Spatiotemporal Variations in the Abundance and Structure of Denitrifier Communities in Sediments Differing in Nitrate Content

David Correa-Galeote[1]*, Germán Tortosa[1], Silvia Moreno[1], David Bru[2], Laurent Philippot[2] and Eulogio J. Bedmar[1]

[1]Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, Granada, Spain.

[2]INRA-Université de Bourgogne, UMR 1229, Microbiologie et Géochimie des Sols, Dijon Cedex, France.

*Correspondence: david.cgaleote@gmail.com

https://doi.org/10.21775/cimb.024.071

Abstract

Spatial and temporal variations related to hydric seasonality in abundance and diversity of denitrifier communities were examined in sediments taken from two sites differing in nitrate concentration along a stream in the Doñana National Park during a 3-year study. We found a positive relationship between the relative abundance of denitrifiers, determined as *narG*, *napA*, *nirK*, *nirS* and *nosZ* denitrification genes, and sediment nitrate content, with similar spatial and seasonal variations. However, we did not find association between denitrification activity and the community structure of denitrifiers. Because *nosZ* showed the strongest correlation with the content of nitrate in sediments, we used this gene as a molecular marker to construct eight genomic libraries. Analysis of these genomic libraries revealed that diversity of the *nosZ*-bearing communities was higher in the site with higher nitrate content. Regardless of nitrate concentration in the sediments, the Bradyrhizobiaceae and Rhodocyclaceae were the most abundant families. On the contrary, Rhizobiaceae was exclusively present in sediments with higher nitrate content. Results showed that differences in sediment nitrate concentration affect the composition and diversity of *nosZ*-bearing communities.

Introduction

Denitrification is the biological process in the biogeochemical nitrogen (N) cycle by which nitrate (NO_3^-) is sequentially reduced to dinitrogen gas (N_2) via the intermediate compounds nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) when oxygen concentrations are limiting. This reduction process is carried out by the sequential activity of the enzymes nitrate (NarG, NapA)-, nitrite (NirK, NirS)-, nitric oxide (cNor, qNor)- and nitrous oxide (NosZ)-reductase, encoded by the *narG*, *napA*, *nirK*, *nirS*, *norC* and *nosZ* genes, respectively (Zumft, 1997; van Spanning *et al.*, 2007; Richardson, 2011; Kraft *et al.*, 2011; Sánchez *et al.*, 2011; Bedmar *et al.*, 2013).

Denitrifiers constitute a taxonomically diverse group of microorganisms included in more than 60 genera of bacteria and some archaea (Philippot *et al.*, 2007; Hayatsu *et al.*, 2008), fungi (Takaya, 2002; Prendergast-Miller *et al.*, 2011), Foraminifera (Risgaard-Petersen *et al.*, 2006) and the amoeboid *Gromia* (Piña-Ochoa *et al.*, 2010). The density of denitrifiers in soils can be up to 10^9 cells per g of soil (Babic *et al.*, 2008; Dandie *et al.*, 2008; Henry *et al.*, 2008), and both cultivation-independent and -dependent methods have shown that the proportion of denitrifiers represents up to 5% of the total soil microbial community (Tiedje, 1988; Henry *et al.*, 2006; Jones *et al.*, 2013).

Several studies have shown that nitrate is the main factor controlling activity, abundance and biodiversity of denitrifier communities in different ecosystems (Wolsing and Priemé, 2004; Enwall *et al.*, 2005; Deiglmayr *et al.*, 2006; Reyna *et al.*, 2010; García-Lledó *et al.*, 2011; Carrino-Kyker *et al.*, 2012; Smith *et al.*, 2015a). However, only a few studies have dealt with the effect of nitrate concentration on the spatial and temporal variations on the abundance and biodiversity of denitrifiers in ecosystems differing in nitrate content. Significant seasonal shifts in the community structure of *nirK*-containing bacteria were found in agricultural soils in Denmark after the application of mineral fertilizer (Wolsing and Priemé, 2004), and variations in the diversity of the *nirS* and *nirK* genes from an agricultural field in Canada were assigned to temporal variations in the physicochemical properties of the soil (Smith *et al.*, 2010). Temporal variations of denitrification activity were dependent on water temperature and nitrate concentration in riverine wetlands (Song *et al.*, 2012) and constructed wetlands (Song *et al.*, 2014). Changes in the relative abundance of *narG*, *napA* and *nirS* genes followed similar spati-temporal patterns than those corresponding to the nitrate concentrations along the estuary of the river Colne (UK) (Smith *et al.*, 2015a) and seasonal changes in the abundance of the *nirK* and *nirS* communities were related to the seasonal variations in nitrogen dynamics in the Elkhorn (USA) tidal estuary (Smith *et al.*, 2015b).

In a previous study (Tortosa *et al.*, 2011), we analysed the biological and physicochemical properties of la Rocina stream, a main natural creek feeding a 60,000 ha wetland, el Rocio, within Doñana National Park. The park is in a marshy area of SW Spain, in the estuary of the Guadalquivir River. Screening of more than 25 points along the course of la Rocina stream (36 km) revealed differences in nitrate concentration in its sediments, most probably due to contamination from agricultural practices allowed in the ecotone of the Park, as no urban areas are located nearby.

Thus, la Rocina stream provides a unique model system to study the effect of nitrate content on abundance and biodiversity of denitrifying communities in sediments as the long term effect related to nitrate content could influence community abundance, composition

and activity. In this study, we determined the spatial and temporal variations in the activity, abundance and biodiversity denitrifying communities between two sites along la Rocina stream with contrasted nitrate content. Denitrification activity was examined as N_2O production and biodiversity was analysed by using the *nosZ* gene as a molecular marker for construction of genomic libraries.

Materials and methods

Site description

Based on the physicochemical properties of the surface waters and sediments along la Rocina stream, which feeds el Rocio marsh in Doñana National Park (Tortosa *et al.*, 2011), two sites, el Acebrón lagoon (S1, UTM coordinates 29S 0718632, 4114294) and la Cañada creek (S2, UTM coordinates 29S 0722653, 4111704) with the lowest and highest nitrate concentrations, respectively, were selected in this study (see Fig. 1 in Tortosa *et al.*, 2011). Sediment samples were taken as indicated earlier (Tortosa *et al.*, 2011) in April and October of years 2008, 2009 and 2010 in order to represent the wet and dry pluvial regimes, respectively. Samples were placed on ice while returned to the laboratory and then stored at −80°C until use.

Denitrification activity

Denitrifying enzyme activity was carried out as previously described (Šimek and Hopkins, 1999; Šimek *et al.*, 2004). Briefly, 25 g of sediment was placed in 125 ml glass bottles containing 25 ml of a solution made of 1 mM glucose, 1 mM KNO_3 and 1 g/l chloramphenicol. The bottles were closed with serum caps and acetylene (10% v/v) was injected into each bottle to inhibit the nitrous oxide reductase (Yoshinari and Knowles, 1976). After incubation for 1 h at 25°C, gas samples (500 µl) were withdrawn from the headspace and injected in a gas chromatograph equipped with an electron capture detector (ECD) and a Porapak Q-packed stainless-steel column (180 × 0.32 cm) (Agilent Technologies, S.L., Madrid, Spain). N_2 at 20 ml/min served as a carrier gas. Oven, detector and injector temperature were 60, 375 and 125°C, respectively. Concentrations of nitrous oxide in each sample were calculated from standards of pure nitrous oxide. The Bunsen coefficient for the N_2O dissolved in water was considered during calculations.

DNA extraction

DNA was extracted from 250 mg of each subsample stored at −80°C according to the ISO standard 11063 'Soil quality – Method to directly extract DNA from soil samples' (Petrić *et al.*, 2011). Briefly, samples were homogenized in 1 ml of extraction buffer (1 M Tris-HCl, 0.5 M EDTA, 1M NaCl, 20% PVP 40, 20% SDS) for 30 s at 1600 rpm in a minibead beater cell disrupter (Mikro-DismembratorS; B. Braun Biotech International, Germany). Soil and cell debris were removed by centrifugation (14,000 × *g* for 1 minute at 4°C). After precipitation with ice-cold isopropanol, nucleic acids were purified using both PVPP and GeneClean Turbo Kit (MP Bio, USA) spin columns. Quality and size of soil DNAs were checked by electrophoresis on 1% agarose. DNA was also quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Germany).

Quantification of the denitrification-associated microbial community

The size of the denitrifier community was estimated by quantitative, real-time PCR (qPCR) of *narG*, *napA*, *nirK*, *nirS* and *nosZ* gene fragments using reaction mixtures, primers and thermal cycling conditions described previously (Bru *et al*., 2011; Correa-Galeote *et al*., 2013a). The total bacterial community was quantified using 16S rRNA gene as molecular marker as described by Correa-Galeote *et al.* (2013b). Reactions were carried out in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Quantification was based on the fluorescence intensity of the SYBR Green dye during amplification. Two independent qPCR assays were performed for each gene. Standard curves were obtained using serial dilutions of linearized plasmids containing cloned *narG*, *napA*, *nirK*, *nirS*, *nosZ* and 16S rRNA genes amplified from bacterial strains. PCR efficiency for the different assays ranged between 90% and 99%. No template controls gave null or negligible values. Presence of PCR inhibitors in DNA extracted from sediments was estimated by (i) diluting soil DNA extract and (ii) mixing a known amount of standard DNA to sediment DNA extract prior to qPCR. In all cases, inhibition was not detected. Methodological evaluation of the real-time PCR assays showed a good reproducibility of 95.0 ± 12% between two runs.

Gene abundances were analysed as absolute and relative abundances (gene copy number/16S rRNA gene Bacteria copy number). As the number of 16S rRNA gene operon per cells is variable (Klappenbach *et al*., 2001), we did not convert the 16S rRNA gene copy data into cells numbers and we expressed our results as gene copy numbers per g of soil.

Clone library construction and DNA sequencing

The construction of genomic libraries using *nosZ* gene as a molecular marker for April and October sampling months and for S1 and S2 sampling sites was limited to years 2009 and 2010.

nosZ amplicons were purified using the QIAquick PCR purification kit (Qiagen, Germany) and cloned using the pGEM-T Easy cloning kit according to the manufacturer's instructions (Promega, USA). The recombinant *Escherichia coli* JM109 cells were inoculated onto solid Luria–Bertani (LB) medium (Miller, 1972) containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and grown overnight at 37°C. White colonies were screened by PCR using the vector primers Sp6 and T7 (Invitrogen). Purity of amplified products was checked by observation of a unique band of the expected size in a 1% agarose gel stained with GelRed as indicated by the manufacturer's (Biotium Inc., USA). Nucleotide sequences of clones containing inserts of the expected size were determined by sequencing with the vector primer Sp6 and the BigDye terminator cycle kit v3.1 (Applied Biosystems, USA) according to the manufacturer's instructions, followed by electrophoresis on an ABI 3100 genetic analyser (Applied Biosystems, USA) at the sequencing facilities of Estación Experimental del Zaidín, CSIC, Granada, Spain.

Phylogenetic analysis

The DNA sequences of *nosZ* gene fragments were aligned by using the ClustalW program available in the Geneious software package (version 6.0.3, Biomatters, New Zealand). Vector sequence was removed and discrepancies in alignment verified manually. The obtained sequences were compared against database sequences using the BLASTN program in Geneious and those showing similarity higher than 80% of those previously deposited for *nosZ* were selected as positives. A distance matrix was calculated according to Kimura's

two-parameter model (Kimura, 1980) using the dnadist Phylip-3.68 package software (University of Washington, USA).

Estimation of the richness as operational taxonomic units (OTUs) and Chao1, Shannon–Weaver and Simpson diversity indexes were calculated using the Mothur program (Schloss *et al.*, 2009). In this study, 3% sequence divergence was used to define OTUs and compare libraries. The Good's coverage index was calculated according to Magurran (2004). A phylogenetic tree was constructed from a matrix of pairwise genetic distances by using the neighbour-joining method available in Geneious. Bootstrap analysis was based on 1000 resamplings.

Statistical analyses

Gene abundances were analysed as absolute or relative abundances (gene copy number/16S rRNA Bacteria copy number) (Correa-Galeote *et al.*, 2013). Absolute abundance of a given denitrification gene was used to analyse changes in the total population containing the gene, while gene relative abundance was used to assess specific changes of the gene with respect to the total bacterial community.

Measured variables in this study were first explored using the Shapiro–Wilk test to check whether they meet the normality assumptions. We used the Mann–Whitney test to compare data between sampling sites and times of sampling, and the Kruskal–Wallis and Conover–Iman combined tests for comparisons among samples. A Spearman correlation matrix was made to study relations between measured variables. A principal component analysis (PCA) was performed to analyse relationships among parameters concerning nitrate content, denitrification activity and denitrification genes relative abundance. A canonical correspondence analysis (CCA) was made to determine the effect of the nitrate content in the structure of the *nosZ*-bearing communities. Multivariate analyses were carried out by the PC-ORD 6.08 version software (MJM). The analysis of molecular variance (AMOVA) to determine population-specific differences among clone libraries was run using MOTHUR (Schloss *et al.*, 2009). Good's coverage, OTUs richness, and diversity (using Chao1, Shannon-Weaver and Simpson indexes) were determined. Different indexes were calculated since they are differentially sensitive to OTUs' rarity or abundance (Morris *et al.*, 2014).

Nucleotide sequence accession numbers

The nucleotide sequences of *nosZ* reported in this study have been deposited in GeneBank under the accession numbers KC936294 to KC936797.

Results

Nitrate content in sediments

For the 3-year study, nitrate content in sediments from site S1 varied between 0.03 and 0.06 mg $N\text{-}NO_3^-$ per kg dry weight, and between 5.73–10.64 mg $N\text{-}NO_3^-$ per kg dry weight from those taken at site S2 (Table 5.1). According to the Mann–Whitney test, nitrate content at S1 was lower than that at S2, regardless of the sampling season and year. Also, the content of nitrate in October was always higher than that found in April for each sampling site, except for year 2008 when no differences were detected.

Table 5.1 Nitrate content and potential denitrification activity in sediments from la Rocina stream. Values of nitrate concentration ($n = 4 \pm SE$) are expressed as mg N-NO_3^- per kg dry sediment. Values of activity ($n = 4 \pm SE$) are expressed as ng N-N_2O per g dry sediment per hour. According to the Mann–Whitney test ($\alpha = 0.05$), letters a and b indicate significant differences between sampling months for a given sampling site and year, and letters A and B show significant differences between sampling sites for a given sampling month and year

Year	Sampling month	Sampling site	Nitrate content (mg N-NO_3^- per kg dry sediment)	Potential denitrification activity (ng N-N_2O per g dry sediment per hour)
2008	April	S1	0.04 ± 0.01 (a, B)	164 ± 8.72 (a, B)
		S2	5.73 ± 0.09 (b, A)	1393 ± 121 (a, A)
	October	S1	0.04 ± 0.01 (a, B)	114 ± 8.08 (a, A)
		S2	7.31 ± 0.12 (a, A)	130 ± 16.46 (b, A)
2009	April	S1	0.05 ± 0.01 (a, B)	164 ± 7.28 (a, B)
		S2	7.02 ± 0.34 (b, A)	1616 ± 122 (a, A)
	October	S1	0.03 ± 0.01 (b, B)	126 ± 9.74 (a, A)
		S2	10.64 ± 0.27 (a, A)	137 ± 9.38 (b, A)
2010	April	S1	0.04 ± 0.01 (b, B)	194 ± 17.48 (a, B)
		S2	6.01 ± 0.33 (b, A)	1134 ± 44.91 (a, A)
	October	S1	0.06 ± 0.02 (a, B)	113 ± 9.88 (a, A)
		S2	7.70 ± 0.26 (a, A)	134 ± 6.96 (b, A)

Denitrification activity

Potential denitrification activity (PDA), determined as N_2O emission, in sediments varied between 113 and 194 ng N-N_2O per g dry sediment per hour and 130–1616 ng N-N_2O per g dry sediment per hour in sediments from S1 and S2, respectively (Table 5.1). For the 3-year study, PDA at S2 was statistically higher than that at S1 for the samples taken in April, and no differences were found in samples taken during October. At S1, PDA detected in April was always similar to that observed in October, while at S2, PDA was higher in April than in October.

Quantification of 16S rRNA, *narG*, *napA*, *nirS*, *nirK* and *nosZ* genes

The number of 16S rRNA genes in the sediment samples ranged from 7.38×10^6 to 2.91×10^9 copies per g dry sediment (Table 5.2). No significant differences between sites were observed regardless of the year and sampling season, except for samples taken in October 2010, when the copy number of the 16S rRNA at S1 was higher than that at S2. Comparing months at the same site, in S1, the number of 16S rRNA genes was higher in October than in April, except for year 2009 when both months had similar values. At S2, however, no differences in the 16S rRNA gene copy number were detected comparing months within the same year, except for October 2009 when the number of target genes was higher than in April 2009.

Among the denitrification genes analysed in the sediments of la Rocina stream, *narG* was the most abundant gene (from 2.19×10^6 to 3.53×10^8 copies per g dry sediment), followed by *napA* (from 1.57×10^6 to 3.84×10^7 copies per g dry sediment), *nirS* (from 3.91×10^5 to 2.72×10^8 copies per g dry sediment), *nirK* (from 1.17×10^5 to 2.22×10^7 copies per g dry sediment), and *nosZ* was the gene with the lowest copy number (from 1.67×10^4 to

Table 5.2 Abundance of 16S rRNA and *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes in sediments from la Rocina stream. Values ($n = 4 \pm$ SE) are expressed as gene copy numbers per g of dry sediment. According to the Mann–Whitney test ($\alpha = 0.05$), letters a and b show statistically significant differences between sampling months for a given sampling site and year, and letters A and B represent statistically significant differences between sampling sites for a given sampling month and year

Year	Sampling month	Sampling site	Gene abundance					
			16S rRNA	*narG*	*napA*	*nirK*	*nirS*	*nosZ*
2008	April	S1	$4.52 \times 10^7 \pm 4.89 \times 10^6$ (b, A)	$4.25 \times 10^6 \pm 4.20 \times 10^5$ (a, A)	$2.69 \times 10^6 \pm 2.42 \times 10^5$ (a, A)	$3.69 \times 10^6 \pm 3.07 \times 10^5$ (a, A)	$7.89 \times 10^5 \pm 8.38 \times 10^4$ (b, B)	$5.03 \times 10^4 \pm 4.02 \times 10^3$ (b, A)
		S2	$2.87 \times 10^7 \pm 4.27 \times 10^6$ (a, A)	$4.76 \times 10^6 \pm 3.22 \times 10^5$ (b, A)	$3.03 \times 10^6 \pm 5.52 \times 10^5$ (b, A)	$2.05 \times 10^6 \pm 1.98 \times 10^5$ (b, B)	$1.51 \times 10^6 \pm 9.97 \times 10^4$ (b, A)	$7.01 \times 10^4 \pm 8.40 \times 10^3$ (b, A)
	October	S1	$5.68 \times 10^7 \pm 2.27 \times 10^6$ (a, A)	$5.10 \times 10^6 \pm 9.16 \times 10^4$ (a, B)	$3.88 \times 10^6 \pm 8.48 \times 10^5$ (a, A)	$4.99 \times 10^6 \pm 3.20 \times 10^5$ (a, A)	$1.80 \times 10^6 \pm 7.77 \times 10^4$ (a, B)	$7.80 \times 10^4 \pm 7.98 \times 10^3$ (a, B)
		S2	$4.33 \times 10^7 \pm 4.62 \times 10^6$ (a, A)	$1.44 \times 10^7 \pm 2.27 \times 10^6$ (a, A)	$9.45 \times 10^6 \pm 1.64 \times 10^6$ (a, A)	$3.87 \times 10^6 \pm 3.27 \times 10^5$ (a, A)	$4.42 \times 10^6 \pm 9.09 \times 10^5$ (a, A)	$2.28 \times 10^5 \pm 1.27 \times 10^4$ (a, A)
2009	April	S1	$8.29 \times 10^7 \pm 1.30 \times 10^7$ (a, A)	$4.32 \times 10^6 \pm 6.32 \times 10^5$ (a, A)	$1.93 \times 10^6 \pm 2.62 \times 10^5$ (a, A)	$4.10 \times 10^6 \pm 9.53 \times 10^5$ (a, A)	$4.13 \times 10^5 \pm 7.02 \times 10^4$ (a, B)	$3.24 \times 10^4 \pm 4.62 \times 10^3$ (a, B)
		S2	$7.38 \times 10^6 \pm 3.44 \times 10^6$ (a, A)	$7.69 \times 10^6 \pm 7.45 \times 10^5$ (a, A)	$3.82 \times 10^6 \pm 4.65 \times 10^5$ (a, A)	$2.35 \times 10^6 \pm 1.24 \times 10^5$ (a, A)	$1.81 \times 10^6 \pm 2.51 \times 10^5$ (a, A)	$2.13 \times 10^5 \pm 8.47 \times 10^3$ (a, A)
	October	S1	$3.76 \times 10^7 \pm 7.96 \times 10^6$ (a, A)	$2.57 \times 10^6 \pm 2.61 \times 10^5$ (a, B)	$1.57 \times 10^6 \pm 4.43 \times 10^5$ (a, B)	$1.17 \times 10^5 \pm 2.12 \times 10^5$ (b, A)	$3.91 \times 10^5 \pm 9.82 \times 10^4$ (a, B)	$1.67 \times 10^4 \pm 3.25 \times 10^3$ (a, B)
		S2	$2.70 \times 10^7 \pm 3.52 \times 10^6$ (b, A)	$9.29 \times 10^6 \pm 1.60 \times 10^6$ (a, A)	$4.60 \times 10^6 \pm 5.93 \times 10^5$ (a, A)	$2.13 \times 10^6 \pm 2.22 \times 10^5$ (a, A)	$3.33 \times 10^6 \pm 3.15 \times 10^5$ (a, A)	$1.35 \times 10^5 \pm 1.21 \times 10^4$ (b, A)
2010	April	S1	$2.49 \times 10^8 \pm 3.24 \times 10^7$ (b, A)	$2.19 \times 10^6 \pm 1.66 \times 10^6$ (b, A)	$1.47 \times 10^7 \pm 2.41 \times 10^6$ (b, A)	$1.35 \times 10^6 \pm 1.39 \times 10^6$ (a, A)	$4.84 \times 10^6 \pm 5.67 \times 10^5$ (b, A)	$3.91 \times 10^5 \pm 9.15 \times 10^4$ (b, B)
		S2	$2.01 \times 10^8 \pm 4.53\ 10^7$ (a, A)	$2.08 \times 10^7 \pm 4.87 \times 10^6$ (a, A)	$1.10 \times 10^7 \pm 4.25 \times 10^6$ (a, A)	$1.21 \times 10^7 \pm 3.56 \times 10^6$ (a, A)	$4.36 \times 10^6 \pm 6.96 \times 10^5$ (b, A)	$5.44 \times 10^5 \pm 9.45 \times 10^4$ (a, A)
	October	S1	$2.91 \times 10^9 \pm 9.68 \times 10^8$ (a, A)	$3.53 \times 10^8 \pm 1.28 \times 10^8$ (a, A)	$3.84 \times 10^7 \pm 8.92 \times 10^6$ (a, A)	$2.15 \times 10^7 \pm 7.17 \times 10^6$ (a, A)	$2.72 \times 10^8 \pm 8.52 \times 10^7$ (a, A)	$4.67 \times 10^6 \pm 1.39 \times 10^6$ (a, A)
		S2	$2.40 \times 10^8 \pm 9.39 \times 10^7$ (a, B)	$5.61 \times 10^7 \pm 2.26 \times 10^7$ (a, A)	$1.52 \times 10^7 \pm 5.98 \times 10^6$ (a, A)	$2.22 \times 10^7 \pm 7.96 \times 10^6$ (a, A)	$5.12 \times 10^7 \pm 2.47 \times 10^7$ (a, A)	$1.80 \times 10^5 \pm 1.63 \times 10^6$ (a, A)

4.67×10^6 copies per g dry sediment) (Table 5.2). In general, spatiotemporal variations were not observed for the *narG*, *napA* and *nirK* genes; however, the Mann–Whitney tests showed that *nirS* and *nosZ* followed the same variations than those in the nitrate content in sites S1and S2 (Table 5.2). It is to note, however, that some exceptions can be found in the general spatio-temporal patterns mainly associated with the *nirS* and *nosZ* genes.

Relative abundance of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes

On average for the 3-year study (Table 5.3), at S1, the mean relative abundances of *narG* (8.09%), *napA* (4.79%), *nirS* (6.16%), *nirK* (1.38%), and *nosZ* (0.10%) genes in April were similar to those of 9.56%, 5.50%, 6.46% 4.61% and 0.12% for the *narG*, *napA*, *nirK*, *nirS* and *nosZ* genes found in October, respectively. In contrast, in April at S2, relative abundances of the *narG* (13.21%), *napA* (7.44%), *nirS* (5.61%), *nirK* (3.54%) and *nosZ* (0.28%) genes

Table 5.3 Relative abundance of *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes in sediments from la Rocina stream. Values ($n = 4 \pm$ SE) are expressed as percentage of the ratio between a given denitrification gene copy number and the 16S rRNA gene copy number. According to the Mann–Whitney test ($\alpha = 0.05$), letters a and b show significant differences between sampling months for a given sampling site and year, and letters A and B represent significant differences between sampling sites for a given sampling month and year

Year	Sampling month	Sampling site	Gene relative abundance (%)				
			narG	*napA*	*nirK*	*nirS*	*nosZ*
2008	April	S1	9.71 ± 1.10 (a, B)	6.11 ± 0.56 (a, B)	8.29 ± 0.32 (a, A)	1.78 ± 0.18 (b, B)	0.11 ± 0.01 (a B)
		S2	17.92 ± 1.93 (a, A)	12.46 ± 1.22 (a, A)	7.81 ± 1.13 (a, A)	5.70 ± 0.61 (b, A)	0.25 ± 0.01 (b, A)
	October	S1	9.08 ± 0.44 (a, B)	8.19 ± 0.79 (a, B)	8.82 ± 0.52 (a, A)	3.23 ± 0.28 (a, B)	0.14 ± 0.01 (a, B)
		S2	34.79 ± 4.73 (a, A)	21.48 ± 2.38 (a, A)	9.33 ± 0.76 (a, A)	9.93 ± 1.45 (a, A)	0.57 ± 0.07 (a, A)
2009	April	S1	5.25 ± 0.06 (a, B)	1.92 ± 0.12 (b, B)	4.69 ± 0.47 (a, B)	0.40 ± 0.04 (b, B)	0.04 ± 0.01 (a, B)
		S2	10.59 ± 1.24 (b, A)	6.46 ± 0.58 (b, A)	3.22 ± 0.22 (a, A)	2.47 ± 0.34 (b, A)	0.29 ± 0.02 (b, A)
	October	S1	8.35 ± 1.38 (a, B)	5.74 ± 0.93 (a, B)	3.28 ± 0.20 (a, B)	1.42 ± 0.24 (a, B)	0.05 ± 0.01 (a, B)
		S2	36.16 ± 6.17 (a, A)	17.05 ± 0.94 (a, A)	8.57 ± 1.35 (b, A)	12.94 ± 1.13 (a, A)	0.53 ± 0.07 (a, A)
2010	April	S1	9.31 ± 0.85 (a, B)	3.41 ± 0.27 (a, A)	5.51 ± 0.28 (b, A)	1.96 ± 0.03 (b, A)	0.15 ± 0.01 (a, B)
		S2	11.26 ± 2.24 (b, A)	6.35 ± 1.13 (b, A)	5.81 ± 0.85 (a, A)	2.45 ± 0.35 (a, A)	0.29 ± 0.02 (b, A)
	October	S1	11.26 ± 0.77 (a, B)	2.57 ± 0.53 (a, B)	7.28 ± 0.26 (a, B)	9.18 ± 0.70 (b, B)	0.17 ± 0.02 (a, B)
		S2	22.47 ± 0.51 (a, A)	15.08 ± 3.60 (a, A)	9.75 ± 0.31 (a, A)	17.20 ± 1.57 (a, A)	0.51 ± 0.09 (a, A)

were lower than those found in October that were 31.14%, 17.87%, 9.22%, 13.36% and 0.57% for the *narG*, *napA*, *nirS*, *nirK* and *nosZ*, respectively (Table 5.3).

Spatial and temporal variations of denitrification genes relative abundances

Generally considered, the relative abundance of each *narG*, *napA*, *nirS* and *nosZ* genes in sediments taken at S2 was higher than in sediments taken at S1; some exceptions, however, were found throughout the 3 yeas study, mainly due to relative abundances of *napA* and *nirS* genes. The relative abundance of *nirK* gene did not show any spatial or temporal variations along the time of study (Table 5.3). During the 3-year study, the sampling month did not affect the relative abundance of the *narG*, *napA*, *nirK* and *nosZ* genes at S1, except for *nirS* whose relative abundance was always higher in October (Table 5.3). At S2, in April, the relative abundances of *narG*, *napA*, *nirS* and *nosZ* were lower than those detected in October, and differences were not observed for the *nirK* gene (Table 5.3).

Correlation tests and multivariate analysis

A Spearman test showed that correlation between nitrate content and abundance of each denitrification gene was very weak (supplementary material Table 5.1S). In contrast, there were some strong significant correlations between the content of nitrate and the relative abundance of *narG*, *napA*, *nirS*, and *nosZ* genes, the last correlation showing the highest value (Table 5.4). There were also significant correlations among relative abundances of *narG*, *napA*, *nirS*, *nirK*, and *nosZ* genes, with high values for some *nosZ* and *narG* (Table 5.4). There were no significant correlations between denitrification activity and either nitrate content or any of the denitrification genes, considering either gene abundances (supplementary material Table 5.1S) or relative gen abundances (Table 5.4).

Fig. 5.1 shows the PCA analysis including the variables nitrate concentration, denitrification activity and relative abundance of denitrification genes. PCA1 accounted for 61.72% of the total variance in the data. Nitrate concentration and the relative abundance of denitrification genes were positively related with PCA1, with the relative abundances of *narG* ($r = 0.927$) and *nosZ* ($r = 0.929$) as main contributors. PCA2 accounted for an additional 17.91% of the variation of the data and this variation is described almost exclusively by the

Table 5.4 Spearman coefficient values between nitrate content, relative abundance of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes and potential denitrification activity in sediments from la Rocina stream. Values followed by asterisk (*) are statistically significant (*P*-value < 0.05), NS: not statistically significant (*P*-value > 0.05)

	Nitrate content	*narG*	*napA*	*nirK*	*nirS*	*nosZ*
narG	0.677*					
napA	0.564*	0.765*				
nirK	0.278^{NS}	0.627*	0.406*			
nirS	0.668*	0.784*	0.634*	0.585*		
nosZ	0.856*	0.817*	0.690*	0.483*	0.793*	
Potential denitrification activity	0.019^{NS}	-0.030^{NS}	0.050^{NS}	-0.127^{NS}	-0.038^{NS}	0.134^{NS}

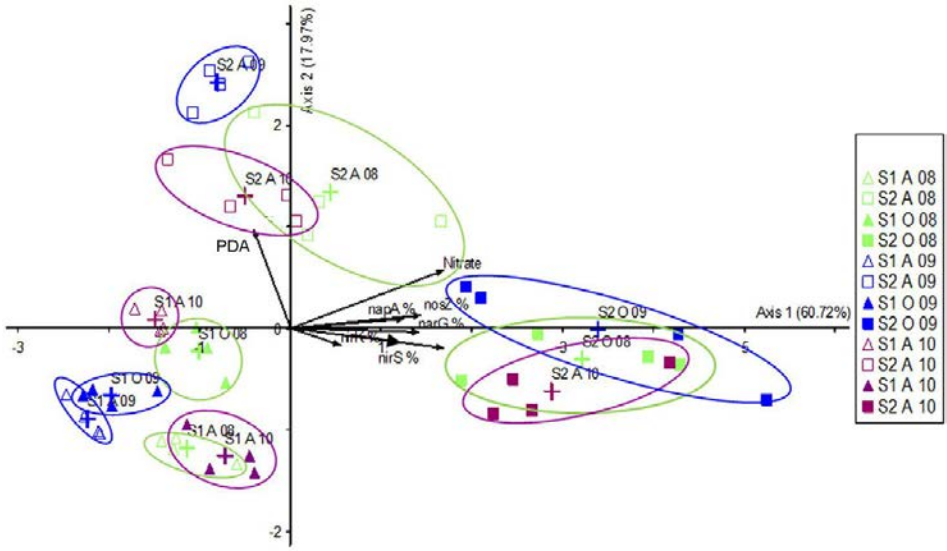

Figure 5.1 Principal components analysis (PCA) of nitrate content, relative abundance of the *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes and potential denitrification activity. Percentages of PCA1 and PCA2 explaining variance are shown. Group centroids are indicated by +. Sediments were taken in April (A) and October (O) 2009 (09) and 2010 (10) at El Acebrón lagoon (S1) and la Cañada creek (S2).

denitrification activity variable ($r = 0.927$). No correlation was found between denitrification activity and the other six variables (supplementary material Table 5.2S). Samples taken at sites S1 and S2 were mostly separated along Axis 1 regardless of the sampling year, and ranked highly negatively and positively for PCA1, respectively. Samples from S2 were also separated according to the sampling season, most of the samples taken in April were associated with DEA activity and separated along PCA2. PCA2 also separated samples S1 from S2, although the percentage of explained variability was low. Samples S1 were not separated by sampling season.

Analysis of clone libraries

The eight *nosZ* libraries contained 504 clones grouped in 109 OTUs (supplementary material Table 5.3S). At S1, 65 and 63 clones were obtained in April 2009 and 2010, respectively, and 61 and 60 in October 2009 and 2010, respectively. Whereas 58 clones were obtained at S2 for April 2009 and 2010, 70 and 69 clones were procured in October 2009 and 2010, respectively.

The Good's coverage index for each library (Table 5.5) was higher than 75%, which indicates that the sampling effort was enough to permit extrapolations for analysis of total *nosZ* biodiversity in the samples. Two libraries, corresponding to October 2009 and 2010 at S2 presented statistically significant higher number of OTUs (35 and 34 OTUs, respectively), and the remaining six libraries contained between 25 and 29 OTUs (Table 5.5 and supplementary material Table 5.3S). The 8 libraries had similar Chao1 and Shannon–Weaver

Table 5.5 Diversity indexes of *nosZ* clone libraries from la Rocina stream sediments as estimated with the Simpson index and Shannon–Weaver and Chao 1 richness estimators computed using Mothur. According to the Kruskal–Wallis test (α = 0.05), letters a and b show significant differences between sampling months and sites

Year	Sampling month	Sampling site	Number of clones	Good's coverage	Number of OTUs	Chao1	Shannon–Weaver	Simpson
2009	April	S1	65	75.38	29 b	53.0 a	3.07 a	0.047 a
		S2	58	77.59	25 b	38.0 a	2.92 a	0.053 a
	October	S1	61	78.69	27 b	36.7 a	3.02 a	0.050 a
		S2	70	75.29	35 a	48.9 a	3.15 a	0.034 b
2010	April	S1	63	76.19	29 b	44.0 a	3.10 a	0.045 a
		S2	58	79.31	26 b	33.3 a	2.96 a	0.055 a
	October	S1	60	76.66	29 b	31.1 a	3.11 a	0.044 a
		S2	69	75.36	34 a	45.3 a	3.29 a	0.031 b

indexes but differed in their Simpson diversity index values, which were statistically lower at S2 in October (Table 5.5).

Construction of a phylogenetic tree based on the 504 *nosZ* sequences showed they distributed into 31 clusters (Fig. 5.2). Overall, members of the Betaproteobacteria class were the most abundant (59.1%) followed by those of the Alphaproteobacteria (39.5%) and the Gammaproteobacteria (1.4%).

Clusters C4, C5, C6, C7 and C10 within the Alphaproteobacteria and clusters C15, C16, C17, C18, C20, C24, C25, C27, C28 and C30 included in the Betaproteobacteria contained clones showing homology with unclassified *nosZ* gene sequences deposited in GenBank (supplementary material Table 5.4S). The number of unclassified clones was clearly higher in libraries from site S1 (56.63%) than that from S2 (23.92%). The more abundant clusters were C1, which contains 52 clones of the family Bradyrhizobiaceae, C22, which includes 44 clones in the Rhodocyclaceae, and clusters C24 (43 clones) and C27 (40 clones) with unidentified families (supplementary material Table 5.4S). Only 14 clones were members of the family Pseudomonadaceae within the Gammaproteobacteria, and they all were found at S1 (supplementary material Table 5.4S).

The unclassified clusters, C4, C5 and C10 from Alphaproteobacteria, C15 and C27 from Betaproteobacteria and members of the family Pseudomonadaceae from Gammaproteobacteria in C14 were found only at S1 (Fig. 5.3). The C2 Bradyrhziobiaceae cluster, the C3 and C12 Rhizobiaceae clusters, the C9 Beijereinckiaceae of the Alphaproteobacteria and the C26 unclassified Burkholderiales and C31 Comamonadaceae of the Betaproteobacteria were present only at S2.

Spatial and temporal variation of denitrifier community diversity

AMOVA of the 504 *nosZ* sequences indicated that total sequence variation was 3.62% among libraries and 96.38% within the clone libraries (supplementary material Table 5.5S), which indicates the existence of a highly randomized diversity within libraries. At S1, pairwise alignments revealed that sequences from April 2009 and 2010 and October 2009 were

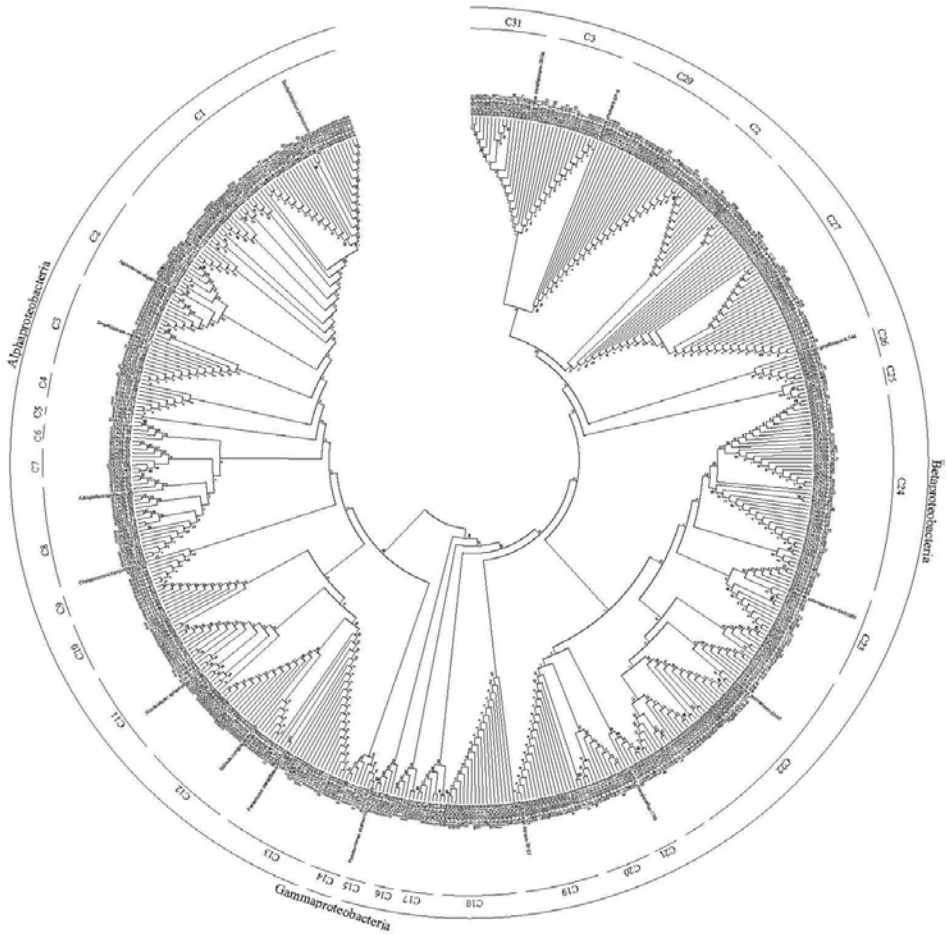

Figure 5.2 Neighbour-joining phylogenetic tree based on 504 *nosZ* DNA sequences cloned from la Rocina stream sediments and other cultured bacteria. Sediments were taken in April (A) and October (O) 2009 (09) and 2010 (10) at El Acebrón lagoon (S1) and la Cañada creek (S2). The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets.

statistically the same population, and that those from October 2010 were significantly different (Table 5.6). On the contrary, at S2, sequences in the clone libraries from April 2009 and 2010 and October 2009 were statistically different populations, but no differences however, were found between sequences in the October 2009 and 2010 clone libraries (Table 5.6).

A CCA sample ordination based on the relative abundance of the *nosZ* clusters within each clone library showed that the eight samples distributed in two clearly separated groups (Fig. 5.4). The two first CCA axes, with canonical coefficients 1.01 and −0.024 for axes 1 and 2, respectively, explained 42.2% of the total variance and revealed that nitrate concentration of the sediments could be responsible for the grouping of the clone libraries along the two axes.

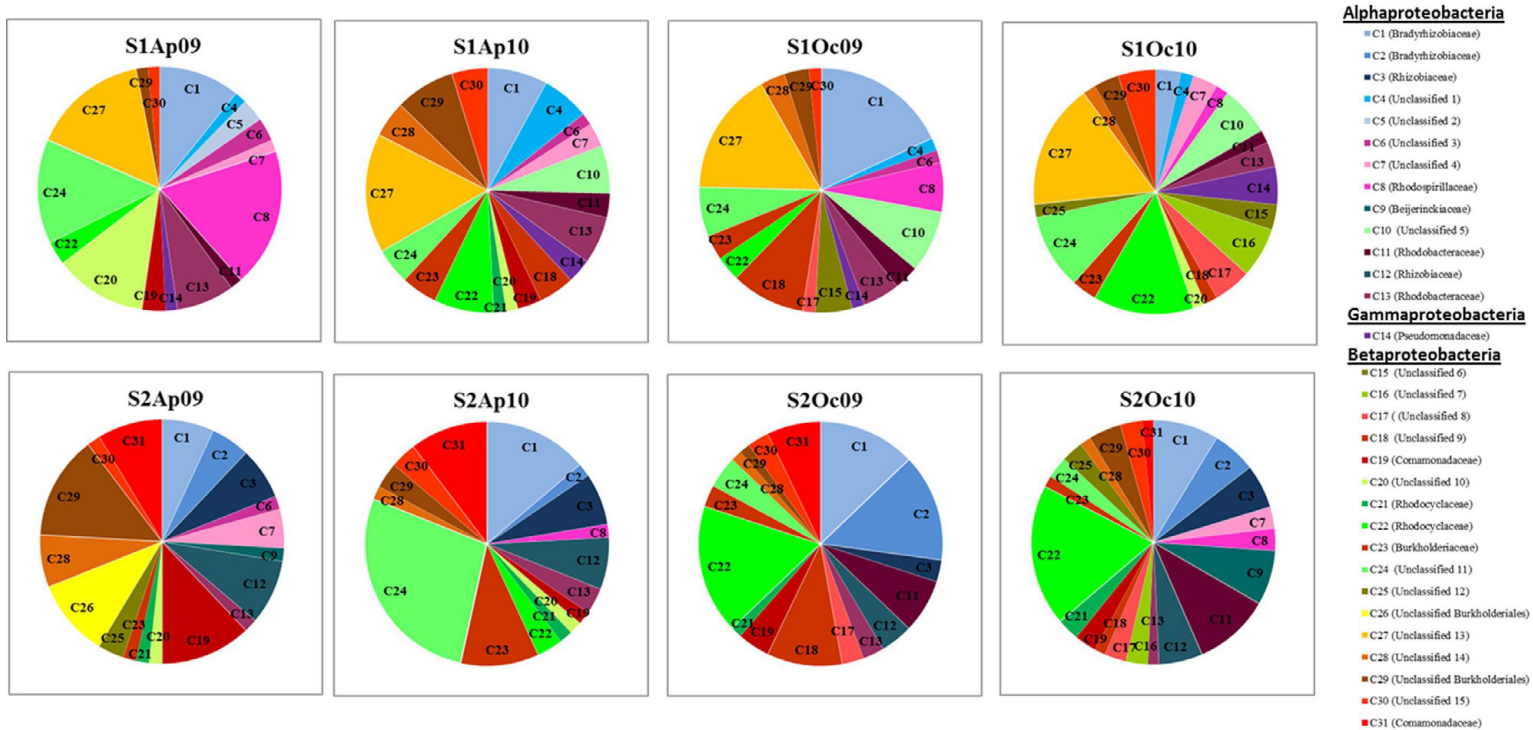

Figure 5.3 Pie charts comparing the *nosZ* communities composition of sediments from la Rocina stream. To facilitate the comparison between clusters colour has been used as an indication of bacterial families and unidentified groups.

Table 5.6 Pairwise dissimilarity indexes (Fst) from AMOVA of *nosZ* clone libraries. Clones from S1 are shown in bold. An asterisk indicates a *P*-value <0.05 observed Fst value compared to Fst value from 1000 randomizations of the sequences. NS, not statistically significant ($P>0.05$). April and October stand for the months of April and October, respectively

	Clone library			
Clone library	Apr09	Oct09	Apr10	Oct10
Apr09		**1.53**^{NS}	**1.99**^{NS}	**4.01***
Oct09	2.66*		**1.25**^{NS}	**3.02***
Apr10	2.60*	3.16*		**1.23**^{NS}
Oct10	2.79*	1.55^{NS}	3.98*	

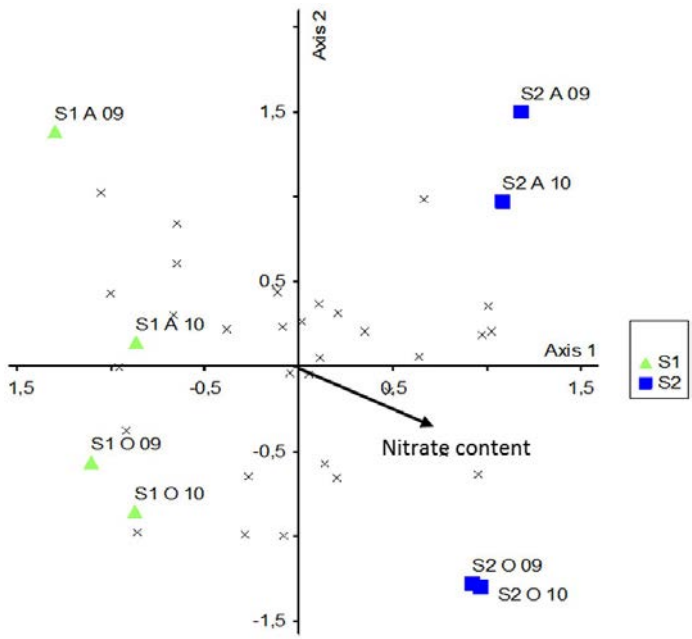

Figure 5.4 Canonical correspondence analysis (CCA) of the composition of the 31 clusters found in the *nosZ* clone libraries. Crosses represent vector scores for the different clusters. Open and closed triangles represent the axes 1 and 2 scores for the clusters found in taken in April (A) and October (O) 2009 (09) and 2010 (10) at el Acebrón lagoon (S1) and la Cañada creek (S2). The arrow represents the biplot vector for the nitrate concentration of the sediments.

Discussion

In this work, the denitrification genes *narG*, *napA*, *nirK*, *nirS*, and *nosZ* were quantified using qPCR to estimate spatio-temporal variations of denitrifiers and to analyse their correlation with nitrate content and denitrification activity in sediments from la Rocina stream taken at two sites with relatively low (S1) and high (S2) nitrate concentration; also, the biodiversity of denitrifiers was analysed using the *nosZ* gene as a molecular marker.

Due to its consideration as a national park, Doñana is subjected to special regulations and, consequently, any anthropogenic effect is mainly derived from agricultural practices allowed in the ecotone of the park, where farming of rice and strawberries is common. In fact, diffuse nitrate contamination in Doñana National Park has been previously recorded (Serrano *et al.*, 2006; Manzano *et al.*, 2009; Espinar and Serrano, 2009; Tortosa *et al.*, 2011). Sediment samples were taken in April and October in order to represent the dry and wet seasons, respectively. During the 3-year study, hydrological dynamics at each sampling site varied with sampling date, which was clearly visible at S2 in October, where stream waters were transformed into swampy waters, and, finally, in almost dry sediments.

With slight differences, the content of nitrate at S1 was similar for the two sampling seasons and lower than those at S2, where nitrate content in October was always higher than in April. All those values, however, were lower than the 50 mg/l defined by the European directive 91/676/CEE as the upper limit for nitrate content in waters (European Commission, 1991).

Values of PDA by sediments were relatively low and remained constant at S1, but those found at S2 were greater and highly variable, and no clear relation was found between the nitrate content and denitrification activity. Shifts in denitrification rates could be due to changes in water content at the end of the dry season in Doñana, when the water flow is scarce or even null as compared with that in April. Woodward *et al.* (2009) proposed that oxygenic conditions remaining in sediments after a drought period would result in inhibition of denitrification activity, and Tortosa *et al.* (2011) showed that the pluvial, seasonal regime of Doñana affected denitrification activity, with the lowest values of N_2O emission found at the end of the dry season. Temporal variations in denitrification activity have been reported in creek sediments (Rich and Myrold, 2004) and agricultural (Dandie *et al.*, 2008) and riparian soils (Deslippe *et al.*, 2014).

The copy number of the 16S rRNA gene was within the ranges previously determined by other authors in soils and sediments samples (Dandie *et al.*, 2007; Bárta *et al.*, 2010; García-Lledó *et al.*, 2011; Keil *et al.*, 2011). Similarly, abundances of denitrification genes were similar to those found for *narG* (Smith *et al.*, 2007; Lindsay *et al.*, 2010), *napA* (Marhan *et al.*, 2011), *nirK* (Henry *et al.*, 2006; Dandie *et al.*, 2008; Su *et al.*, 2010; Attard *et al.*, 2011), *nirS* (Yoshida *et al.*, 2009; Attard *et al.*, 2011; Deslippe *et al.*, 2014) and *nosZ* (Torrentó *et al.*, 2011; Ma *et al.*, 2011; Deslippe *et al.*, 2014) genes from soils and sediments under different environmental conditions.

Whereas spatial differences in gene abundances were not detected for any of the denitrification genes, the copy number of *nirS* and *nosZ* showed seasonal variations, being October the month with the highest abundance of those genes. Using hybridization techniques, the abundance of the *narG, nirK, nirS* and *nosZ* genes in a forest soil was higher in the autumn season (Merget *el al.*, 2001) and so was the abundance of the *narG, napA* and *nirS* genes determined by qPCR in samples collected in October from estuarine sediments during 1-year study (Smith *et al.*, 2015a).

Relative abundances of denitrification genes in sediments from la Rocina stream are within the range of those reported for *narG* (Henry *et al.*, 2006; Čuhel *et al.*, 2010), *napA* (Kandeler *et al.*, 2009; Bru *et al.*, 2011; Wieder *et al.*, 2013), *nirK* (Chen *et al.*, 2012a; Palmer *et al.*, 2012), *nirS* (Chon *et al.*, 2011; Chen *et al.*, 2012a; Ligi *et al.*, 2014a,b) and *nosZ* (Chen *et al.*, 2012a; Ligi *et al.*, 2014a,b) from different environmental samples after qPCR determination. In this study, relative abundances of *narG*/*napA* were always higher than

those of *nirK*/*nirS* which, in turn, widely exceeded those of *nosZ*. These results suggest that incomplete denitrifiers are more abundant than those able to carry out the complete denitrification process in sediments from la Rocina stream. They also agree with those found in constructed wetlands (García-Lledó *et al.*, 2011), aquifer's water and sediment (Torrentó *et al.*, 2011) and paddy (Chen *et al.*, 2012a) and riparian soils (Deslippe *et al.*, 2014).

When expressed as gene copy number, spatio-temporal variations of most denitrification genes were not clearly shown; however, both temporal and spatial changes were observed when their relative abundance was calculated. Spatio-temporal shifts in the denitrification communities have been described to occur in constructed wetlands (Song *et al.*, 2012), crop fields (Enwall *et al.*, 2010; Novinscak *et al.*, 2013) and estuarine sediments (Magalhães *et al.*, 2008; Smith *et al.*, 2015a).

Correlations among variables analysed in this study revealed that nitrate content correlated best with the relative abundance of the *narG*, *napA*, *nirS* and *nosZ* genes, the highest positive correlation corresponding to the *nosZ* relative abundance. Similar results were published by Song *et al.* (2012) and Smith *et al.* (2015a) in their studies on the effects of nitrate content on the relative abundance of denitrification genes in constructed wetlands and estuarine sediments, respectively. However, the size of *nosZ* populations was independent of the nitrate content in constructed wetlands (García-Lledó *et al.*, 2011), grassland soils (Keil *et al.*, 2011) and river's biofilms (Lyautey *et al.*, 2013).

The PCA analysis showed a positive relationship between the content of nitrate and the relative abundance of the *narG*, *napA*, *nirK*, *nirS* and *nosZ* genes.

Because *nosZ* showed a strong correlation with the content of nitrate in sediments we used it as a molecular marker to analyse diversity of denitrifiers in the sediment samples taken in years 2009 and 2010. We are aware that the primers used in our study capture only part of the *nosZ* diversity as other primers have been designed that amplify the so called *nosZ* clade II (Sanford *et al.*, 2012; Jones *et al.*, 2013; Ligi *et al.*, 2015). A total of 504 sequences were obtained from the 8 genomic libraries showing homology with *nosZ* genes deposited in the DataBank, of which 202 sequences corresponded to unclassified bacteria, which indicates the presence of hitherto uncultured bacterial groups in the sediments. Similar results were found in marine sediments by Scala and Kherkoff (1999) when analysing *nosZ* denitrifiers and Chen *et al.* (2010) and Smith and Ogram (2008) during studies on bacterial diversity based on the *nirK* and *nirS* genes communities in soil and sediments.

Betaproteobacteria in la Rocina stream sediments dominated over the Alphaproteobacteria, and was followed far behind by Gammaproteobacteria whose presence was restricted to S1. Circumscription of Gammaproteobacteria to specific sites has been previously reported in paddy soils (Chen *et al.*, 2012a).

Generally considered, the dominant clusters found in this study include members of families Bradyrhizobiaceae (cluster C1) and Rhodocyclaceae (cluster C22), and clusters C24 and 27 which contain unclassified sequences. Members of those clusters have been reported to be the most abundant in eutrophic lake sediments (Wang *et al.*, 2013), ephemeral wetland soils (Ma *et al.*, 2011), wastewater treatment plants (Chon *et al.*, 2010), paddy soils (Ishii *et al.*, 2011) and activated sludge (Srinandan e*t al.*, 2011).

The AMOVA test revealed that the structure of the denitrifier communities at S1 remained relatively constant during the 2-year study and changed at S2 associated to the sampling dates and sampling years. OTUs richness and Simpson diversity index also indicated a seasonal effect on diversity at S2. Changes in biodiversity of denitrifier communities

in natural ecosystems following re-wetting after prolonged drought periods have been already published (Groffman *et al.*, 2009), and temporal and spatial variation in their structures were described for bacterial communities bearing *nirK* (Wolsing and Priemé, 2004; Yoshida *et al.*, 2009; Smith, 2010; Tatti *et al.*, 2015), *nirS* (Yoshida *et al.*, 2009; Smith *et al.*, 2010; Hussain *et al.*, 2011; Song *et al.*, 2012; Tatti *et al.*, 2015; Zheng *et al.*, 2015; Pan *et al.*, 2016), and *nosZ* (Smith *et al.*, 2010; Tatti *et al.*, 2015) genes. Nevertheless, other works have shown that denitrifier communities are both spatially and temporally stable (Zhou *et al.*, 2011; Dandie *et al.*, 2011; Chen *et al.*, 2012b; Clark *et al.*, 2012).

Despite the scarce differences in the numbers of clusters and their distribution among the 8 genomic libraries, the CCA analysis confirmed the spatio-temporal variations found in the AMOVA test and suggests that the structure of the *nosZ* populations was affected by the content of nitrate. Other studies, however, suggest that the community structure of denitrifiers is less or not related to the content of nitrate (Wolsing and Priemé, 2004; Zhou *et al.*, 2011; Carrino-Kyker *et al.*, 2012; Chen *et al.*, 2012b; Vilar-Sanz *et al.*, 2013). Nitrate content also affected the diversity of denitrifiers when it was analysed using the *narG* (Reyna *et al.*, 2010) and the *nirK*/*nirS* (Santoro *et al.*, 2006) genes.

Taken together, our results suggest the existence of spatio-temporal variations linked to seasonality in the structure (abundance and diversity) of the denitrifier communities in sediments of two locations differing in nitrate concentration within Doñana National Park. We showed that sediment nitrate content is a main factor structuring these communities, although the effect of other habitat variables cannot be discarded.

Acknowledgements

This study was supported by the ERDF-cofinanced grants RNM-4746 and AGR2012–1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain).

Table 5.1S Values of Spearman coefficient between nitrate content, abundance of the 16S rRNA gene and the *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes and PDA in sediments from la Rocina stream. Values followed by asterisk (*) are statistically significant (*P*-value < 0.05)

Variables	Nitrate content	16S rRNA	*narG*	*napA*	*nirK*	*nirS*	*nosZ*
16S rRNA	–0.046						
narG	0.409*	0.697*					
napA	0.275	0.594*	0.832*				
nirK	0.005	0.775*	0.782*	0.660*			
nirS	0.476*	0.571*	0.891*	0.821*	0.671*		
nosZ	0.442*	0.717*	0.907*	0.781*	0.707*	0.911*	
PDA	0.019	0.115	0.094	0.053	0.007	0.078	0.238

Table 5.2S Factor loadings of sediment nitrate concentration, relative abundance of *narG*, *napA*, *nirS*, *nirK* and *nosZ* denitrification genes and PDA as derived from the principal components analysis (PCA). Spearman's correlation test was used to calculate F1 and F2. Values followed by an asterisk are significant at $\alpha = 0.01$

Variable	F1	F2
Nitrate	0.813*	–0.168
narG (%)	0.927*	–0.070
napA (%)	0.808*	–0.084
nirK (%)	0.645*	–0.390
nirS (%)	0.898*	–0.079
nosZ (%)	0.929*	–0.186
PDA	0.011	0.927*

Table 5.3S Grouping into OTUs and phylogenetic classification of the 504 clones isolated from la Rocina stream. Sediments were taken in April (A) and October (O) 2009 (09) and 2010 (10) at S1 (el Acebrón lagoon) and S2 (la Cañada creek)

OTU	Number of clones	Clones	Closest relative clones and strains based on *nosZ* gene sequence	Homology (%)
1	2	S2A09-5.S2A09-61	Uncultured bacterium clone Nancy126	93.6
2	2	S2O10-17.S2O10-30	Uncultured bacterium clone DCSN-C1	96.3
3	24	S2A09-11.S2A09-28.S2A09-45.S2A09-60. S2A09-86.S2A09-90.S1A09-143.S1O09-110. S1A10-24.S1A10-57.S1A10-93.S1A10-94. S2O10-1.S2O10-8.S2O10-19.S1O10-49. S2A09-91.S2A09-82.S2O09-172.S1O10-29. S1O09-109.S2A10-57.S2A10-61.S1A10-51	*Leptothrix cholodnii* SP-6	97.8
4	12	S2A09-21.S2O09-45.S2A09-92.S2O09-17. S2O09-171.S2O09-174.S2O10-18.S2O09-10. S2A10-15.S2A10-17.S2A10-22.S2A10-37	*Ralstonia eutropha* H16	98.8
5	8	S2A09-41.S2O09-26.S2O09-33.S2A10-6. S2A10-7.S1A10-111.S2O10-12.S2O10-32	Uncultured bacterium clone DCSN-C48	99.3
6	7	S1A09-5.S1O10-35.S1O09-158.S1A10-41. S1A10-3.S1O10-110.S1O10-115	Uncultured bacterium clone Z30K44(12)	98.3
7	40	S1A09-8.S1A09-158.S1A10-2.S1A10-67. S1A10-97.S1O10-59.S1A09-26.S1A09-61. S1O09-67.S1O09-99.S1O09-143.S1O09-150. S1A10-34.S1O10-30.S1O10-41.S1O10-52. S1O10-82.S1O09-40.S1A10-66.S1O10-8. S1A10-69.S1A10-83.S1A10-109.S1O10-116. S1A10-68.S1A09-16. S1A10-48.S1O10-54. S1O10-109.S1O09-149.S1O09-159. S1O10-17.S1A09-10.S1A09-12.S1A09-31. S1O09-206.S1O09-216.S1A09-27.S1O09-93. S1A09-23	Uncultured bacterium clone DaS67	95.5
8	11	S2A09-42.S2A09-57.S2A09-46.S2A09-53. S2O09-101.S1O09-80.S1O09-224.S1A10-87. S2A10-31.S1O10-80.S2O10-4	Uncultured bacterium clone DCSN-C23	98.9
9	2	S1A10-21.S1A10-105	Uncultured bacterium clone AH-Z9	99.2
10	6	S2A09-44.S2A09-70.S2A09-113.S2A09-55. S2A09-67.S2A09-85	*Rubrivivax gelatinosus* IL144	97.0
11	5	S2A09-47.S2A09-58.S2A09-62.S2A10-59. S2A10-63	Uncultured bacterium clone DCSN-C52	90.3
12	5	S2A09-65.S2A10-54.S2A10-66.S2A10-87. S2A10-69	*Ralstonia solanacearum* GMI1000	99.2
13	6	S2A09-52.S2A10-73.S1A10-100.S2O10-83. S2O10-87.S2O09-30	*Thauera phenylacetica* TN9	98.5
14	7	S2A09-38.S1A09-62.S1A09-67.S1A09-113. S2A10-72.S1A09-91.S1A09-84	Uncultured bacterium clone DMZb1-2017	96.9
15	5	S1A09-20.S1A09-37.S1A09-104.S1A10-112. S1O10-26	Uncultured bacterium clone DCSN-C24	90.3

Table 5.3S Continued

OTU	Number of clones	Clones	Closest relative clones and strains based on *nosZ* gene sequence	Homology (%)
16	5	S1A09-19.S1A10-90.S1A10-114.S1O10-73. S1O10-83	Uncultured bacterium clone DCSN-C18	97.8
17	1	S2O09-81	Uncultured bacterium clone DCSN-W54	98.9
18	2	S1A10-107.S2O10-26	Uncultured bacterium clone 2-73	93.6
19	7	S2O10-10.S2O10-70.S2O10-85.S2O10-89. S2O10-92.S2O10-93.S2O10-94	Uncultured bacterium clone DCSN-C36	93.9
20	7	S1A09-39.S1A10-56.S1A10-74.S1O10-1. S1O10-39.S1O10-27.S1O10-40	Uncultured bacterium clone 2-85	97.7
21	8	S2O09-96.S2O09-132.S2O09-148. S2O09-154.S2O09-97.S2O09-136. S2O09-138. S2O09-141	Uncultured bacterium clone 4-37	97.0
22	2	S2A10-86.S2O10-62	Uncultured bacterium clone ISA00346	98.5
23	1	S2O10-25	Uncultured bacterium clone 1-2	97.0
24	2	S2O10-29.S2O10-56	Uncultured bacterium clone 2-31	95.3
25	1	S2A10-19	Uncultured bacterium clone 1-8	99.6
26	2	S2O10-13.S1O10-23	Uncultured bacterium clone DCSN-C46	99.3
27	4	S2O09-99.S1O09-78.S1O09-232.S1O10-74	Uncultured bacterium clone 1-61	94.0
28	7	S2O09-151.S2O09-177.S1O09-79. S1O09-226.S1A10-82.S1O10-112.S1O10-72	Uncultured bacterium clone AH-Z7	92.8
29	2	S1A10-1.S1A10-113	Uncultured *Azospirillum* sp. clone 3-68	85.7
30	2	S2O09-37.S2O09-178	Uncultured bacterium clone 5_10	99.6
31	2	S1A10-110.S2O10-81	Uncultured *Azospirillum* sp. clone 1-66	85.3
32	2	S1A09-15.S1A10-19	Uncultured *Azospirillum* sp. clone 4-36	97.8
33	3	S2O09-179.S2A10-21.S2A10-85	Uncultured *Azospirillum* sp. clone 3-68	98.9
34	12	S1A09-41.S1A09-95.S2O09-27.S1O10-108. S1A09-86.S1O09-115.S1A10-26. S1A10-20. S1O10-107.S1O09-96.S1O10-79.S1O10-28	Uncultured *Azospirillum* sp. clone 1-87	98.5
35	2	S2A10-49.S2A10-50	Uncultured bacterium clone A55_67	96.6

Table 5.3S Continued

OTU	Number of clones	Clones	Closest relative clones and strains based on *nosZ* gene sequence	Homology (%)
36	20	S1A09-51.S2A10-35.S2A10-2.S2A10-12. S2A10-55.S2A10-62.S2A10-71.S2O10-7. S1O10-51.S2A10-79.S1A09-128.S1A09-142. S2O09-2.S1O09-157.S2A10-80. S2O10-28. S2A10-36.S2A10-53.S2A10-46.S1O10-25	Uncultured bacterium clone SNZ44	93.3
37	1	S1A09-32	Uncultured *Azospirillum* sp. clone 3-52	97.3
38	1	S1A09-101	*Herbaspirillum* sp. TSA29	98.1
39	3	S2A10-82.S2O10-16.S2A10-84	Uncultured bacterium clone ISA00276	96.6
40	1	S1O10-48	Uncultured bacterium clone DMZb2-2817	98.1
41	15	S2A09-16.S2A09-17.S2A09-23.S2A09-59. S2A09-66.S2O09-84.S2O09-86.S2O09-121. S2O10-42.S2A09-18.S2A09-20.S1A09-33. S1A09-48.S1A10-104.S2A10-67	*Acidovorax* sp. JS42	97.0
42	5	S1O09-20.S1O09-34.S1O09-47.S1O10-64. S1O10-85	Uncultured *Azospirillum* sp. clone 3-49	84.9
43	19	S2O09-8.S2O09-134.S2A10-83.S2O10-15. S2O09-20.S2O09-92.S2O09-100.S2O09-109. S1O09-64.S1O09-83.S1O09-91.S1O09-225. S1O09-227.S1O09-229.S1A10-46.S1A10-73. S1A10-103.S1O10-104.S2O09-25	Uncultured bacterium clone DCSN-W15	95.1
44	4	S1O09-71.S1O10-4.S1O10-6.S1O10-58	Uncultured bacterium clone Ly-S-B	89.8
45	2	S1A10-72.S1O10-57	Uncultured bacterium clone 2-80	90.8
46	2	S2O09-91.S2O09-94	Uncultured bacterium clone SNZ39	83.9
47	2	S1O09-84.S1O10-65	Uncultured bacterium clone HEb-A036	90.0
48	4	S2O10-2.S1O10-61.S1O10-24.S2O10-5	Uncultured bacterium clone MS_cDNA_017	95.8
49	2	S2O10-3.S2O10-80	Uncultured bacterium clone DMZb1-2188	98.8
50	2	S2A09-10.S2A09-12	Uncultured bacterium clone Nancy58	97.8
51	10	S2A09-56.S2A09-69.S2O09-28.S2O09-46. S2O10-54.S2O10-96.S2A10-29.S2A10-43. S2O10-60.S2A10-39	Uncultured bacterium clone O_149	99.2
52	15	S1A09-9.S1A09-25.S1A10-71.S1A09-87. S1A10-102.S1O09-92.S1A09-102.S1O09-6. S1O09-10.S1O10-16.S1O10-68.S1O09-68. S1A10-33.S1A10-43.S1A09-115	*Bradyrhizobium japonicum* USDA 110	99.6

Table 5.3S Continued

OTU	Number of clones	Clones	Closest relative clones and strains based on *nosZ* gene sequence	Homology (%)
53	2	S2O10-63.S2O10-64	Uncultured bacterium clone ISA00282	99.6
54	1	S1A09-28	Uncultured bacterium clone PS_280	100.0
55	3	S2O09-12.S1O09-55.S1O09-77	Unidentified bacterium clone 7z43	100.0
56	1	S2A10-32	Unidentified bacterium clone 6z6	97.7
57	3	S1A09-107.S1O09-70.S1O09-9	Uncultured bacterium clone B19_26	100.0
58	1	S2O09-110	Unidentified bacterium clone 7z38	97.7
59	1	S2A10-14	Uncultured bacterium clone Nancy80	97.8
60	1	S1A10-98	Uncultured bacterium clone Champ66	99.6
61	3	S2O09-129.S2O09-142.S2O09-153	Uncultured bacterium clone B42_142	93.5
62	1	S2A10-16	Uncultured bacterium clone J33_79	98.5
63	1	S1O09-76	Uncultured bacterium clone J33_22	99.2
64	1	S2O09-98	Uncultured bacterium clone A55_104	94.8
65	1	S2O09-11	Unidentified bacterium clone 19z8	98.9
66	1	S1O09-2	Uncultured bacterium L_189	98.9
67	1	S1O09-25	Uncultured bacterium clone Champ54	98.5
68	3	S2A10-4.S2A10-27.S2O10-73	Uncultured bacterium clone Champ67	97.8
69	10	S2A09-14.S2A10-25.S2O09-144.S2A10-70. S2A10-96.S2O10-31.S2O09-155.S2A10-68. S2A09-115.S2A09-43	*Sinorhizobium fredii* USDA 257	99.2
70	6	S1A09-50.S1O09-37.S1A10-58.S1A10-95. S1O10-114.S1A10-32	Uncultured bacterium clone Nancy1	86.5
71	3	S2O10-9.S2O10-51.S2O10-55	Rhizobiales bacterium N21	91.3
72	2	S1A09-11.S1A09-46	Uncultured bacterium clone ZC55	93.9
73	1	S2A09-73	Uncultured bacterium clone B42_5	98.5

Table 5.3S Continued

OTU	Number of clones	Clones	Closest relative clones and strains based on *nosZ* gene sequence	Homology (%)
74	6	S2A09-84.S2O10-6.S2O10-58.S2O10-84. S2O10-91.S2O10-95	*Chelatococcus daeguensis* strain TAD1	95.9
75	5	S2A09-25.S2O10-71.S2A09-64.S1A10-31. S1A09-24	Uncultured bacterium clone DCSN-C42	95.8
76	1	S2O10-79	Uncultured bacterium clone Sali.nosZ.09	98.9
77	4	S1A09-30.S1A09-59.S1A09-82.S1A09-90	*Azospirillum* sp. A1-3	99.6
78	1	S2A09-81	Uncultured bacterium clone Ljubljana7-71	98.5
79	1	S1A09-108	Uncultured bacterium clone J33_6	95.9
80	3	S1A10-106.S2O10-11.S2O10-48	Uncultured bacterium clone 4-5	90.9
81	1	S1O10-63	Uncultured bacterium clone Ljubljana7-59	98.9
82	1	S1A09-152	Uncultured bacterium clone ISA00274	99.2
83	1	S2A10-9	Unidentified bacterium clone 18z8	98.1
84	4	S1A09-14.S1A09-92.S1O09-4.S1A10-101	Uncultured bacterium 878	91.7
85	6	S2A09-36.S2A09-50.S2A09-63.S2O09-16. S2A09-54.S1O09-66	*Shinella zoogloeoides* BC026	97.7
86	12	S2A09-93.S2O09-133.S2O09-140. S2O09-147.S2A10-60.S2A10-64.S2A10-81. S2A10-89. S2O10-14.S2O10-69.S2O10-97. S2O10-86	Uncultured bacterium clone Champ98	96.9
87	19	S2A09-40.S1A09-55.S1A10-65.S1A10-88. S1A10-96.S2O10-22.S1O10-77.S1O10-111. S1A09-7.S1O09-60.S1O09-148.S1O09-222. S2A10-48.S2O09-23.S1A10-16.S1A09-6. S1A09-114.S1A09-144.S2A10-77	*Paracoccus denitrificans* PD1222	98.1
88	12	S1O09-90.S1O09-230.S1O09-233.S1A10-70. S1A10-84.S1A10-85.S1O09-95.S1A10-92. S1O10-62.S1O10-75.S1O10-76.S1O10-81	Uncultured bacterium clone 1_87	95.5
89	2	S1A09-66.S1A10-108	*Rhodobacter sphaeroides* KD131	96.5
90	3	S2O09-6.S2O09-88.S2O10-21	Uncultured bacterium clone Dianchi-N11	97.7
91	3	S2O09-83.S1A10-99.S2O09-85	Uncultured bacterium clone 5_11	91.8
92	6	S2O10-23.S2O10-27.S2O10-35.S2O10-90. S2O10-98.S2O10-72	Uncultured bacterium clone DCSN-C25	96.3

Table 5.3S Continued

OTU	Number of clones	Clones	Closest relative clones and strains based on *nosZ* gene sequence	Homology (%)
93	2	S1O09-212.S1O10-70	*Dinoroseobacter shibae* DFL 12	87.6
94	1	S1O09-205	*Rhodobacter capsulatus* SB 1003	94.4
95	1	S2O09-89	Uncultured bacterium clone 1_58	99.3
96	1	S1A09-38	Uncultured bacterium clone ISA00249	98.9
97	3	S1A09-53.S1A09-60.S1A09-96	Uncultured bacterium clone 1-77	95.5
98	3	S2A09-29.S2O09-21.S2O09-22	Uncultured bacterium clone ISA00234	93.3
99	5	S2A09-48.S2O09-90.S2A10-75.S2O10-36. S2O10-82	*Bradyrhizobium* sp. BTAi1	92.8
100	1	S2O10-37	Uncultured bacterium clone ISA00207	88.1
101	1	S2O09-14	Uncultured bacterium clone ISA00187	95.8
102	1	S2O10-38	Uncultured bacterium 13DNB	98.3
103	2	S2A09-114.S2O09-13	Uncultured bacterium clone ISA00182	99.6
104	3	S2O09-131.S2O09-146.S2O09-152	Uncultured bacterium clone ISA00317	93.6
105	3	S2O09-175.S2O09-176.S1O10-12	Uncultured bacterium clone ISA00193	92.0
106	7	S1A09-13.S1A09-21.S1O09-135.S1O09-142. S1O09-228.S1O10-103.S1O09-204	Uncultured bacterium clone C57_51	98.5
107	4	S1A09-47.S1A10-14.S1A10-17.S1O10-106	*Pseudomonas stutzeri* CCUG 29243	95.8
108	3	S1O09-231.S1O10-105.S1O10-78	*Pseudomonas stutzeri* LSMN2	99.6
109	1	S2A09-83	Uncultured bacterium clone DCSN-W50	96.2

Table 5.4S Clustering of the 504 *nosZ* clones isolated from la Rocina stream sediments. Values represent the number of sequences in each library that belong to the same cluster followed by the percentage (%) of clones in a given library. Sediments were taken in April (A) and October (O) 2009 (09) and 2010 (10) at S1 (el Acebrón lagoon) and S2 (la Cañada creek)

Cluster	Class	S1A09	S1A10	S1O09	S1O10	S2A09	S2A10	S2O09	S2O10
C1 (Bradyrhizobiaceae)	Alphaproteobacteria	10.77	7.94	18.03	3.33	6.90	13.79	12.86	8.70
C2 (Bradyrhizobiaceae)	Alphaproteobacteria	0.00	0.00	0.00	0.00	5.17	1.72	14.29	5.80
C3 (Rhizobiaceae)	Alphaproteobacteria	0.00	0.00	0.00	0.00	6.90	6.90	2.86	5.80
C4 (Unclassified 1)	Alphaproteobacteria	1.54	6.35	1.64	1.67	0.00	0.00	0.00	0.00
C5 (Unclassified 2)	Alphaproteobacteria	3.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C6 (Unclassified 3)	Alphaproteobacteria	3.08	1.59	1.64	0.00	1.72	0.00	0.00	0.00
C7 (Unclassified 4)	Alphaproteobacteria	1.54	3.17	0.00	3.33	5.17	0.00	0.00	2.90
C8 (Rhodospirillaceae)	Alphaproteobacteria	18.46	0.00	6.56	1.67	0.00	1.72	0.00	2.90
C9 (Beijerinckiaceae)	Alphaproteobacteria	0.00	0.00	0.00	0.00	1.72	0.00	0.00	7.25
C10 (Unclassified 5)	Alphaproteobacteria	0.00	6.35	8.20	6.67	0.00	0.00	0.00	0.00
C11 (Rhodobacteraceae)	Alphaproteobacteria	1.54	3.17	3.28	1.67	0.00	0.00	7.14	10.14
C12 (Rhizobiaceae)	Alphaproteobacteria	0.00	0.00	0.00	0.00	8.62	6.90	4.29	5.80
C13 (Rhodobacteraceae)	Alphaproteobacteria	7.69	6.35	4.92	3.33	1.72	3.45	2.86	1.45
C14 (Pseudomonadaceae)	Gammaproteobacteria	1.54	3.17	1.64	5.00	0.00	0.00	0.00	0.00
C15 (Unclassified 6)	Betaproteobacteria	0.00	0.00	4.92	3.33	0.00	0.00	0.00	0.00
C16 (Unclassified 7)	Betaproteobacteria	0.00	0.00	0.00	6.67	0.00	0.00	0.00	2.90
C17 (Unclassified 8)	Betaproteobacteria	0.00	0.00	1.64	5.00	0.00	0.00	2.86	2.90
C18 (Unclassified 9)	Betaproteobacteria	0.00	4.76	9.84	1.67	0.00	0.00	10.00	1.45
C19 (Comamonadaceae)	Betaproteobacteria	3.08	3.17	0.00	0.00	12.07	1.72	4.29	2.90
C20 (Unclassified 10)	Betaproteobacteria	12.31	1.59	0.00	1.67	1.72	1.72	0.00	0.00
C21 (Rhodocyclaceae)	Betaproteobacteria	0.00	1.59	0.00	0.00	1.72	1.72	1.43	2.90
C22 (Rhodocyclaceae)	Betaproteobacteria	3.08	7.94	3.28	13.33	0.00	3.45	17.14	18.84

Table 5.4S Continued

Cluster	Class	S1A09	S1A10	S1O09	S1O10	S2A09	S2A10	S2O09	S2O10
C23 (Burkholderiaceae)	Betaproteobacteria	0.00	4.76	3.28	3.33	1.72	10.34	2.86	1.45
C24 (Unclassified 11)	Betaproteobacteria	13.85	4.76	6.56	10.00	0.00	27.59	4.29	2.90
C25 (Unclassified 12)	Betaproteobacteria	0.00	0.00	0.00	1.67	3.45	0.00	0.00	2.90
C26 (Unclassified Burkholderiales)	Betaproteobacteria	0.00	0.00	0.00	0.00	10.34	0.00	0.00	0.00
C27 (Unclassified 13)	Betaproteobacteria	15.38	15.87	16.39	16.67	0.00	0.00	0.00	0.00
C28 (Unclassified 14)	Betaproteobacteria	0.00	4.76	3.28	1.67	6.90	1.72	1.43	1.45
C29 (Unclassified Burkholderiales)	Betaproteobacteria	1.54	7.94	3.28	3.33	13.79	3.45	1.43	4.35
C30 (Unclassified 15)	Betaproteobacteria	1.54	4.76	1.64	5.00	1.72	3.45	2.86	2.90
C31 (Comamonadaceae)	Betaproteobacteria	0.00	0.00	0.00	0.00	8.62	10.34	7.14	1.45

Table 5.5S Pairwise comparisons of the sequence variation among the different clone libraries and within the clone libraries according to the MANOVA test

	% variation	
Clone libraries	Among clone libraries	Within clone libraries
All clone libraries	3.62	96.38
S1A09 VS S1O09	1.23	98.77
S1A09 VS S1A10	1.55	98.45
S1A09 VS S1O10	3.16	96.84
S1A10 VS S1O09	1.01	98.99
S1A10 VS S1O10	1.01	98.99
S1O09 VS S1O10	2.46	97.54
S2A09 VS S2O09	2.07	97.93
S2A09 VS S2A10	2.24	97.76
S2A09 VS S2O10	2.18	97.82
S2A10 VS S2O09	2.44	97.56
S2A10 VS S2O10	3.08	96.92
S2O09 VS S2O10	1.11	98.89

References

Attard, E., Recous, S., Chabbi, A., de Berranger, C., Guillaumaud, N., Labreuche, J., Philippot, L., Schmid, B., and Le Roux, X. (2011). Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Glob. Change Biol. *17*, 1975–1989. http://dx.doi.org/10.1111/j.1365-2486.2010.02340.x

Babic, K.H., Schauss, K., Hai, B., Sikora, S., Redzepovic, S., Radl, V., and Schloter, M. (2008). Influence of different *Sinorhizobium meliloti* inocula on abundance of genes involved in nitrogen transformations in the rhizosphere of alfalfa (*Medicago sativa* L.). Environ. Microbiol. *10*, 2922–2930. http://dx.doi.org/10.1111/j.1462-2920.2008.01762.x

Bárta, J., Melichová, T., Vaněk, D., Picek, T., and Šantrůčková, H. (2010). Effect of pH and dissolved organic matter on the abundance of *nirK* and *nirS* denitrifiers in spruce forest soil. Biogeochemistry.101, 123–132. http://dx.doi.org/10.1007/s10533-010-9430-9

Bedmar, E.J., Bueno, E., Correa-Galeote, D., Torres, M.J., Delgado, M.J., and Mesa, S. (2013). Ecology of denitrification in soils and plant-associated bacteria. In Beneficial Plant-Microbial Interactions: Ecology and Applications, M.B. Rodelas and J. Gonzalez-López, eds. (Boca Raton, USA: CRC Press), pp. 164–182.

Bru, D., Ramette, A., Saby, N.P., Dequiedt, S., Ranjard, L., Jolivet, C., Arrouays, D., and Philippot, L. (2011). Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. ISME. J. *5*, 532–542. http://dx.doi.org/10.1038/ismej.2010.130

Carrino-Kyker, S.R., Smemo, K.A., and Burke, D.J. (2012). The effects of pH change and NO3- pulse on microbial community structure and function: a vernal pool microcosm study. FEMS Microbiol. Ecol. *81*, 660–672. http://dx.doi.org/10.1111/j.1574-6941.2012.01397.x

Chen, Z., Luo, X., Hu, R., Wu, M., Wu, J., and Wei, W. (2010). Impact of long-term fertilization on the composition of denitrifier communities based on nitrite reductase analyses in a paddy soil. Microb. Ecol. *60*, 850–861. http://dx.doi.org/10.1007/s00248-010-9700-z

Chen, Z., Hou, H., Zheng, Y., Qin, H., Zhu, Y., Wu, J., and Wei, W. (2012a). Influence of fertilisation regimes on a *nosZ*-containing denitrifying community in a rice paddy soil. J. Sci. Food Agric. *92*, 1064–1072. http://dx.doi.org/10.1002/jsfa.4533

Chen, Z., Liu, J., Wu, M., Xie, X., Wu, J., and Wei, W. (2012b). Differentiated response of denitrifying communities to fertilization regime in paddy soil. Microb. Ecol. *63*, 446–459. http://dx.doi.org/10.1007/s00248-011-9909-5

Chon, K., Kim, Y., Chang, N.I., and Cho, J. (2010). Evaluating wastewater stabilizing constructed wetland, through diversity and abundance of the nitrite reductase gene *nirS*, with regard to nitrogen control. Desalination. *264*, 201–205. http://dx.doi.org/10.1016/j.desal.2010.05.010

Chon, K., Chang, J.S., Lee, E., Lee, J., Ryu, J., and Cho, J. (2011). Abundance of denitrifying genes coding for nitrate (*narG*), nitrite (*nirS*), and nitrous oxide (*nosZ*) reductases in estuarine versus wastewater effluent-fed constructed wetlands. Ecol. Eng. 37, 64–69. http://dx.doi.org/10.1016/j.ecoleng.2009.04.005

Clark, I.M., Buchkina, N., Jhurreea, D., Goulding, K.W., and Hirsch, P.R. (2012). Impacts of nitrogen application rates on the activity and diversity of denitrifying bacteria in the Broadbalk Wheat Experiment. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *367*, 1235–1244. http://dx.doi.org/10.1098/rstb.2011.0314

Correa-Galeote, D., Tortosa, G., and Bedmar, E.J. (2013a). Determination of denitrification genes abundance in environmental samples. Metagenomics.2, article ID 235702. http://dx.doi.org/10.4303/mg/235702

Correa-Galeote, D., Marco, D.E., Tortosa, G., Bru, D., Philippot, L., and Bedmar, E.J. (2013b). Spatial distribution of N-cycling microbial communities showed complex patterns in constructed wetland sediments. FEMS Microbiol. Ecol. *83*, 340–351. http://dx.doi.org/10.1111/j.1574-6941.2012.01479.x

Čuhel, J., Simek, M., Laughlin, R.J., Bru, D., Chèneby, D., Watson, C.J., and Philippot, L. (2010). Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. Appl. Environ. Microbiol. *76*, 1870–1878. http://dx.doi.org/10.1128/AEM.02484-09

Dandie, C.E., Miller, M.N., Burton, D.L., Zebarth, B.J., Trevors, J.T., and Goyer, C. (2007). Nitric oxide reductase-targeted real-time PCR quantification of denitrifier populations in soil. Appl. Environ. Microbiol. *73*, 4250–4258. http://dx.doi.org/10.1128/AEM.00081-07

Dandie, C.E., Burton, D.L., Zebarth, B.J., Henderson, S., Trevors, J.T., and Goyer, C. (2008). Changes in bacterial denitrifier community abundance over time in an agricultural field and their relationship with denitrification activity. Appl. Environ. Microbiol. *74*, 5997–6005. http://dx.doi.org/10.1128/AEM.00441-08

Dandie, C.E., Wertz, S., Leclair, C.L., Goyer, C., Burton, D.L., Patten, C.L., Zebarth, B.J., and Trevors, J.T. (2011). Abundance, diversity and functional gene expression of denitrifier communities in adjacent riparian and agricultural zones. FEMS Microbiol. Ecol. *77*, 69–82. http://dx.doi.org/10.1111/j.1574-6941.2011.01084.x

Deiglmayr, K., Philippot, L., and Kandeler, E. (2006). Functional stability of the nitrate-reducing community in grassland soils towards high nitrate supply. Soil Biol. Biochem. *38*, 2980-2984. http://dx.doi.org/10.1016/j.soilbio.2006.04.034

Deslippe, J.R., Jamali, H., Jha, N., and Saggar, S. (2014). Denitrifier community size, structure and activity along a gradient of pasture to riparian soils. Soil Biol. Biochem. *71*, 48–60. http://dx.doi.org/10.1016/j.soilbio.2014.01.007

Espinar, J.L., and Serrano, L. (2009). A quantitative hydrogeomorphic approach to the classification of temporary wetlands in the Donana National Park (SW Spain). Aquatic Ecol. 43, 323–334. http://dx.doi.org/10.1007/s10452-007-9162-7

European Commision (1991). Council directive 91/676/EEC of 12 December 1991, concerning the protection of wasters against pollution caused by nitrates from agricultural sources. Official J. Eur. Communities. L. *375*, 31/12/1991.

Enwall, K., Philippot, L., and Hallin, S. (2005). Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. Appl. Environ. Microbiol. *71*, 8335–8343. http://dx.doi.org/10.1128/AEM.71.12.8335-8343.2005

Enwall, K., Throbäck, I.N., Stenberg, M., Söderström, M., and Hallin, S. (2010). Soil resources influence spatial patterns of denitrifying communities at scales compatible with land management. Appl. Environ. Microbiol. *76*, 2243–2250. http://dx.doi.org/10.1128/AEM.02197-09

García-Lledó, A., Vilar-Sanz, A., Trias, R., Hallin, S., and Bañeras, L. (2011). Genetic potential for N2O emissions from the sediment of a free water surface constructed wetland. Water Res. *45*, 5621–5632. http://dx.doi.org/10.1016/j.watres.2011.08.025

Groffman, P.M., Butterbach-Bahl, K., Fulweiler, R.W., Gold, A.J., Morse, J.L., Stander, E.K., Tague, C., Tonitto, C., and Vidon, P. (2009). Challenges to incorporating spatially and temporally explicit phenomena (hotspots and hot moments) in denitrification models. Biogeochemistry 93, 49–77. http://dx.doi.org/10.1007/s10533-008-9277-5

Hayatsu, M., Tago, K., and Saito, M. (2008). Various players in the nitrogen cycle: diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Sci. Plant. Nutr. *54*, 33–45. http://dx.doi.org/10.1111/j.1747-0765.2007.00195.x

Henry, S., Bru, D., Stres, B., Hallet, S., and Philippot, L. (2006). Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. Appl. Environ. Microbiol. *72*, 5181–5189. http://dx.doi.org/10.1128/AEM.00231-06

Henry, S., Texier, S., Hallet, S., Bru, D., Dambreville, C., Chèneby, D., Bizouard, F., Germon, J.C., and Philippot, L. (2008). Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: insight into the role of root exudates. Environ. Microbiol. *10*, 3082–3092. http://dx.doi.org/10.1111/j.1462-2920.2008.01599.x

Hussain, Q., Liu, Y., Jin, Z., Zhang, A., Pan, G., Li, L., Crowley, D., Zhang, X., Song, X., and Cui, L. (2011). Temporal dynamics of ammonia oxidizer (*amoA*) and denitrifier(*nirK*) communities in the rhizosphere of a rice ecosystem from Tai Lake region, China. Appl. Soil Ecol. *48*, 210–218. http://dx.doi.org/10.1016/j.apsoil.2011.03.004

Ishii, S., Ohno, H., Tsuboi, M., Otsuka, S., and Senoo, K. (2011). Identification and isolation of active N2O reducers in rice paddy soil. ISME. J. *5*, 1936–1945. http://dx.doi.org/10.1038/ismej.2011.69

Jones, C.M., Graf, D.R., Bru, D., Philippot, L., and Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. ISME. J. *7*, 417–426. http://dx.doi.org/10.1038/ismej.2012.125

Kandeler, E., Brune, T., Enowashu, E., Dörr, N., Guggenberger, G., Lamersdorf, N., and Philippot, L. (2009). Response of total and nitrate-dissimilating bacteria to reduced N deposition in a spruce forest soil profile. FEMS Microbiol. Ecol. *67*, 444–454. http://dx.doi.org/10.1111/j.1574-6941.2008.00632.x

Keil, D., Meyer, A., Berner, D., Poll, C., Schützenmeister, A., Piepho, H.P., Vlasenko, A., Philippot, L., Schloter, M., Kandeler, E., *et al.* (2011). Influence of land-use intensity on the spatial distribution of N-cycling microorganisms in grassland soils. FEMS Microbiol. Ecol. *77*, 95–106. http://dx.doi.org/10.1111/j.1574-6941.2011.01091.x

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. *16*, 111–120. http://dx.doi.org/10.1007/BF01731581

Klappenbach, J.A., Saxman, P.R., Cole, J.R., and Schmidt, T.M. (2001). rrndb: the Ribosomal RNA Operon Copy Number Database. Nucleic Acids Res. *29*, 181–184. http://dx.doi.org/10.1093/nar/29.1.181

Kraft, B., Strous, M., and Tegetmeyer, H.E. (2011). Microbial nitrate respiration – genes, enzymes and environmental distribution. J. Biotechnol. *155*, 104–117. http://dx.doi.org/10.1016/j.jbiotec.2010.12.025

Ligi, T., Oopkaup, K., Truu, M., Preem, J.K., Nõlvak, H., Mitsch, W.J., Mander, Ü., and Truu, J. (2014a). Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16S rRNAamplicon sequencing. Ecol. Eng. *72*, 56–66. http://dx.doi.org/10.1016/j.ecoleng.2013.09.007

Ligi, T., Truu, M., Truu, J., Nõlvak, H., Kaasik, A., Mitsch, W.J., and Mander, Ü. (2014b). Effects of soil chemical characteristics and water regime on denitrification genes (*nirS*, *nirK*, and *nosZ*) abundances in a created riverine wetland complex. Ecol. Eng. *72*, 47–55. http://dx.doi.org/10.1016/j.ecoleng.2013.07.015

Ligi, T., Truu, M., Oopkaup, K., Nõlvak, H., Mander, Ü., Mitsch, W.J., and Truu, J. (2015). The genetic potential of N_2 emission via denitrification and ANAMMOX from the soils and sediments of a created riverine treatment wetland complex. Ecol. Eng. *80*, 181–190. http://dx.doi.org/10.1016/j.ecoleng.2014.09.072

Lindsay, E.A., Colloff, M.J., Gibb, N.L., and Wakelin, S.A. (2010). The abundance of microbial functional genes in grassy woodlands is influenced more by soil nutrient enrichment than by recent weed invasion or livestock exclusion. Appl. Environ. Microbiol. *76*, 5547–5555. http://dx.doi.org/10.1128/AEM.03054-09

Lyautey, E., Hallin, S., Teissier, S., Iribar, A., Compin, A., Philippot, L., and Garabetian, F. (2013). Abundance, activity and structure of denitrifier communities in phototrophic river biofilms (River Garonne, France). Hydrobiologia *716*, 177–187. http://dx.doi.org/10.1007/s10750-013-1561-2

Ma, W.K., Farrell, R.E., and Siciliano, S.D. (2011). Nitrous Oxide Emissions from Ephemeral Wetland Soils are Correlated with Microbial Community Composition. Front. Microbiol. *2*, 110. http://dx.doi.org/10.3389/fmicb.2011.00110

Magalhães, C., Bano, N., Wiebe, W.J., Bordalo, A.A., and Hollibaugh, J.T. (2008). 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. Microb. Ecol. 55, 259–269. http://dx.doi.org/10.1007/s00248-007-9273-7

Magurran, A.E. (2004). Measuring biological diversity. Afr. J. Aquat. Sci. *29*, 285–286.

Manzano, M., Custodio, E., Higueras, H., Puig, R., and Soler, A. (2009). Influencia de la gestión del acuífero sobre los humedales del manto eólico de Doñana. Boletín Geológico y Minero. *120*, 377–392.

Marhan, S., Philippot, L., Bru, D., Rudolph, S., Franzaring, J., Högy, P., Fangmeier, A., and Kandeler, E. (2011). Abundance and activity of nitrate reducers in an arable soil are more affected by temporal variation and soil depth than by elevated atmospheric [CO2.] FEMS Microbiol. Ecol. *76*, 209–219. http://dx.doi.org/10.1111/j.1574-6941.2011.01048.x

Mergel, A., Schmitz, O., Mallmann, T., and Bothe, H. (2001). Relative abundance of denitrifying and dinitrogen-fixing bacteria in layers of a forest soil. FEMS Microbiol. Ecol. *36*, 33–42. http://dx.doi.org/10.1111/j.1574-6941.2001.tb00823.x

Miller, J.H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, USA: Cold Spring Harbor Laboratory).

Morris, E.K., Caruso, T., Buscot, F., Fischer, M., Hancock, C., Maier, T.S., Meiners, T., Müller, C., Obermaier, E., Prati, D., *et al.* (2014). Choosing and using diversity indices: insights for ecological applications from the German Biodiversity Exploratories. Ecol. Evol. *4*, 3514–3524. http://dx.doi.org/10.1002/ece3.1155

Novinscak, A., Goyer, C., Dandie, C.E., and Filion, M. (2013). Abundance, diversity and spatio-temporal dynamics of *nirS* gene-harbouring denitrifiers in a potato field over the course of a growth season. Syst. Appl. Microbiol. *36*, 112–115. http://dx.doi.org/10.1016/j.syapm.2012.10.005

Palmer, K., Biasi, C., and Horn, M.A. (2012). Contrasting denitrifier communities relate to contrasting N2O emission patterns from acidic peat soils in arctic tundra. ISME. J. *6*, 1058–1077. http://dx.doi.org/10.1038/ismej.2011.172

Pan, H., Li, Y., Guan, X., Li, J., Xu, X., Liu, J., Zhang, Q., Xu, J., and Di, H. (2016). Management practices have a major impact on nitrifier and denitrifier communities in a semiarid grassland ecosystem. J. Soils Sediments. *16*, 896–908. http://dx.doi.org/10.1007/s11368-015-1321-1

Petric, I., Philippot, L., Abbate, C., Bispo, A., Chesnot, T., Hallin, S., Laval, K., Lebeau, T., Lemanceau, P., Leyval, C., *et al.* (2011). Inter-laboratory evaluation of the ISO standard 11063 'Soil quality - Method to directly extract DNA from soil samples'. J. Microbiol. Methods *84*, 454–460. http://dx.doi.org/10.1016/j.mimet.2011.01.016

Philippot, L., Hallin, S., and Schloter, M. (2007). Ecology of denitrifying prokaryotes in agricultural soil. Adv. Agron. *96*, 249–305. http://dx.doi.org/10.1016/S0065-2113(07)96003-4

Piña-Ochoa, E., Høgslund, S., Geslin, E., Cedhagen, T., Revsbech, N.P., Nielsen, L.P., Schweizer, M., Jorissen, F., Rysgaard, S., and Risgaard-Petersen, N. (2010). Widespread occurrence of nitrate storage and denitrification among Foraminifera and Gromiida. Proc. Natl. Acad. Sci. U.S.A. *107*, 1148–1153. http://dx.doi.org/10.1073/pnas.0908440107

Prendergast-Miller, M.T., Baggs, E.M., and Johnson, D. (2011). Nitrous oxide production by the ectomycorrhizal fungi *Paxillus involutus* and *Tylospora fibrillosa*. FEMS Microbiol. Lett. *316*, 31–35. http://dx.doi.org/10.1111/j.1574-6968.2010.02187.x

Reyna, L., Wunderlin, D.A., and Genti-Raimondi, S. (2010). Identification and quantification of a novel nitrate-reducing community in sediments of Suquía River basin along a nitrate gradient. Environmental Pollut. *158*, 1608–1614. http://dx.doi.org/10.1016/j.envpol.2009.12.014

Rich, J.J., and Myrold, D.D. (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. http://dx.doi.org/10.1016/j.soilbio.2004.03.008

Richardson, D.J. (2011). Redox complexes of the nitrogen cycle. In Nitrogen Cycling in Bacteria: Molecular analysis, J.W.B. Moir, ed. (Norfolk, U.K.: Caister Academic Press), pp. 23–37.

Risgaard-Petersen, N., Langezaal, A.M., Ingvardsen, S., Schmid, M.C., Jetten, M.S., Op den Camp, H.J., Derksen, J.W., Piña-Ochoa, E., Eriksson, S.P., Nielsen, L.P., *et al.* (2006). Evidence for complete denitrification in a benthic foraminifer. Nature *443*, 93–96. http://dx.doi.org/10.1038/nature05070

Sánchez, C., Bedmar, E.J., and Delgado, M.J. (2011). Denitrification in legume-associated endosymbiotic bacteria. In Nitrogen Cycling in Bacteria: Molecular analysis, J.W.B. Moir, ed. (Norfolk, U.K.: Caister Academic Press), pp. 195–210.

Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K.K., Ritalahti, K.M., *et al.* (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U.S.A. *109*, 19709–19714. http://dx.doi.org/10.1073/pnas.1211238109

Santoro, A.E., Boehm, A.B., and Francis, C.A. (2006). Denitrifier community composition along a nitrate and salinity gradient in a coastal aquifer. Appl. Environ. Microbiol. *72*, 2102–2109. http://dx.doi.org/10.1128/AEM.72.3.2102-2109.2006

Scala, D.J., and Kerkhof, L.J. (1999). Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl. Environ. Microbiol. *65*, 1681–1687.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., *et al.* (2009). Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Envrion. Microbiol. *75*, 7537–7541. http://dx.doi.org/10.1128/AEM.01541-09

Serrano, L., Bravo, M.A., Jiménez, A.J., Prenda Marín, J., Martín, M.I., Pidre, J.R., and Salas Rodríguez, J.J. (2006). La contaminación por eutrofización en arroyos que vierten a la Marisma de Doñana. In V Congreso Ibérico de Gestión y Planificación del Agua (Cuencas Compartidas, Claves para la Gestión Sostenible del Agua y del Territorio). Faro. Vol. *4*, 8.

Šimek, M., and Hopkins, D.W. (1999). Regulation of potential denitrification by soil pH in long-term fertilized arable soils. Biol. Fert. Soils. 30, 41–47. http://dx.doi.org/10.1007/s003740050585

Šimek, M., Elhottová, D., Klimeš, F., and Hopkins, D.W. (2004). Emissions of N_2O and CO_2, denitrification measurements and soil properties in red clover and ryegrass stands. Soil Biol. Biochem. *36*, 9–21. http://dx.doi.org/10.1016/j.soilbio.2003.08.010

Smith, C.J., Nedwell, D.B., Dong, L.F., and Osborn, A.M. (2007). Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. Appl. Environ. Microbiol. *73*, 3612–3622. http://dx.doi.org/10.1128/AEM.02894-06

Smith, J.M., and Ogram, A. (2008). Genetic and functional variation in denitrifier populations along a short-term restoration chronosequence. Appl. Environ. Microbiol. *74*, 5615–5620. http://dx.doi.org/10.1128/AEM.00349-08

Smith, J., Wagner-Riddle, C., and Dunfield, K. (2010). Season and management related changes in the diversity of nitrifying and denitrifying bacteria over winter and spring. Appl. Soil Ecol. 44, 138–146. http://dx.doi.org/10.1016/j.apsoil.2009.11.004

Smith, C.J., Dong, L.F., Wilson, J., Stott, A., Osborn, A.M., and Nedwell, D.B. (2015a). Seasonal variation in denitrification and dissimilatory nitrate reduction to ammonia process rates and corresponding key functional genes along an estuarine nitrate gradient. Front. Microbiol. *6*, 542. http://dx.doi.org/10.3389/fmicb.2015.00542

Smith, J.M., Mosier, A.C., and Francis, C.A. (2015b). Spatio-temporal relationships between the abundance, distribution, and potential activities of ammonia-oxidizing and denitrifying microorganisms in intertidal sediments. Microb. Ecol. *69*, 13–24. http://dx.doi.org/10.1007/s00248-014-0450-1

Song, K., Kang, H., Zhang, L., and Mitsch, W.J. (2012). Seasonal and spatial variations of denitrification and denitrifying bacterial community structure in created riverine wetlands. Ecol. Eng. *38*, 130–134. http://dx.doi.org/10.1016/j.ecoleng.2011.09.008

Song, K., Hernandez, M.E., Batson, J.A., and Mitsch, W.J. (2014). Long-term denitrification rates in created riverine wetlands and their relationship with environmental factors. Ecol. Eng. *72*, 40–46. http://dx.doi.org/10.1016/j.ecoleng.2013.06.041

van Spanning, R.J., Richardson, D.J., and Ferguson, S.J. (2007). Introduction to the biochemistry and molecular biology of denitrification. In Biology of the nitrogen cycle, H. Bothe, S.J. Ferguson and W.E. Newton, eds. (Amsterdam, The Netherlands: Elsevier), pp. 83–93.

Srinandan, C.S., Shah, M., Patel, B., and Nerurkar, A.S. (2011). Assessment of denitrifying bacterial composition in activated sludge. Bioresour. Technol. *102*, 9481–9489. http://dx.doi.org/10.1016/j.biortech.2011.07.094

Su, M., Kleineidam, K., and Schloter, M. (2010). Influence of different litter quality on the abundance of genes involved in nitrification and denitrification after freezing and thawing of an arable soil. Biol. Fert. Soils. *46*, 537–541. http://dx.doi.org/10.1007/s00374-010-0449-9

Takaya, N. (2002). Dissimilatory nitrate reduction metabolisms and their control in fungi. J. Biosci. Bioeng. *94*, 506–510. http://dx.doi.org/10.1016/S1389-1723(02)80187-6

Tatti, E., Goyer, C., Burton, D.L., Wertz, S., Zebarth, B.J., Chantigny, M., and Filion, M. (2015). Tillage Management and Seasonal Effects on Denitrifier Community Abundance, Gene Expression and Structure over Winter. Microb. Ecol. *70*, 795–808. http://dx.doi.org/10.1007/s00248-015-0591-x

Tiedje, J.M. (1988). Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In Biology of anaerobic microorganisms, A.J.B. Zehnder, ed. (New York, USA: John Wiley & Sons, Inc.) pp. 179–244.

Torrentó, C., Urmeneta, J., Otero, N., Soler, A., Viñas, M., and Cama, J. (2011). Enhanced denitrification in groundwater and sediments from a nitrate-contaminated aquifer after addition of pyrite. Chem. Geol. *287*, 90–101. http://dx.doi.org/10.1016/j.chemgeo.2011.06.002

Tortosa, G., Correa-Galeote, D., Sánchez-Raya, A.J., Delgado, A., Sánchez-Monedero, M.A., and Bedmar, E.J. (2011). Effects of nitrate contamination and seasonal variation on the denitrification and greenhouse gas production in La Rocina stream (Doñana National Park, SW Spain). Ecol. Eng. *37*, 539–548. http://dx.doi.org/10.1016/j.ecoleng.2010.06.029

Vilar-Sanz, A., Puig, S., García-Lledó, A., Trias, R., Balaguer, M.D., Colprim, J., and Bañeras, L. (2013). Denitrifying bacterial communities affect current production and nitrous oxide accumulation in a microbial fuel cell. PLOS ONE *8*, e63460. http://dx.doi.org/10.1371/journal.pone.0063460

Wang, C., Zhu, G., Wang, Y., Wang, S., and Yin, C. (2013). Nitrous oxide reductase gene (*nosZ*) and N2O reduction along the littoral gradient of a eutrophic freshwater lake. J. Environ. Sci. *25*, 44–52. http://dx.doi.org/10.1016/S1001-0742(12)60005-9

Wieder, W.R., Cleveland, C.C., Taylor, P.G., Nemergut, D.R., Hinckley, E.L., Philippot, L., Bru, D., Weintraub, S.R., Martin, M., and Townsend, A.R. (2013). Experimental removal and addition of leaf litter inputs reduces nitrate production and loss in a lowland tropical forest. Biogeochemistry *113*, 629–642. http://dx.doi.org/10.1007/s10533-012-9793-1

Wolsing, M., and 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. http://dx.doi.org/10.1016/j.femsec.2004.02.002

Woodward, K.B., Fellows, C.S., Conway, C.L., and Hunter, H.M. (2009). Nitrate removal, denitrification and nitrous oxide production in the riparian zone of an ephemeral stream. Soil Biol. Biochem. *41*, 671–680. http://dx.doi.org/10.1016/j.soilbio.2009.01.002

Yoshida, M., Ishii, S., Otsuka, S., and Senoo, K. (2009). Temporal shifts in diversity and quantity of *nirS* and *nirK* in a rice paddy field soil. Soil Biol. Biochem. *41*, 2044–2051.

Yoshinari, T., and Knowles, R. (1976). Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. *69*, 705–710. http://dx.doi.org/10.1016/0006-291X(76)90932-3

Zheng, Y., Hou, L., Liu, M., Gao, J., Yin, G., Li, X., Deng, F., Lin, X., Jiang, X., Chen, F., *et al.* (2015). Diversity, Abundance, and Distribution of *nirS*-Harboring Denitrifiers in Intertidal Sediments of the Yangtze Estuary. Microb. Ecol. *70*, 30–40. http://dx.doi.org/10.1007/s00248-015-0567-x

Zhou, Z.F., Zheng, Y.M., Shen, J.P., Zhang, L.M., and He, J.Z. (2011). Response of denitrification genes *nirS*, *nirK*, and *nosZ* to irrigation water quality in a Chinese agricultural soil. Environ. Sci. Pollut. Res. Int. *18*, 1644–1652. http://dx.doi.org/10.1007/s11356-011-0482-8

Zumft, W.G. (1997). Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. *61*, 533–616.