, $P < 0.001$) and ammonium concentrations ($r = –0.776$, $P < 0.001$), while neither nitrate nor ammonium concentration correlated with temperature. In contrast, oligohaline wetlands from the Doñana region were characterized by rather low nitrate concentrations (<0.1 mg/l).

Both the TOC and the DOC in the water were highly correlated with temperature (sampling time) when the sampled sites were considered altogether ($r = 0.525$, $P < 0.001$ and $r = 0.508$, $P < 0.001$, respectively). Sediment TC and TN were

also related to temperature ($r = 0.630$, $P < 0.001$, $r = 0.515$, $P < 0.001$, respectively) regardless of the region sampled. Water pH was always above 7.0 and was as high as 10.1 in Túries.

Nitrate + nitrite reduction activities in the rhizosphere. Nitrate + nitrite reduction rates varied significantly depending on the sampled wetland and date (Fig. 2). Measured values ranged from almost undetectable, as in Fra Ramon (the Empordà), to 0.22 ± 0.11 mg N h^{-1} (g DW)$^{-1}$ in
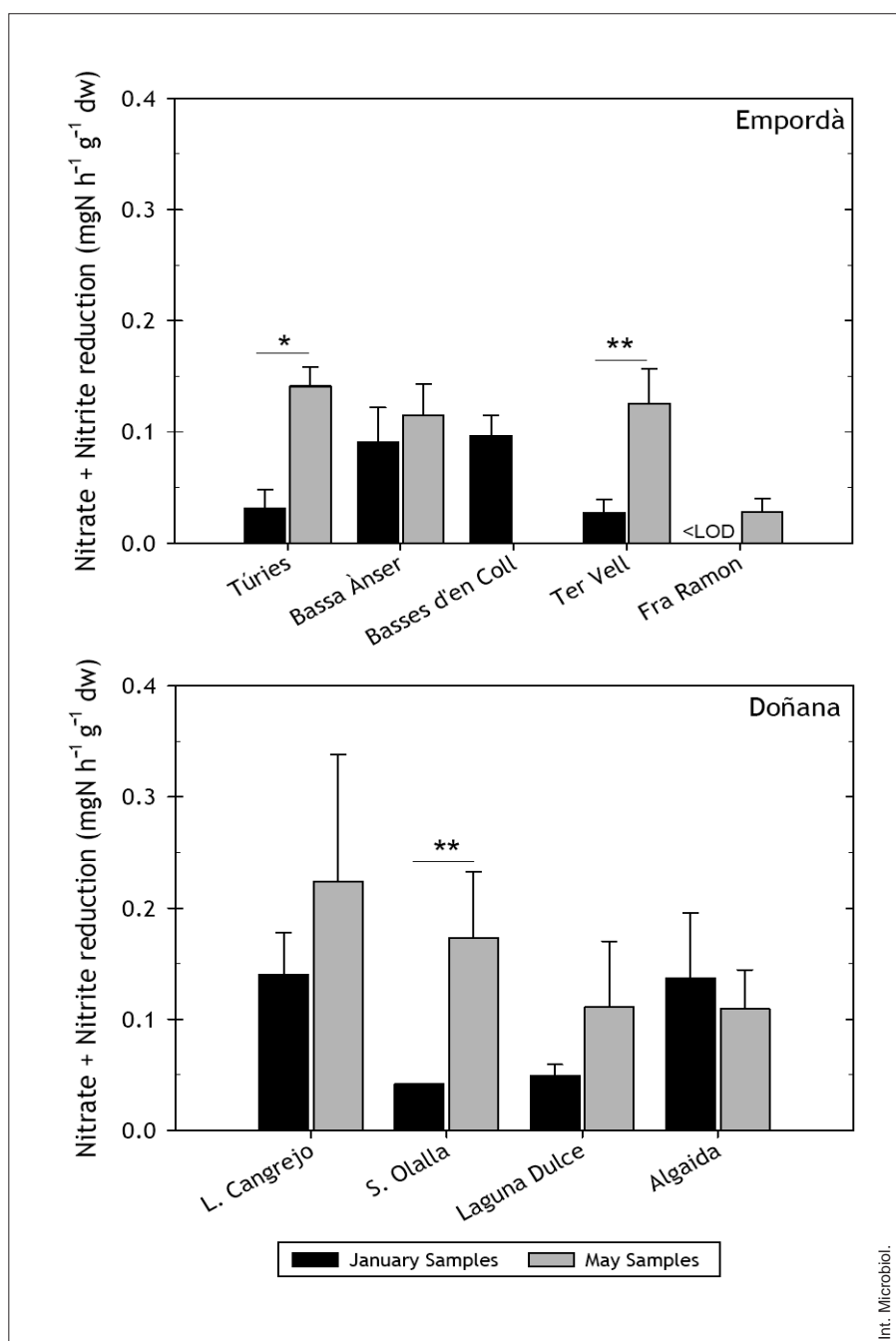
Fig. 2. Potential nitrate + nitrite reduction rates (mean ± standard deviation, n = 3) in the rhizosphere of the dominating plant species in the lagoons in January and May, in the Empordà and Doñana areas. Symbols above the bars from the same environment indicate significant differences between the two sampling occasions according to a Welch test (* $P < 0.05$, ** $P < 0.01$). <LOD, activity below detection limit.

Lucio del Cangrejo (Doñana). The largest differences in nitrate reduction rates between the two sampling dates were mainly found among environments with low nitrate concentrations. Significant differences ($P < 0.05$) were observed between Ter Vell and Túries in Empordà and Santa Olalla in Doñana. With reference to the plant species, significantly higher rates were measured in *Phragmites* sp. rhizosphere samples in the Empordà region (Welch test 4.77, $P < 0.05$), whereas the differences in the Doñana region were not significant.

The log-transformed potential nitrate+nitrite reduction rates (PNR) and the physicochemical characteristics of the water and sediments were subjected to pairwise regression analyses to determine the main contributor to the changes observed in rhizosphere activities. Water pH (Pearson correlation coefficient $r = -0.408$, $P < 0.01$) was the only variable that correlated when all samples from the two geographical areas were considered together. When the analysis was restricted to samples of the Empordà region, ammonium ($r = 0.637$, $P < 0.001$) and nitrate

($r = 0.465$, $P < 0.05$) concentrations correlated with PNR, whereas negative correlation coefficients were determined for conductivity ($r = -0.452$, $P < 0.01$), pH ($r = -0.603$, $P < 0.01$) and the DOC/nitrate ratio in water ($r = -0.395$, $P < 0.05$). In the Doñana region, positive correlations were established for TN ($r = 0.539$, $P < 0.050$) and total carbon ($r = 0.830$, $P < 0.001$) in sediments. The only significant negative correlations were TN ($r = -0.499$, $P < 0.05$) and DOC in the overlying water ($r = -0.507$, $P < 0.05$).

T-RFLP analysis of denitrifier communities.

Partial *nirS*, *nirK* and *nosZ* genes were successfully PCR-amplified from nearly all rhizosphere samples. Amplifications failed for all three genes in four samples: two replicate samples from Túries (Empordà) and one from Laguna Dulce and Lucio del Cangrejo (Doñana), all obtained in January. The T-RFLP profiles included 211, 163 and 252 different peaks for *nirK*, *nirS* and *nosZ* genes, respectively. The average numbers of TRFs for single samples were 43, 60 and 71 for *nirS*, *nirK* and *nosZ* genes, respectively. Broadly distributed T-RFLP peaks, i.e., found in more than 75 % of the samples regardless of the origin, included only five fragments for *nirK*, but 26 and 33 fragments for *nirS* and *nosZ*, respectively. T-RFLP profiles dominated by a single fragment accounting for a relative area above 40 % of the total area were commonly determined for *nirK* with all three endonucleases. Dominant peaks accounted for 90% of the area and were particularly frequent in samples from Doñana and in the *Ruppia* spp. rhizospheres in Fra Ramon and Túries in the Empordà. By contrast, individual peak areas for *nirS* and *nosZ* genes rarely exceeded 40 % of the total area.

Environmental variables affecting the *nirS*, *nirK* and *nosZ* community structure.

In analyses of the T-RFLP data, a two dimensional solution was sufficient to meet the test requirements for the NMS ordination of *nirK* genes, whereas three-dimensional solutions were needed for

Fig. 3. Non-metric multidimensional (NMS) ordinations obtained from T-RFLP profiles of *nirK* (upper) *nirS* (center) and *nosZ* (bottom) genes. The two most informative combinations of NMS axis are presented. Joint plots of environmental variables were restricted to those showing a Pearson correlation coefficient $r^2 > 0.250$ with the NMS defined axis. The percentage of variation is represented in parentheses for each NMS axis. Stress (S) values for all ordinations are indicated at the bottom. Open symbols refer to samples collected in January and filled symbols to samples collected in May. Different symbols indicate plant species (squares: *Phragmites australis*; circles: *Ruppia* sp.; triangles: *Paspalum* sp.). Names of sampled lagoons are indicated to locate clusters of replicate samples.
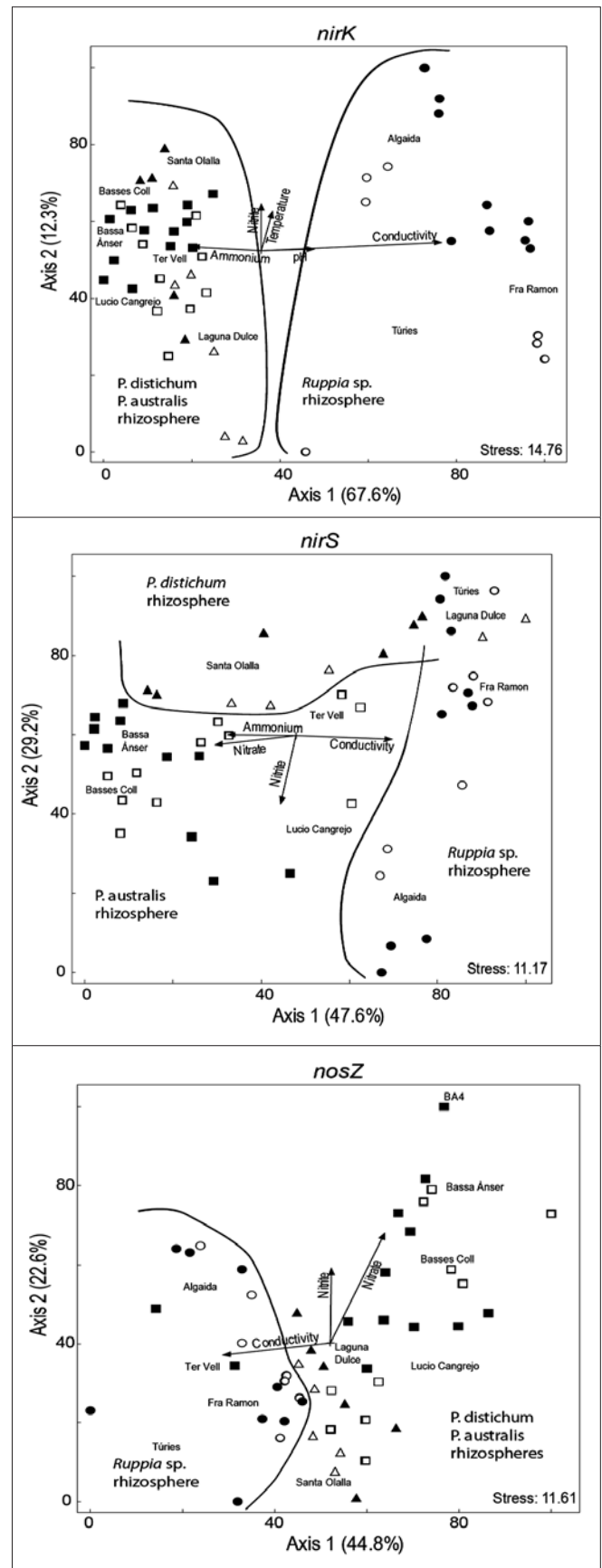
Table 2. Values of the Mantel statistic (*r*) obtained from the comparisons between dissimilarity matrices of the *nirS*, *nirK* and *nosZ* denitrifier communities and environmental and activity variables. (Statistical significances of the analyses are indicated as: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001)

	nirK		*nirS*		*nosZ*	
Water characteristics						
Temperature	0.194	***	0.158	***	−0.003	
Conductivity	0.672	***	0.487	***	0.235	***
pH	0.318	***	0.302	***	−0.031	
$N-NH_4^+$	0.467	***	0.343	***	0.030	
$N-NO_3^-$	0.275	***	0.412	***	0.319	***
DIN	0.382	***	0.453	***	0.268	***
TOC	0.030		0.033		0.122	*
DOC	0.001		0.044		0.140	**
NT	0.049		−0.001		0.138	*
Pore water and sediment						
Nitrate (pore water)	0.165	**	0.208	***	0.235	***
pH (pore water)	0.129	*	0.213	***	0.176	*
TN (%)	0.186	***	0.107	*	0.113	*
TC (%)	0.207	**	0.102		−0.013	
C/N ratio	0.328	***	0.072		−0.046	
Plant species[a]	0.582	***	0.470	***	0.234	***
Nitrate reduction activity	0.243	***	0.005		−0.027	

[a]Values were calculated from a matrix consisting of dummy variables indicating the presence (1) or absence (0) of the three plant species considered.

the *nirS* and *nosZ* genes. All three genetic markers largely grouped the samples according to plant species, although with some differences between them (Fig. 3). In addition, samples from the same lagoon and sampling time were located in proximity to each other in the ordinations, demonstrating a greater similarity among replicates than between sampling sites and occasions. Ordinations of the *nirK* community reflected the distribution of samples in two separate groups related to conductivity and plant species. The correlation between water conductivity and NMS axis 1 was strong ($r^2 = 0.816$), distinguishing this variable as the main abiotic contributor to sample separation in the *nirK* ordination. One of the groups included all samples from both geographic areas

derived from the rhizospheres of the ditch grass *Ruppia* spp. (Túries, Fra Ramon and Algaida). Rhizosphere communities derived from *Phragmites australis* and *Paspalum distichum* appeared in a heterogeneous group. Nevertheless, *Phragmites australis* replicates clustered while the *Paspalum* sp. rhizospheres were scattered, indicating that factors other than salinity and plant species have a large effect on the structure of the *nirK* denitrifiers at those sites. MRPP analyses supported the overall differentiation between plant species (A = 0.404, *P* < 0.00001), with the pairwise comparisons indicating the poorer separation between *Phragmites* sp. and *Paspalum* sp. rhizospheres (A = 0.133, *P* = 0.00004). Water temperature ($r^2 = 0.204$), a proxy for sam-

pling time, and nitrite concentration ($r^2 = 0.243$) were the main contributors to NMS axis 2. Thus, temporal differences in the denitrifier community composition in samples retrieved from the same site at different dates were indicated along axis 2. The differences depending on sampling time were significantly different in all cases according to the MRPP.

The NMS ordinations of *nirS* and *nosZ* data divided the samples into two groups as observed in the *nirK* ordination. For *nirS*, water conductivity and nitrate concentration correlated in opposite directions with the NMS axis 1 ($r^2 = 0.433$ and 0.366), while samples from plant rhizospheres of *Ruppia* sp. and *Phragmites australis* appeared as two separated groups. Significant differences between groups of different plant species were detected according to the MRPP (A = 0.310, $P < 0.00001$). Nitrite concentration was the only variable that significantly correlated with NMS axis 2 ($r^2 = 0.344$) for *nirS*, whereas temperature and pH correlated with the NMS axis 3 ($r^2 = 0.330$ and 0.295, respectively). Thus, although salinity contributed to the community structure of NirS-type denitrifiers, it was less important than for NirK-type denitrifiers. In the NMS ordination of *nosZ*, the samples were more scattered, although there was a tendency for the separation of *Phragmites australis* and *Ruppia* sp. samples along axis 1, which correlated with conductivity ($r^2 = 0.464$). The MRPP resulted in a significant separation of samples in relation to plant species, but the value of the test statistic A (A = 0.196, $P < 0.00001$) was lower than that obtained for *nirS* and *nirK* genes. The influence of nitrate was observed along NMS axis 2 ($r^2 = 0.555$) and a pattern of separation related to nutrient content was suggested.

The relative importance of environmental variables for the structure of the denitrifier community was confirmed by the results of the Mantel test (Table 2). The *nirS* and *nirK* communities were similarly affected by the same variables; the exception was TC and the C/N ratio in sediments, which were only significant for the *nirK* denitrifiers. By contrast, the differences among *nosZ*-harboring communities were influenced by differences in TOC, DOC and TN, which had no significant correlation with differences among *nirK* or *nirS* communities. For all three genes, significant correlations were found when dissimilarity matrices were compared with the presence or absence of the studied emergent plant species, which indicates that plant species influenced the denitrifier community structure.

The differences in denitrifier community composition based on the three different T-RFLP profiles correlated with each other, such that all three marker genes were able to capture the major differences among samples. However, only differences among *nirK* denitrifiers significantly correlated with differences in the potential nitrate reduction activity on the rhizosphere.

Discussion

In the present study, the contribution of ecological drivers in natural coastal wetlands to the nitrate reduction capacity and the structure of denitrifying bacterial communities in the rhizosphere of emergent aquatic vegetation were explored. The variation in nitrate reduction capacity in the rhizospheres was partly explained by nitrate content in the water and sediment although differences between the two sampled areas were determined. The activity was significantly higher in May in environments with relatively low nitrate levels and higher DOC content, i.e., Ter Vell and Túries in Empordà and Santa Olalla in Doñana. Temporal differences in nitrate reduction potential were reflected by the denitrifier community structure. A closer look at the NMS ordinations suggested a temporal effect on the community composition in the rhizosphere samples, which was supported by the significant results of the MRPP when all lagoons were considered separately.

Seasonal or temporal effects on the composition of denitrifying communities have been documented both in soils and wetland sediments [3,5,32,37]. Nevertheless, most soil studies have not been able to link the structure and function of denitrifier communities, and recent studies have instead shown that denitrification enzyme activities correlated with the size of the denitrifier community [14,28].

Similar to our findings, effects of different aquatic plants on the structure of denitrifying community in the rhizosphere have been reported in constructed freshwater wetlands [1,32]. The rhizosphere effect on the composition of the denitrifying communities could be due to differences in organic root exudates, which have been shown to influence the abundance and diversity of microorganisms [26]. Nevertheless, when using different artificial root exudates, Henry et al. [15] found no major effect on the composition of the denitrifying community. In the present study, the observed plant species-specific effect was likely driven by the large differences in salinity, since salinity determines the presence of specific wetland vegetation [25]. However, salinity has also been shown to have a significant effect on the denitrifier community structure [18]. The significant correlation between nitrate and ammonia concentrations in water and the community structure indicates that eutrophication, which is a major

threat to coastal wetlands, can be an additional selective factor acting on denitrifying communities in these ecosystems.

The simultaneous analysis of the denitrifier community using three independent functional genes revealed dissimilarities that reflected the different contributions of the same ecological drivers on the communities of the *nirS* and *nirK* type denitrifiers as well as those carrying *nosZ*. Plant species was related to the structure of all three communities. However, the number of TRFs shared among more than 75 % of the samples was rare in the *nirK* communities and they were often dominated by a single genotype, whereas the *nirS* and *nosZ* communities displayed a more even distribution, and individual genotypes were frequently shared among samples. This indicates a stronger plant species effect on the *nirK* communities than on the *nirS* and *nosZ* communities. Accordingly, *nirK* genes are preferentially found among denitrifiers belonging to typical rhizosphere bacteria. NirS and NirK nitrite reductases are functionally equivalent, but mutually exclusive [38]. It is therefore a matter of debate whether *nirS* or *nirK* type denitrifiers are selected by environmental factors according to differences in their ecology [9,18,28]. Habitat selection on the two different nitrite reductases was observed in soil when the spatial distribution of *nirK* and *nirS* communities and the *nirS:nirK* abundance ratio were mapped and related to soil properties across arable fields [9]. Also, Knapp et al. [21] have shown that the spatial distribution of *nirK* and *nirS* gene abundance reflects different habitat preferences in a stream. In the samples analyzed here, the community structures of both *nirK* and *nirS* type denitrifiers were influenced by almost the same variables, which does not support different community assembly rules for co-existing *nirK* and *nirS* denitrifier communities.

In water, the carbon content, either organic or inorganic, was a significant factor defining the structure of the *nosZ*-harboring community, but no effect could be detected on the *nirK* and *nirS* communities. In agreement with this observation, the abundances of these three genes in soils have been shown to be affected by environmental factors in different ways, indicating that growth and selection of denitrifying bacteria may occur as a heterogeneous response to the same variable [16,28]. Many bacterial isolates that harbor either *nirS* or *nirK* genes lack the *nosZ* gene [7,19], and the *nosZ* gene is frequently detected in lower abundances than *nir* genes in the environment [4,11,27]. This may have ramifications for emissions of the greenhouse gas nitrous oxide. Further work is needed to determine whether there are significant differences in the ecological drivers acting on the denitrifiers carrying *nosZ* as compared to the total denitrifying community, as the sum of *nirK* and *nirS* communities.

If so, their potential implications for nitrous oxide emissions from coastal wetlands should be explored as well.

In conclusion, the nitrate+nitrite reduction potential in the rhizosphere of emergent aquatic plants from coastal lagoons was found to be mainly governed by the nitrate concentration in water, while the community composition of denitrifying bacteria showed a plant species-specific effect. Nevertheless, this effect could not be completely separated from water or sediment characteristics, such as salinity and carbon and nitrogen contents. The results obtained by using three molecular markers to study the community composition of *nirS* and *nirK* type denitrifiers in addition to those also carrying *nosZ* suggests that there are different populations with different responses to environmental conditions. This was most evident when the results obtained for the nitrite reductase genes were compared with *nosZ*.

Competing interests: None declared.

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