Microb Ecol (2008) 55:259–269
DOI 10.1007/s00248-007-9273-7

ORIGINAL ARTICLE

Dynamics of Nitrous Oxide Reductase Genes (*nosZ*) in Intertidal Rocky Biofilms and Sediments of the Douro River Estuary (Portugal), and their Relation to N-biogeochemistry

C. Magalhães · N. Bano · W. J. Wiebe · A. A. Bordalo · J. T. Hollibaugh

Received: 12 September 2006 / Accepted: 6 May 2007 / Published online: 1 July 2007

Abstract In this study, temporal variability of *nosZ* genotypes was evaluated in two intertidal rocky biofilms and two intertidal sediment sites of the Douro River estuary, Portugal. The results were compared to rates of key N-cycle processes and environmental variables to examine possible links between denitrifier community dynamics and N biogeochemistry. Genetic heterogeneity of the *nosZ* gene was evaluated by terminal restriction fragment length polymorphism analysis (T-RFLP) and by sequencing cloned *nosZ* gene fragments. Phylogenetic analysis showed that the majority of the *nosZ* genes detected were most similar to *nosZ* genes from isolates affiliated with alpha-subclass of the class Proteobacteria. Results revealed low *nosZ* genotype richness, and hierarchical cluster analysis showed significant differences in the composition of denitrifier communities that inhabit different intertidal environments of the Douro River estuary. Monthly surveys of *nosZ* genotypes from sandy sediments showed that, while the same T-RFLP peaks were present in all samples, shifts in the relative peak areas of the different *nosZ* genotypes occurred. Canonical correspondence analysis, based on data from the monthly survey, revealed a strong relationship between the relative peak areas of some T-RFLP operational taxonomic units (OTUs) with denitrification rate and NO_3^- availability. Results suggest that denitrifiers with specific *nosZ* genotypes (OTUs) have competitive advantage over others when NO_3^- fluctuates in the system; these fluctuations reflect, in turn, variability in denitrification rates.

C. Magalhães (✉) · A. A. Bordalo
Laboratory of Hydrobiology, Institute of Biomedical Sciences, University of Porto,
Largo Professor Abel Salazar, No 2,
4099-003 Porto, Portugal
e-mail: c_magalhaes@yahoo.com

C. Magalhães · A. A. Bordalo
Centre of Marine and Environmental Research,
Rua dos Bragas, no 289,
4050-123 Porto, Portugal

N. Bano · W. J. Wiebe · J. T. Hollibaugh
Department of Marine Sciences, University of Georgia,
Athens, GA 30602, USA

Introduction

Denitrification involves the stepwise reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to produce the gaseous products nitric oxide (NO); nitrous oxide (N_2O); and, mainly, dinitrogen (N_2) under suboxic conditions. In marine ecosystems, this process is of global concern because it directly impacts nitrogen availability to primary producers and may ultimately contribute to the global warming through formation of N_2O [12, 13, 31]. Denitrification is also responsible for nitrogen losses from agricultural soils and is important in wastewater treatment as the process primarily responsible for removing fixed nitrogen. Given the importance of denitrification, much attention has been given to evaluating the biogeochemical dynamics of this process in a wide variety of environments [e.g., 1, 4, 11, 30].

Because denitrification is mediated by physiologically diverse groups of prokaryotes [42, 43], determining the factors limiting the distribution and controlling the dynamics of denitrifiers is essential for understanding the ecosystem-level controls on the biogeochemical process of denitrification. Previous efforts have been directed

toward amplification of functional genes from environmental samples involved in denitrification, mainly nitrite reductase genes (*nirS* and *nirK*) [6, 8, 33, 40, 41], the gene encoding nitrous oxide reductase (*nosZ*) [14, 25, 28, 34] and nitric oxide reductase (*norB*) [5]. These PCR-based approaches have revealed the presence of diverse denitrifier communities in different natural ecosystems, including continental shelf and estuarine sediments [7, 15, 21, 29], the marine water column [8], meadow and forest soils [23, 25, 26], activated sludge systems [33, 41], and cultivated soils [32, 34, 36, 39]. Most of these studies emphasize the distribution in time and space of denitrifier assemblages [3, 8, 15, 41]. Although knowledge of the environmental controls on denitrifying communities is emerging, [3, 8, 25, 39, 41], little is known about the relationship between denitrifier community structure and denitrification rates or N_2O fluxes in natural environments [14, 25]. We have examined these questions using samples from the Douro River estuary, Portugal, to gain insights into the functional significance of denitrifier diversity.

We hypothesized that different denitrifiers, as indicated by *nosZ* genes, would be associated with different intertidal environments of the Douro River estuary and that denitrifier community structure, environmental variables, and rates of N-processing would be related. We sought to test this hypotheses by investigating temporal variability of *nosZ* genotypes at four different estuarine habitats of the Douro River estuary: two intertidal rocky biofilm sites and two intertidal sediment sites; then comparing assemblage composition with rates of nitrogen biogeochemical processes (net NO_3^-, NO_2^-, N_2O, and N_2 fluxes) and environmental variables, using data from a previous study [18, 19].

Materials and Methods

Site Description

The Douro River estuary is a 21.6-km-long mesotidal estuary [37] on the NW coast of Portugal. Physical and chemical characteristics of this estuary have been presented previously [17]. The total area of the estuary is 7.5 km^2, of which approximately 1.5 km^2 is intertidal sand and muddy sediment and 0.39 km^2 is intertidal natural and man-made hard surfaces. The hard surfaces support extensive microbial biofilms overlain by macroalgal canopies of *Enteromorpha* spp. and/or *Fucus* spp. This study was conducted in the four dominant intertidal environments of the lower estuary: two intertidal rocky areas colonized by *Enteromorpha* spp. and *Fucus* spp. (EZ and FZ, respectively), and two intertidal sediments (sandy and muddy; SZ and MZ, respectively).

Sampling

Quarterly (summer, fall, winter, and spring) sampling was performed at all sites between 2001 and 2002; an additional monthly survey was conducted at SZ between February 2002 and January 2003. During these sampling programs, two cores (3 cm in diameter and 10 cm long) were collected at the sediment stations and biofilm samples (approximately 80 g wet weight) were retrieved from the rocky sites by scraping the rocks. Samples from each site were homogenized, stored in sterile plastic bags, transported to the laboratory in the dark in refrigerated ice chests, and then immediately frozen and stored at −70°C until DNA extraction. During the same period of time and at the same sites, denitrification, net N_2O fluxes, net inorganic nitrogen fluxes (NO_3^-, NO_2^-), N_2O/N_2 flux ratios (ratio of the production of N_2O to N_2), and some environmental variables were evaluated (Table 1) [18, 19]. These data were presented in a different context in previous papers where the details of the methods used are given [18, 19].

DNA Extraction and PCR Amplification

DNA was extracted from 1 to 1.5 g wet weight of homogenized sediment or biofilm samples using the Ultra Clean Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA). Fragments of *nosZ* genes were amplified with the primer set Nos661F (5′-CGGCTGGGGGCTGACCAA), fluorescently labeled at the 5′ end with 6-carboxyfluorescein [7], and Nos1773R (5′-ATRTCGATCARCTGBTCGTT), which amplify ~1,100 bp of the *nosZ* gene [27]. Primers used in PCR were synthesized by Operon Technologies (Oakland, CA, USA). DNA sample amplification was performed using Ready-to-Go PCR beads (Amersham Biosciences, Buckinghamshire, UK) in reactions containing 20 ng of template DNA and 2.4 μM of each primer in a final volume of 25 μl. All PCRs were performed with initial denaturation at 95°C for 5 min, followed by 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min. DNA from *Pseudomonas stutzeri* was used as a positive control; a PCR reaction mixture with all reagents except template DNA served as the negative control. PCR products were separated by agarose gel electrophoresis; the gels were stained with ethidium bromide, and a band of the correct size (~1,100 bp) was extracted and purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). The concentration of the resulting PCR purified product was measured by fluorometry, using the Hoechst dye assay [22].

Terminal Restriction Fragment Length Polymorphism Analysis

Approximately 300 ng of purified PCR product from environmental samples and 50 ng of PCR product from

plasmid DNA from the clone libraries were digested for 6 h at 37°C in a final volume of 10 μl with 10 U of *Hin*P1 restriction enzyme (5′...G¡CGC...3′; New England Biolabs, Ipswich, MA, USA). The digested DNA was precipitated in 0.1 vol of 3 M sodium acetate and 2 vol of ice-cold ethanol followed by centrifugation at 12,000×*g* for 15 min. The DNA pellet was dried in a DNA SpeedVac (Savant). Immediately before analysis, the pellet was resuspended in a mixture of 12 μl of deionized formamide and 1 μl of GeneScan 2500 TAMRA size standard (Applied Biosystems, Foster City, CA, USA) then denatured for 5 min at 95°C and immediately placed on ice for at least 3 min. To assess the level of variation of terminal restriction fragment length polymorphism (T-RFLP) technique, two T-RFLP profiles were generated from independent PCRs for each clone and environmental sample. Comparison of these duplicates confirmed high reproducibility of peak number and relative height.

Restriction fragments were separated and detected using an ABI Prism 310 genetic analyzer in Gene-Scan mode, which displays the various 5′ terminal restriction fragments as a series of peaks. Each terminal restriction fragment, represented as a peak in the electropherogram, was treated as an operational taxonomic unit (OTU) identified by the length, in base pairs, of the fragment. The area of the peak for each fragment was expressed as a percentage of the total area of all peaks in each T-RFLP profile and used to estimate the relative abundance of each terminal restriction fragment in the sample. Fragments with a signal below 1.5% of the total area were removed from the analysis [25].

Clone Libraries and Phylogenetic Analysis

To identify the *nosZ* sequences that correspond to the OTUs identified in T-RFLP profiles, *nosZ* gene fragments from seven environmental samples (EZ summer, fall, winter, and spring; FZ spring; MZ spring; and SZ spring) were amplified and the PCR product obtained was gel purified as described above. The amplicons were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with the exception of slight modifications (vector DNA and salt solution was decreased to 0.5 μl and chemically competent cells decreased to 23 μl). Approximately 10 colonies were selected randomly from the library of each sample, and a total of 69 clones were sequenced on an automated sequencer in the core sequencing facility of the University of Georgia. The clones were also screened using *Hin*P1 T-RFLP analysis as described above. Nucleotide sequences were aligned and converted to inferred amino acid sequences using the Genetic Computer Group package (Madison, WI, USA). Nucleotide and amino acid sequences were compared to known sequences using BLASTX [2]. The inferred amino acid tree was constructed with PHYLIP using Kimura distances and the neighbor-joining method with a bootstrap analysis of 100 replicates (MEGA package version 2). The clone sequences have been deposited in GenBank under the accession numbers DQ311680 to DQ311686.

Statistical Analysis

Hierarchical cluster analysis was used to detect intersite differences in denitrifier assemblages. Data on relative area of the *nosZ* gene T-RFLP peaks was 4th-root transformed, and then Bray–Curtis similarities were calculated. The ANOSIM test [10] was used to test the statistical significance of differences between the different clusters generated. Principal components analysis (PCA) was applied to the environmental and biogeochemical variables measured during the monthly sampling program (Table 1). The software package PRIMER version 5 [10] was used to perform the latter multivariate statistical analysis. Relationships between *nosZ* gene assemblages, environmental variables, and rates of N-processes measured during the monthly sampling program (Table 1) were analyzed with canonical correspondence

Table 1 Sediment net N_2 and N_2O fluxes, N_2O/N_2 ratios, and water column $NO_3^- + NO_2^-$ concentrations and salinity during the monthly sampling survey at the SZ site (mean± SDV of three replicates)

	Net N_2 fluxes (nmol g wet sed^{-1} h^{-1})	Net N_2O fluxes (nmol g wet sed^{-1} h^{-1})	N_2O/N_2	$NO_3^- + NO_2^-$ (μM)	Salinity (psu)
Feb 2002	2.81±0.21	0.19±0.02	6.03±0.27	34.5±1.1	22.7
Mar 2002	2.62±0.05	0.20±0.06	7.02±0.12	31.9±0.9	15.1
Apr 2002	3.09±0.31	0.03±0.03	1.11±0.10	39.6±0.3	16.0
May 2002	3.07±0.05	1.08±0.50	0.66±0.03	21.5±0.1	24.0
Jun 2002	0.96±0.02	0.01±0.00	0.65±0.03	5.2±0.3	26.8
July 2002	0.74±0.04	0.00±0.00	0.65±0.03	12.8±0.2	25.0
Aug 2002	6.17±0.47	0.08±0.04	0.14±0.03	65.0±0.6	13.9
Sep 2002	3.05±0.18	0.02±0.02	0.78±0.06	36.0±0.5	13.2
Oct 2002	0.85±0.04	0.01±0.01	0.78±0.06	20.6±1.1	22.7
Nov 2002	4.41±0.33	0.01±0.01	0.30±0.00	59.1±1.8	9.0
Dec 2002	8.36±0.57	0.02±0.02	0.24±0.03	59.9±1.0	3.4
Jan 2003	8.75±0.39	0.13±0.13	1.48±0.07	92.0±2.8	1.5

Data from Magalhães *et al.* [18].

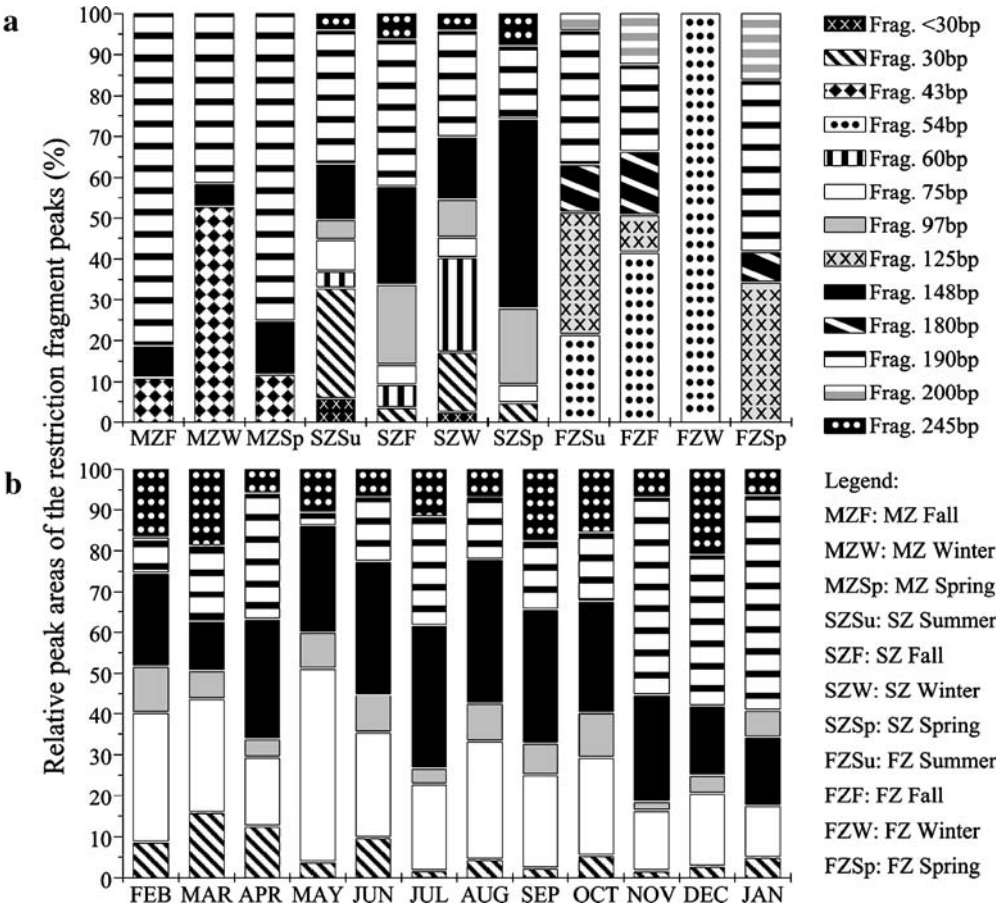

Figure 1 Spatial and seasonal variation of OTU distributions derived from replicate T-RFLP analysis of *nosZ* amplicons digested with *Hin*P1 restriction enzyme. **a** Seasonal (summer, fall, winter, spring) sampling program performed at MZ, SZ, and FZ sites during 2000/2001. **b** Monthly sampling program performed at SZ site during 2002/2003

analysis (CCA) [35], using the software package CANOCO (version 4.5, Microcomputer Power, Ithaca, NY, USA) [35]. For CCA, the relative areas of T-RFLP peaks for each sample were log transformed, and the environmental variables entered into the CCA in normalized form (i.e., adjusted for a mean of 0 and SD of 1). We used a Monte Carlo permutation test to assess the statistical significance of relationships. In the CCA ordination diagram, the angle and length of the arrow relative to a given axis reveals the extent of correlation between the variable and the canonical axis (environmental gradient). The position of different T-RFLP peaks on an axis reveals their association with the environmental gradient (i.e., increasing

Table 2 Correspondence between *nosZ* T-RFL, T-RFLP relative peak area in spring samples from 2000/2001 sampling program, *nosZ* clone libraries from spring samples, and their relative percentage in clone libraries [(number of clones with the same DNA sequence/total number of clones)×100]

T-RFL (bp)	T-RFLP frequency (%)			Clone identifier	Clone libraries (%)			Cluster identifier
	SZsp	MZsp	FZsp		SZsp	MZsp	FZsp	
<30	–	–	–	–	–	–	–	–
30	5	–	–	SZ3, SZ5, SZ6	27	–	–	B
43	–	12	–	MZ1, MZ4	–	22	–	A
54	–	–	–	–	–	–	–	–
60	–	–	–	–	–	–	–	–
75	3	–	–	–	–	–	–	–
97	19	–	–	–	–	–	–	–
125	–	–	34	FZ2, FZ5, FZ7, FZ9	–	–	45	F
148	46	13	–	SZ1, SZ2, SZ4, SZ7, SZ10, SZ11, MZ3	55	11	–	E
180	–	–	8	–	–	–	–	–
190	18	75	41	MZ2, MZ5, MZ6, MZ7, MZ8, MZ9, SZ8, FZ3, FZ8	9	67	22	G
200	–	–	16	FZ1, FZ4, FZ6	–	–	33	C
245	9	–	–	SZ9	9	–	–	D

relative abundance of the T-RFLP peak with high or low values for certain environmental variables).

Results

Distribution of *nosZ* OTUs and Genotypes

A total of 13 distinct OTUs, defined as constrained above, were identified in our T-RFLP profiles from all stations and times. Samples from the SZ site always contained more OTUs (between 6 to 8, Fig. 1a,b) than the MZ and FZ sites, which contained three and one to five per sample, respectively (Fig. 1a). OTUs 148 and 190 were detected in the greatest number of samples (Fig. 1a). OTUs 54, 125, 180, and 200 were unique to the FZ site and were present in all FZ samples except those collected in winter, when only OTU 54 was detected (Fig. 1a). OTUs <30, 30, 60, 75, 97, and 245 were only found in samples from the SZ site (Fig. 1a,b), whereas OTU 43 was only observed in MZ samples (Fig. 1a).

Phylogenetic analysis of sequences from cloned *nosZ* amplicons and comparison with *nosZ* sequences available

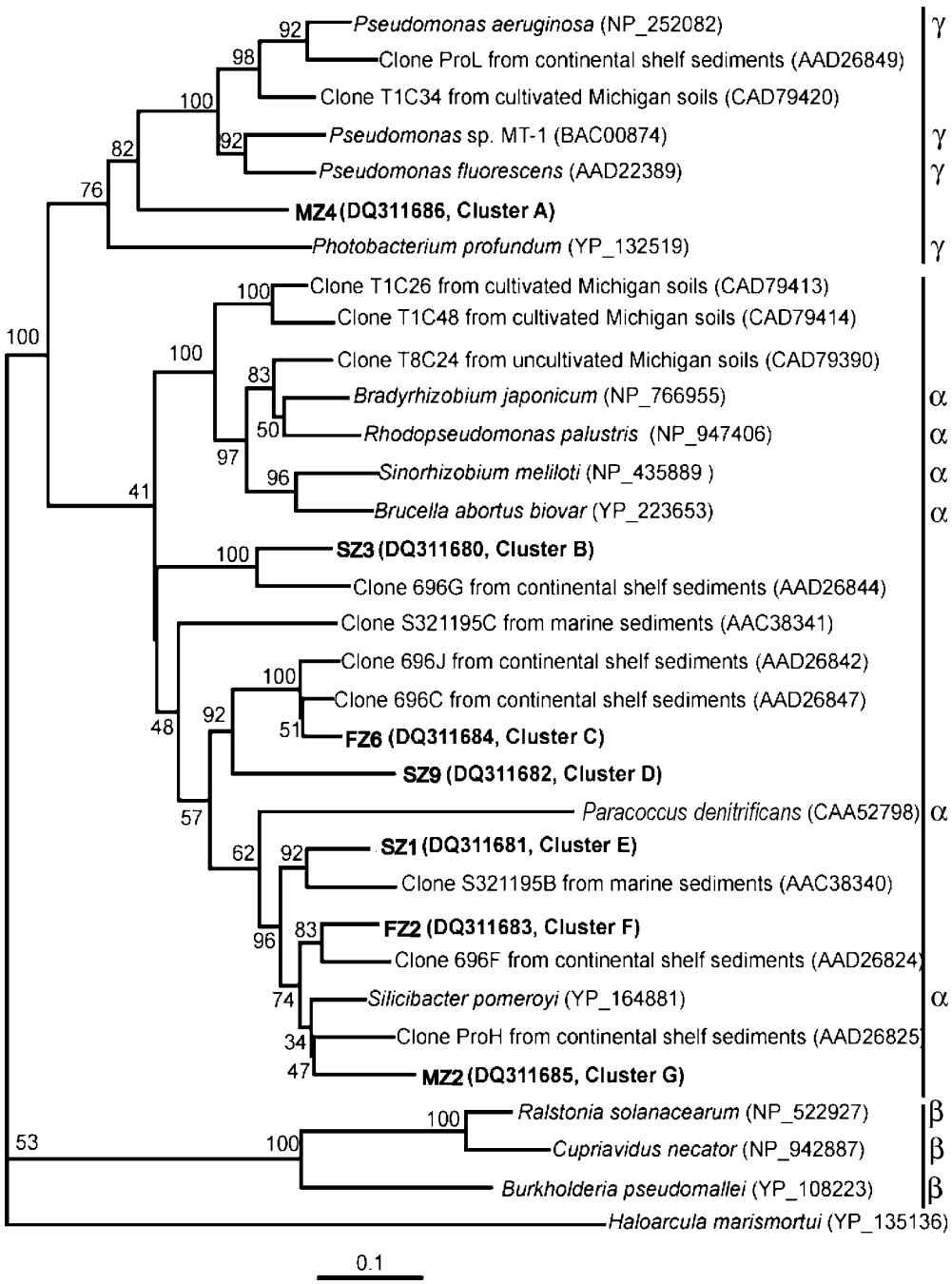

Figure 2 Phylogenetic analysis of partial sequences of *nosZ* genes retrieved from our samples. Major clusters are identified by *capital letters* and are related to T-RFLP OTUs in Table 2. The tree is based on sequences of 341 deduced amino acids and was constructed based on Kimura distances and the neighbor-joining method, with *Haloarcula marismortui* as the outgroup. Clones obtained from this study are shown in *boldface*. A single sequence was chosen to represent all sequences in clusters consisting of >99.5% identical sequences. Bootstrap values (100 iterations) greater than 50% are shown

in the GenBank revealed that they were homologous to known *nosZ* sequences and fell into seven distinct clusters of sequences (clusters A to G, Table 2 and Fig. 2). The sequences in each cluster were remarkably similar, between 99.5 and 100% identity (data not shown). We considered amino acid sequence divergences less than 0.5% to be error inherent to PCR or cloning and thus not indicative of a novel *nosZ* gene. Thus, we considered that each cluster of cloned sequences (Table 2) represented a distinct *nosZ* phylotype. Similarities of different *nosZ* gene clusters (A to G) ranged between 55 and 84%. *NosZ* genes detected in this study were most similar to *nosZ* from bacterial isolates affiliated with the alpha subclass of the class Proteobacteria (Fig. 2). Exceptions were two 99.9% similar DNA sequences (100% similar amino acid sequences) from the MZ site (cluster A) that fell into the group of *nosZ* genes from the gamma-subclass of the class Proteobacteria (Fig. 2). Cluster A sequences were 71–73% similar to *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* and two environmental sequences PROL and T1C34 (Fig. 2), retrieved from samples of continental shelf sediments and cultivated Michigan soils [28, 34]. The *nosZ* clade, where several cultured bacterial sequences from alpha subclass of the class Proteobacteria are included, contains three main subclusters; one includes sequences from *Brucella abortus*, *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, and *Rhodopseudomonas palustris* and environmental sequences retrieved from cultivated Michigan soil [34]. The second includes cluster B sequences and does not contain any cultured alpha-Proteobacteria, and the third contains sequences from *Silicibacter pomeroyi* and *Paracoccus denitrificans*, as well as environmental sequences retrieved predominantly from Atlantic and Pacific Ocean sediments [28]. Most of the Douro River estuary *nosZ* gene sequences were included in the third subcluster (Fig. 2).

Seven of the 13 distinct peaks in T-RFLP profiles from all environmental samples were identified by clone analysis. These sequences represented 54% of the total number of different T-RFLP peaks found, and, generally, the relative occurrence of different phylotypes in clone libraries agree with the relative abundance of T-RFLP fragments from the same environmental samples (Table 2). The remaining unidentified OTUs were found in samples from SZ and FZ sites (Table 2 and Fig. 1a). None of the PCR products of the correct length (1,100-bp fragment; a total of 40 sequences was examined) cloned from samples collected at the EZ site was identified as a *nosZ* gene sequence by BLASTX search; therefore, no T-RFLP results for EZ were shown.

Inter- and Intrasite Distribution of *nosZ* Genotypes

Hierarchical cluster analysis of OTU distributions showed that denitrifier assemblages differed among sites (Fig. 3a). The ANOSIM test revealed statistically significant differences between the three clusters generated ($n=11$, $R=0.76$, $p=0.002$). Hierarchical cluster analysis showed that denitrifier assemblages from the intertidal sediment sites were more similar to each other than to the FZ samples; the FZ samples clustered separately at a similarity level of 41%, whereas the MZ and SZ samples were 50% similar (Fig. 3a). Analysis of monthly variability of *nosZ* genotypes over a year at the SZ site indicated that the composition of the denitrifier assemblage at this site is stable over time (Fig. 1b); the same T-RFLP peaks were observed in all samples, although in different relative proportions (Fig. 1b).

N-biogeochemistry and Intrasite Variation in *nosZ* Genotypes

Two-dimensional PCA was applied to environmental and N-processing variables (Fig. 4) measured at the SZ site during the monthly sampling program (Table 1). PCA1 and PCA2 together explained 96% of the total variability in the variables included in the analysis (Fig. 4). While salinity, $NO_3^- + NO_2^-$ concentrations, and denitrification rates were weighted heavily in PCA 1 (with eigenvectors of −0.55, 0.57, and 0.56, respectively), the N_2O/N_2 flux ratio, which evaluate the magnitude of the N_2O accumulation during denitrification, and net N_2O fluxes were weighted heavily in PCA 2 (with eigenvectors of 0.72 and 0.67, respective-

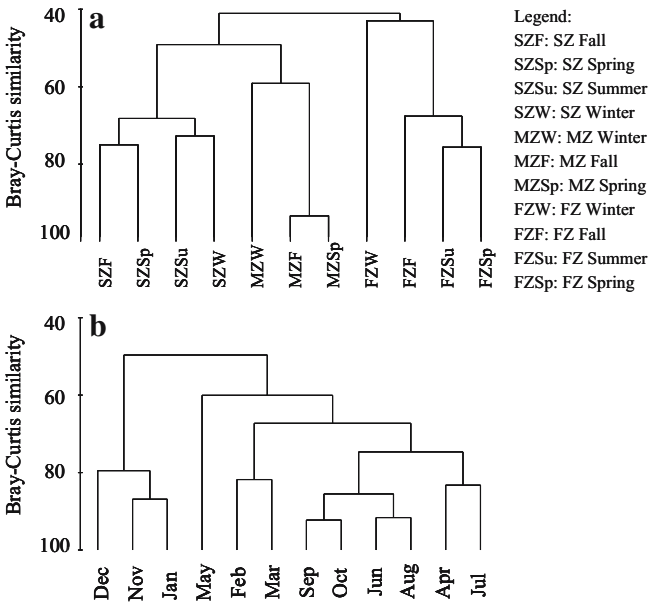

Figure 3 Dendrogram for hierarchical clustering analysis based on group-average linking of Bray–Curtis similarities calculated from 4th-root-transformed relative area of the *nosZ* gene T-RFLP peaks. **a** Seasonal (summer, fall, winter, spring) sampling program performed at MZ, SZ, and FZ sites during 2000/2001. **b** Monthly sampling program performed at SZ site during 2002/2003

ly). Superimposition of denitrification rates (Fig. 4a), NO_3^- + NO_2^- availability (Fig. 4b), and salinity (Fig. 4c), as circles of different sizes, on the PCA plot shows that samples from August, November, December, and January were associated with higher denitrification rates, higher NO_3^- + NO_2^- concentrations, and lower salinity relative to the other samples (Table 1). Samples from February and March were characterized by higher net N_2O fluxes (Fig. 4d) and N_2O/N_2 flux ratios (higher accumulation of N_2O in relation to N_2; Fig. 4e) and May, June, July, and October samples by low denitrification rates and low NO_3^- + NO_2^- concentrations (Fig. 4a,b). The ANOSIM test revealed statistically significant differences between these three different groups generated by PCA analysis (n=12, R=0.74, p=0.002). Hierarchical cluster analysis based on the relative area of *nosZ* gene T-RFLP peaks (Fig. 3b) grouped November, December, and January samples together.

Correlations between environmental variables, N biogeochemistry, and *nosZ* assemblages were examined using CCA (Fig. 5). All T-RFLP data from the monthly samplings at site SZ (Fig. 1b) were included in the CCA. The first two CCA axes explained 90.5% of the total cumulative *nosZ* genotype variance and accounted for 90.2% of the cumulative variance of the *nosZ* genotype–environment relation. In this analysis, all individual environmental variables contributed significantly to explaining *nosZ* genotype distributions, and the contribution of the combined variables was significant as well (F=2.628 and p=0.026, Monte Carlo permutation test). The variables that correlated most strongly with CCA 1 were denitrification rates and N_2O/N_2 flux ratios, whereas salinity, NO_3^- + NO_2^- concentration, and net N_2O fluxes correlated best with CCA 2 (Fig. 5). The relative abundance of *nosZ* genotypes from cluster G was highly correlated with denitrification rates

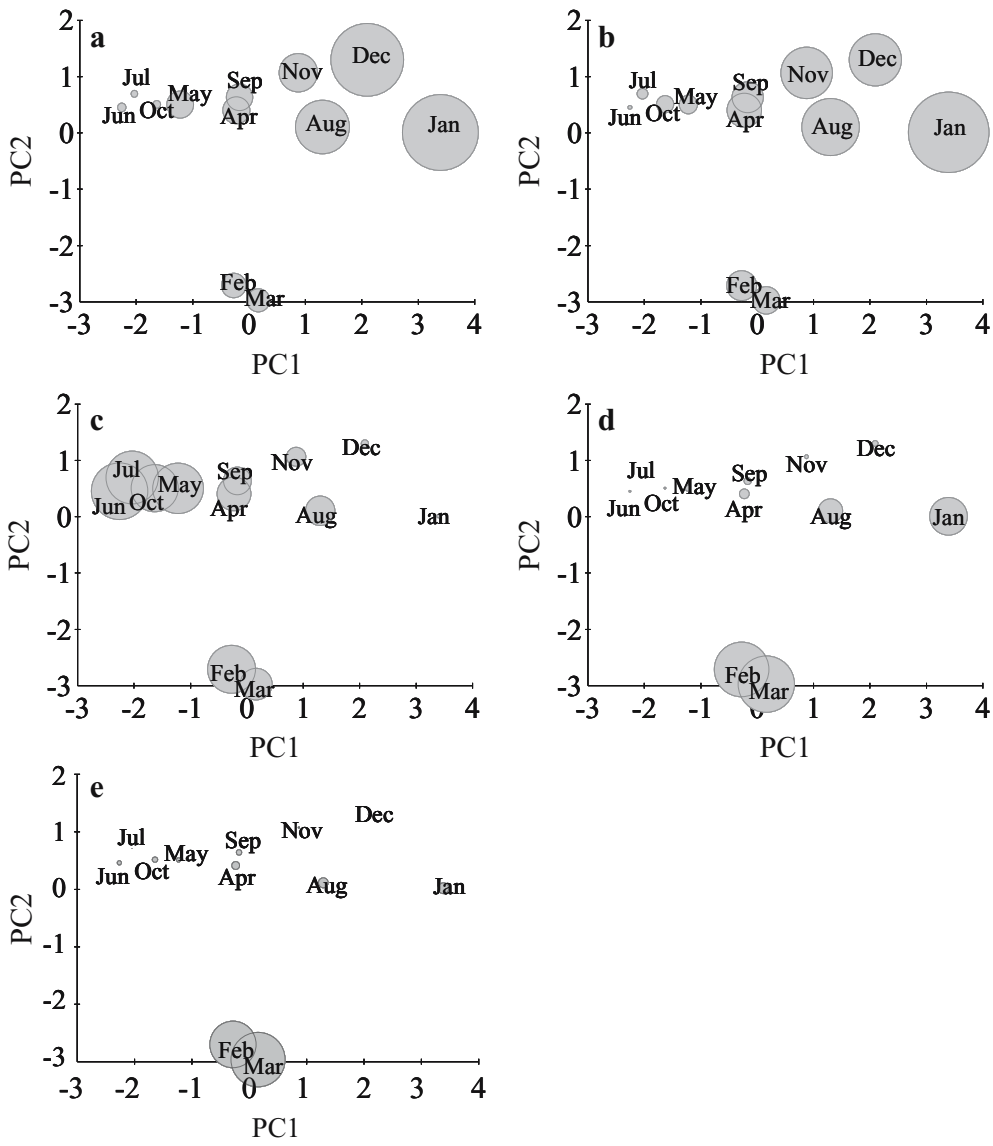

Figure 4 PCA of five nitrogen biogeochemistry and environmental variables measured monthly at the SZ site during 2002/2003 [18] (**a**–**d**). Values of denitrification rates (**a**), NO_3^- + NO_2^- concentrations (**b**), salinity (**c**), net N_2O fluxes (**d**), and N_2O/N_2 flux ratios (**e**) for each sample are represented as *circles* of a diameter proportional to the magnitude of the value

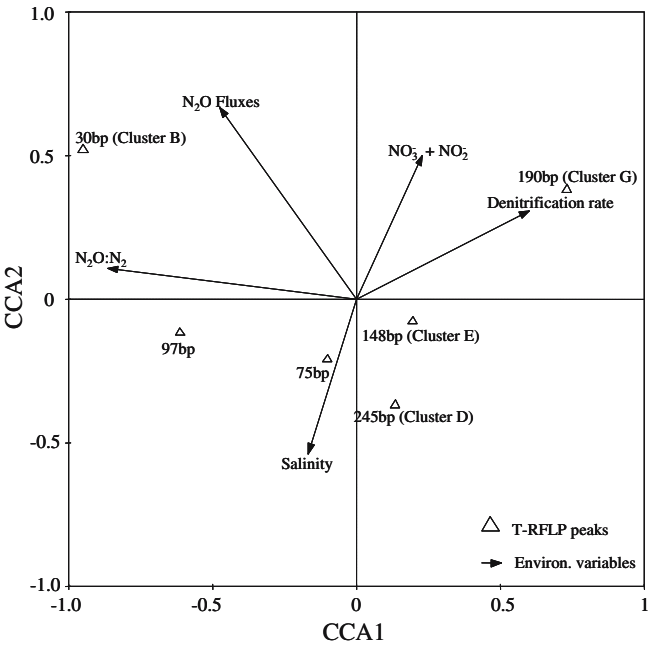

Figure 5 CCA ordination plot for the first two dimensions of a CCA of the relationship between the distribution of T-RFLP peaks and denitrification rates, net N_2O fluxes, N_2O/N_2 ratios, water column NO_3^- + NO_2^- concentration, and salinity. Correlations between environmental variables and CCA axes are represented by the length and angle of *arrows*

and NO_3^- + NO_2^- concentrations (Fig. 5). CCA analysis also suggests that the organisms carrying the *nosZ* gene corresponding to OTU 75 (not identified in the clone libraries) were most abundant at high salinities and low NO_3^- + NO_2^- concentrations (Fig. 5).

Discussion

T-RFLP analysis indicated low *nosZ* gene diversity in the denitrifying communities that inhabit MZ, SZ, and FZ sites, as only 13 distinct T-RFLP peaks were detected in all profiles. In contrast, Scala and Kerkhof [29] found a total of 71 distinct *nosZ* T-RFLP peaks in continental shelf sediment samples. These authors showed that the endonuclease *Hin*PI is able to produce a large number of restriction fragments from environmental samples, facilitating high resolution of sequence variability. In contrast, low diversity of *nirS* genes was also noted in samples from the South Pacific Ocean oxygen minimum zone (19 *nirS* phylotypes) [8] and in *nirK* transcripts from the rhizospheres of legumes (12 *nirK* phylotypes) [32].

It is surprising to note that no *nosZ* genes were retrieved from samples taken at the rocky biofilm site colonized by *Enteromorpha* spp. (EZ site). The quality of the DNA harvested from this site was suitable for PCR amplification because we successfully amplified the 16S rRNA genes of ammonia-oxidizing bacteria from these same samples [20]. Because complete denitrification (N_2 production) was measured at the EZ site [18, 19], denitrifiers able to reduce nitrous oxide must have been present at this site. Low abundance of *nosZ* genes in this habitat could also be an explanation for the unsuccessful amplification; however, we would not expect the high rates of denitrification measured at this site (21.1–42.3 μmol N_2 m^{-2} h^{-1}) to correspond to such apparently low denitrifier abundance. By inference then, the denitrifier assemblage present at this site must contain *nosZ* genes that are divergent from the denitrifiers upon which the primer set we used is based. The primers we used were based on *nosZ* sequences from cultured denitrifiers and from environmental sequences retrieved from denitrifier assemblages found in continental shelf marine sediments off the mid-Atlantic states of the US [27]. This primer set has yielded useful sequence information in previous studies [28, 34]. Throbäck *et al.* [36] evaluated several *nosZ* primer combinations, including the one used in this study (nos661F/nos1773R), and concluded that it successfully amplifies *nosZ* genes from environmental samples. However, they also reported that another primer set, nosZ-F/nosZ1622R, amplified *nosZ* genes from a broader range of denitrifying isolates [36]. We attempted to amplify *nosZ* genes from EZ samples using this primer set; however, the same results were obtained; no *nosZ* genes were retrieved. Isolating and characterizing the unknown denitrifiers that appear to inhabit the EZ site, and possibly similar environments, would be a fruitful target for future research. Several authors have reported failing to detect *nirS* genes in environmental samples [3, 32, 39], attributing this to the presence of divergent *nirS* genes in those samples. Results from our previous research [18, 19] revealed that denitrification at the EZ site is independent of the NO_3^- availability, which differs from denitrification at the SZ, MZ, and FZ sites. At this time, it is not clear whether EZ denitrifiers are adapted to the specific characteristics of sites colonized by *Enteromorpha* spp. or if they are cosmopolitan microorganisms adapted to some characteristics of the EZ site that have simply not yet been described. However, the apparent divergence of *nosZ* genes at the EZ site suggests surprising differences in denitrifier assemblages from two physically similar and adjacent sites (FZ and EZ) and points to an ecological control of denitrifier community composition related to the dominant macroalgal cover in these biofilm habitats.

In this study, only a few OTUs were found to be shared between different sites, and a number of genotypes were found to be restricted to each location, suggesting that the microenvironmental conditions at each site constrain assemblages of denitrifying bacteria. Several other authors have reported that different denitrifier populations develop under different environmental conditions [7, 14, 25, 39]. The high reduced sulfur concentrations inherent to muddy

sediments may have contributed to lower *nosZ* richness in them. Oxygen penetration is another environmental variable that differs between MZ (Eh=−45.4±5.1 mV, within 0.5 cm depth) and SZ (Eh=144.9±20.1 mV, within 0.5 cm depth) sites and most probably between FZ, whereas Eh data are not available for this site. Cavigelli and Robertson [9] found substantial differences in the sensitivity to oxygen of nitrous oxide reductase enzymes from 156 denitrifying soil isolates. The MS, SZ, and FZ sites function quite differently from each other with respect to denitrification [19]. FZ samples always had the highest denitrification rates, net NO_2^-, and net N_2O fluxes [19]. The differences in denitrifier assemblages together with differences in environmental factors, like inorganic nitrogen availability, that have previously been found to be important in regulating denitrification rates and net N_2O fluxes at these sites [18, 19], may contribute to a more active denitrifying community at the rocky sites. In agreement, Rich *et al.* [25] demonstrated that site-specific differences in denitrifier community composition, as well as in environmental factors, contribute to site-to-site variability of denitrification rates in meadow and forest soils. In contrast, denitrifier community composition and activity were found to be uncoupled in agroecosystems [24] and in arable soil with different long-term fertilizing treatments [14].

It is difficult to relate intrasite dynamics of denitrifier communities to N biogeochemistry with samples from just four samples. Thus, relationships between denitrifier community structure and N-biogeochemistry were evaluated at the SZ site, which was sampled 12 times over the course of a year. Relationships between *nosZ* gene assemblages, environmental variables, and rates of N-processes were evaluated using the relative areas of T-RFLP peaks for each sample. However, T-RFLP is PCR-based, and because of potential PCR bias and possible variability of gene copy number between microbial genomes, the quantitative interpretation of fingerprinting data requires caution [38]. Lueders and Friedrich [16] evaluated PCR amplification bias by T-RFLP in pure cultures and soil samples and demonstrated that T-RFLP analysis can give a quantitative view of the template pool. The good reproducibility observed between T-RFLP replicates generated from independent PCRs (data not shown), and the fact that samples used to analyze intra-site variability were subject to exactly the same analytical protocols and conditions, gives us confidence in our use of the relative abundance of terminal restriction fragments in the CCA and hierarchical cluster analysis.

Two-dimensional PCA based on the N-biogeochemistry data and hierarchical cluster analysis based on the relative area of T-RFLP peaks indicates that samples characterized by higher water column NO_3^- + NO_2^- concentrations and lower salinity (November, December, and January) have similar *nosZ* assemblages and higher denitrification rates. In agreement, denitrification rates at the SZ site were positively correlated with water column NO_3^- + NO_2^- concentrations (r=0.92), p<0.001, n=12), and salinity (r=0.89, p<0.001, n=12). However, in the Douro estuarine water column, NO_3^- concentrations covary with salinity [19] and previous NO_3^- and salt addition experiments [19] revealed that denitrification rates at the SZ site are a function of NO_3^- availability and that salinity does not have a direct effect on the denitrification process. These findings suggest that water column NO_3 + NO_2^- concentration and not salinity, is an important variable governing denitrifier community structure. Specifically, CCA analysis showed that high relative abundance of OTU 190 was correlated with high NO_3^- + NO_2^- concentrations and high denitrification rates; however, other *nosZ* genotypes (OTU 75) were more abundant when NO_3^- + NO_2^- concentrations were low. These results together suggest that different groups of denitrifiers may have a competitive advantage when NO_3^- fluctuates in the system. NO_3^- was identified previously as an important environmental factor governing shifts in denitrifier communities of the oxygen minimum zone in the South Pacific [8] and in continental margin sediments of the Pacific coast of Mexico [15]. Other biogeochemical factors, especially NO_2^-, O_2, NH_4^+, and pH, were also found to be important in controlling the structure of denitrifier communities in different environments [3, 8, 15]. Salinity was also identified as the factor responsible for decreased nitrite reductase gene abundance in a wastewater treatment system [41].

In conclusion, this study revealed that all intertidal environments of the Douro River estuary are characterized by low richness of *nosZ* genotypes, compared with other environments that have been studied using a similar approach [26, 29]. We observed significant differences between sites in the composition of denitrifier assemblages as indicated by *nosZ* sequence diversity, and some *nosZ* sequences have greater similarity to sequences retrieved from Pacific and Atlantic Ocean sediments [28] than with *nosZ* sequences from other sites in the Douro River estuary. This intersite variability in *nosZ* genotypes contrasts with stable assemblages at a given site, as exemplified by the SZ site, where clear relationships were observed between variability in *nosZ* genotype abundance and environmental variables (NO_3^- + NO_2^- availability) or biogeochemical processes. This study represents an important step in establishing the relationship between environmental factors and the distribution of denitrifier genotypes, with consequences for N biogeochemistry.

Acknowledgements We thank M. J. Magalhães, I. Azevedo, S. Ramos, R. Moreira, and L. Torgo for their assistance in fieldwork and during the incubations, and two anonymous reviewers for their extremely helpful comments on the manuscript. This study was funded by the Portuguese Science and Technology Foundation through a Ph.D. fellowship to C.M.M. (SFRH/BD/1397/2000) and a

research grant to A.A.B (POCTI/CTA/39034/2001). J.T.H. and N.B.'s contribution to this research was supported by a grant from the US Environmental Protection Agency's Science to Achieve Results Estuarine and Great Lakes Coastal Initiative through funding to the Pacific Estuarine Ecosystem Indicator Research Consortium, US EPA Agreement #EPA/R-82867601.

References

1. Ahn Y-H (2006) Sustainable nitrogen elimination biotechnologies: a review. Process Biochem 41:1709–1721
2. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
3. Avrahami S, Conrad R, Braker G (2002) Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. Appl Environ Microbiol 68:5685–5692
4. Barton L, McLay CDA, Schipper LA, Smith CT (1999) Annual denitrification rates in agricultural and forest soils: a review. Aust J Soil Res 37:1073–1094
5. Braker G, Tiedje JM (2003) Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. Appl Environ Microbiol 69:3476–3483
6. Braker G, Zhou J, Wu L, Devol AH, Tiedje JM (2000) Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. Appl Environ Microbiol 66:2096–2104
7. Braker G, Ayala-del-Río HL, Devol AH, Fesefeldt A, Tiedje JM (2001) Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. Appl Environ Microbiol 67:1893–1901
8. Castro-González M, Braker G, Farías L, Ulloa O (2005) Communities of *nirS* type denitrifiers in the water column of the oxygen minimum zone in the eastern South Pacific. Environ Microbiol 7:1298–1306
9. Cavigelli MA, Robertson GP (2001) Role of denitrifier diversity in rates of nitrous oxide consumption in a terrestrial ecosystem. Soil Biol Biochem 33:297–310
10. Clarke KR, Warwick RM (1994) Change in Marine Communities: An Approach to Statistical Analysis and Interpretation. Plymouth Marine Laboratory, Plymouth
11. Cornwell JC, Kemp WM, Kana TM (1999) Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquat Ecol 33:41–54
12. Corredor JE, Morell JM, Bauza J (1999) Atmospheric nitrous oxide fluxes from mangrove sediments. Mar Pollut Bull 38:473–478
13. Devol AH (1991) Direct measurements of nitrogen gas fluxes from continental sediments. Nature 349:319–321
14. Enwall K, Philippot L, Hallin S (2005) Activity and composition of denitrifying bacterial community respond differently to long-term fertilization. Appl Environ Microbiol 71:8335–8343
15. Liu X, Tiquia SM, Holguin G, Wu L, Nold SC, Devol AH, Luo K, Palumbo AV, Tiedje JM, Zhou J (2003) Molecular diversity of denitrifying genes in continental margin sediments within the oxygen-deficient zone off Pacific coast of Mexico. Appl Environ Microbiol 69:3549–3560
16. Lueders T, Friedrich MW (2003) Evaluating of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and *mcrA* genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Appl Environ Microbiol 69:320–326
17. Magalhães CM, Bordalo AA, Wiebe WJ (2002) Temporal and spatial patterns of intertidal sediment-water nutrient and oxygen fluxes in the Douro River estuary, Portugal. Mar Ecol Prog Ser 233:55–71
18. Magalhães CM, Joye SB, Moreira RM, Wiebe WJ, Bordalo AA (2005) Effect of salinity and inorganic nitrogen concentrations on nitrification and denitrification rates in intertidal sediments and rocky biofilms of the Douro River estuary, Portugal. Water Res 39:1783–1794
19. Magalhães CM, Wiebe WJ, Joye SB, Bordalo AA (2005) Inorganic nitrogen dynamics in intertidal rocky biofilms and sediments of the Douro River estuary (Portugal). Estuaries 28:592–606
20. Magalhães CM, Bano N, Hollibaugh JT, Wiebe WJ, Bordalo AA (2007) Composition and activity of betaproteobacteria ammonia-oxidizing communities associated with intertidal rocky biofilms and sediments of the Douro River Estuary, Portugal. J Appl Microbiol (in press) DOI 10.1111/j.1365-2672.2007.03390.x
21. Nogales B, Timmis KN, Nedwell DB, Osborn AM (2002) Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. Appl Environ Microbiol 68:5017–5025
22. Paul JH, Myers B (1982) Fluorometric determination of DNA in aquatic microorganisms by use of Hoechst 33258. Appl Environ Microbiol 43:1393–1399
23. Priemé A, Braker G, Tiedje JM (2002) Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetlend soils. Appl Environ Microbiol 68:1893–1900
24. Rich JJ, Myrold DD (2004) Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. Soil Biol Biochem 36:1431–1441
25. Rich JJ, Heichen RS, Bottomley PJ, Cromack K Jr, Myrold DD (2003) Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Appl Environ Microbiol 69:5974–5982
26. Rösch C, Mergel A, Bothe H (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl Environ Microbiol 68:3818–3829
27. Scala DJ, Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene-specific PCR primers detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol Lett 162:61–68
28. Scala DJ, Kerkhof LJ (1999) Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl Environ Microbiol 65:1681–1687
29. Scala DJ, Kerkhof LJ (2000) Horizontal heterogeneity of the denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis. Environ Microbiol 66:1980–1986
30. Seitzinger SP (1988) Denitrification in freshwater and coastal marine ecosystems: Ecological and geochemical significance. Limnol Oceanogr 33:702–724
31. Seitzinger SP (2000) Scaling up: site-specific measurements to global-scale estimates of denitrification. In: Hobbie JE (ed) Estuarine Science: A Synthetic Approach to Research and Practice. Island Press, Montague, pp 211–241
32. Sharma S, Aneja MK, Mayer J, Munch JC, Schloter M (2005) Diversity of transcripts of nitrite reductase genes *nirK* and *nirS* in Rhizospheres of grain legumes. Appl Environ Microbiol 71:2001–2007
33. Song B, Ward BB (2003) Nitrite reductase genes in halobenzoate degrading denitrifying bacteria. FEMS Microbiol Ecol 43:349–357
34. Stres B, Mahne I, Avguštin G, Tiedje JM (2004) Nitrous oxide reductase (*nosZ*) gene fragments differ between native and cultivated Michigan soils. Appl Environ Microbiol 70:301–309

35. ter Braak CJF, Smilaeur P (2002) CANOCO reference manual and CanoDraw for Windows user's guide: software for Canonical Community Ordination (version 4.5). Microcomputer Power, Ithaca
36. Throbäck IN, Enwall K, Jarvis, A, Hallin S (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol 49:401–417
37. Vieira MEC, Bordalo AA (2000) The Douro estuary (Portugal): a mesotidal salt wedge. Oceanol Acta 23:585–594
38. von Wintzingerode F, Göbel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213–329
39. Wolsing M, Priemé A (2004) Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. FEMS Microbiol Ecol 48:261–271
40. Yan T, Fields MW, Wu L, Zu Y, Tiedje JM, Zhou J (2003) Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. Environ Microbiol 5:13–24
41. Yoshie S, Noda N, Tsuneda S, Hirata A, Inamori Y (2004) Salinity decreases nitrite reductase gene diversity in denitrifying bacteria of wastewater treatment systems. Appl Environ Microbiol 70:3152–3157
42. Zumft WG (1992) The denitrifying prokaryotes. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, Vol. 1. Springer-Verlag, New York, pp 554–582
43. Zumft WG (1997) Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev 61:533–616