Environmental Pollution xxx (2010) 1–7

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Identification and quantification of a novel nitrate-reducing community in sediments of Suquía River basin along a nitrate gradient

Luciana Reyna, Daniel Alberto Wunderlin, Susana Genti-Raimondi*

Universidad Nacional de Córdoba-CONICET, Facultad de Ciencias Químicas, Departamento de Bioquímica Clínica-CIBICI, Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina

A novel narG community present in Suquía River sediments was quantified; values were in line with the water quality index.

ARTICLE INFO

Article history:
Received 17 August 2009
Received in revised form
1 December 2009
Accepted 6 December 2009

Keywords:
narG gene
Pollution
Nitrate gradient

ABSTRACT

We evaluated the molecular diversity of *narG* gene from Suquía River sediments to assess the impact of the nitrate concentration and water quality on the composition and structure of the nitrate-reducing bacterial community. To this aim, a library of one of the six monitoring stations corresponding to the highest nitrate concentration was constructed and 118 *narG* clones were screened. Nucleotide sequences were associated to *narG* gene from alpha-, beta-, delta-, gammaproteobacteria and *Thermus thermophilus*. Remarkably, 18% of clones contained *narG* genes with less than 69% similarity to *narG* sequences available in databases. Thus, indicating the presence of nitrate-reducing bacteria with novel *narG* genes, which were quantified by real-time PCR. Results show a variable number of *narG* copies, ranging from less than 1.0×10^2 to 5.0×10^4 copies per ng of DNA, which were associated with a decreased water quality index monitored along the basin at different times.

1. Introduction

Nitrate reduction sustained by bacteria, fungus and archaea constitutes one of the main branches of the global nitrogen cycle, influencing soil and water conservation, atmospheric chemistry as well as wastewater treatment (Zumft, 1997; Takaya, 2009; Cabello et al., 2004). Nitrate pollution of surface water is of high concern as it may have negative impacts on water supply and on freshwater and coastal ecosystems. Bacteria having nitrate-reducing capacity are widely distributed in diverse polyphyletic groups in nature (Zumft, 1997). Nitrate is reduced to nitrite by three different bacterial nitrate-reducing enzymes: cytoplasmic assimilatory nitrate reductases (Nas), membrane-bound respiratory nitrate reductases (Nar), and periplasmic dissimilatory nitrate reductases (Nap). Membrane-bound nitrate reductase is associated with denitrification and anaerobic nitrate respiration (Zumft, 1997; Moreno-Vivian et al., 1999; González et al., 2006; Morozkina and Zvyagilskaya, 2007). The Nar reductase is important since the produced nitrite is then reduced to N_2O or N_2 gases, which can lead to considerable nitrogen losses in agriculture and emissions of greenhouse gases (Conrad, 1996). The Nar enzyme consists of three subunits, the two cytoplasmic NarGH and the membrane-bound NarI subunits. Subunit NarG coded by *narG* gene contains the catalytic site on which the nitrate reduction takes place (Zumft, 1997).

Several authors have used molecular biology techniques targeting the *narG* gene to study the genetic diversity of nitrate-reducing communities in a variety of natural habitats as well as in laboratory settings, including glacier forelands (Deiglmayr et al., 2006; Kandeler et al., 2006), arable soils (Chèneby et al., 2000; Enwall et al., 2005), freshwater lake sediments (Gregory et al., 2003), rhizosphere sediments (Delorme et al., 2003; Philippot et al., 2002), estuarine sediments (Nogales et al., 2002; Smith et al., 2007) and extreme saline alkaline soils (Alcántara-Hernández et al., 2009).

The aim of the present work was to analyze the *narG* molecular diversity of the nitrate-reducing community from sediments of Suquía River basin along a nitrate gradient and a decreased water quality index, by direct PCR amplification, cloning, and restriction fragment length polymorphism and sequence analysis.

2. Material and methods

2.1. Environmental samples

The Suquía River basin is located in a semi-arid region of Córdoba province (Argentina). It begins at the San Roque dam and flows for about 28 km across Córdoba city; downstream, near the eastern edge of the city, the river receives the city sewage discharge and then continues up to Mar Chiquita Lake crossing an area with intensive agriculture practice (Fig. 1). This basin presents a marked pollution

* Corresponding author. Tel.: +54 351 4344973; fax: +54 351 4333048.
E-mail address: sgenti@fcq.unc.edu.ar (S. Genti-Raimondi).

0269-7491/$ – see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.envpol.2009.12.014

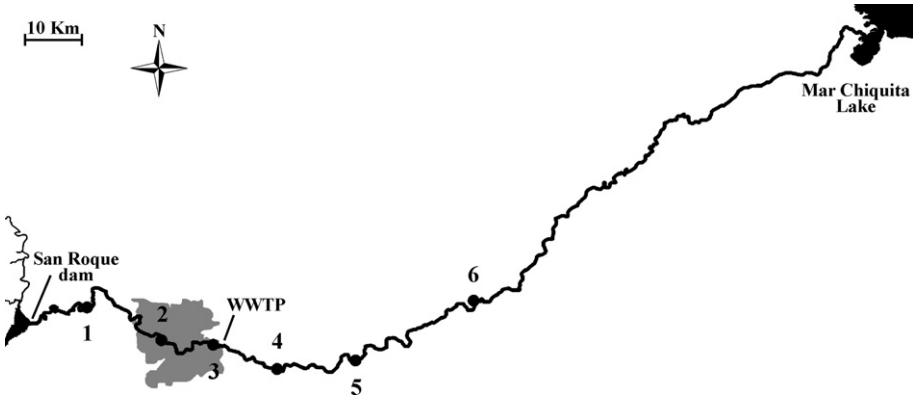

Fig. 1. Map of the Suquía River basin (Province of Córdoba, Argentina), showing the sampling sites (station 1: La Calera, station 2: Isla de los Patos, station 3: Bajo Grande, station 4: Corazón de María, station 5: Capilla de los Remedios, station 6: Río Primero). WWTP indicates where the wastewater treatment plant is located.

gradient, determined by the quality water index, characterized by areas with low pollution upstream from Córdoba City and heavy pollution downstream from this city (Pesce and Wunderlin, 2000; Wunderlin et al., 2001). This pollution gradient causes changes in fish biodiversity (Hued and Bistoni, 2005) as well as changes in physiological and morphological aspects of fish (Cazenave et al., 2005; Hued et al., 2006). Also aquatic plants are affected by exposure to polluted sediments within the Suquía basin (Nimptsch et al., 2005). Table 1 shows the nitrate, nitrite and ammonium concentrations and also the water quality index determined in the Suquía River water at each monitoring station. Water samples were taken 20 cm below the water surface in clean glass bottles. Sediment was obtained from the first 10 cm of the top layer using a handle dredge.

2.2. Extraction of DNA

Total DNA from the sediment samples was extracted from 250 mg aliquots of wet sediments using UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) as specified by the manufacturer. Sample DNA concentrations were determined using the Quant-iT™ Broad-Range DNA Assay Kit (Invitrogen) according to manufacturer's protocol, and sample aliquots were standardized to contain 5–10 ng/μL in Tris–EDTA buffer.

2.3. PCR amplification of narG fragments, cloning and sequencing

Amplification of *narG* gene fragments (ca. 650 pb) from 10 ng of DNA from sediment samples were amplified using *narG*1960 F (5′-TAYGTSGGSCARGARAA-3′) and *narG*2650R (5′-TTYTCRTACCABGTBGC-3′) primers as described by Philippot et al. (2002). The PCR amplification was done in a total volume of 50 μL with 5 μL 10 × PCR buffer (500 mM KCl, 20 mM $MgCl_2$, 200 mM Tris–HCl [pH 8.4]), 200 μM concentration of each deoxyribonucleoside triphosphate, 0.5 U of *Taq* polymerase (Invitrogen), and 25 pmol of each primer. The following PCR conditions were used for amplification. A denaturation step of 5 min at 95 °C was followed by a touchdown PCR of 8 cycles with denaturation at 94 °C for 30 s, primer annealing at 59 °C–55 °C for 30 s (decreased by 0.5 °C each cycle) and extension at 72 °C for 45 s, and then by 30 cycles with annealing at 55 °C. The amplification ended with 10 min at 72 °C (Enwall et al., 2005). The PCR products were analyzed on 1.5% agarose gels. To avoid potential sample biases and to obtain enough PCR products for cloning, three replicate amplifications from station 6 DNA were carried out. Aliquots of pooled and purified PCR products were cloned by using the pGEM-T Easy cloning kit (Promega) according to the manufacturer's recommendations. Transformants were selected on Luria agar plates containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White colonies were screened by PCR using vector primers. Clones with correct insert sizes (approximately 650 bp) were amplified and the PCR products were digested with *Alu*I 2 h at 37 °C and separated by gel electrophoresis on 7% acrylamide gels. In the case of those clones that could not to be cut with *Alu*I restriction enzyme, they were digested with *Msp*I restriction enzyme. The clones were grouped into RFLP pattern types as determined by similarity in RFLP patterns. The inserts were sequenced on both strands by Macrogen Inc. (Korea).

2.4. Phylogenetic analysis

narG nucleotide sequences were translated into amino acid sequences by using the National Center for Biotechnology Information tBLASTN program. DNA and protein sequences were compared with sequences in the GenBank database. Nucleotide sequences were aligned by using ClustalX (Thompson et al., 1997). To construct the phylogenic tree based on nucleotide alignments, gene sequence distances were inferred by the neighbor-joining method (Saitou and Nei, 1987). For each calculation, 1000 bootstrap resamplings were performed. Phylogenetic trees were drawn using NJPlot 2.1 (Perriere and Gouy, 1996).

2.5. Primer design for real-time PCR assay

Sequences obtained from the 21 *narG* clones isolated from the library constructed from the station 6 and grouped in the cluster III (Fig. 2) were aligned using ClustalX (Thompson et al., 1997) and scanned for conserved regions that could provide suitable primer target sites. Two primers, *nar*G328f (5′-GAC AAA CTT CGC AGC GG-3′) and *nar*G497r (5′-TCA CCC AGG ACG CTG TTC-3′), were designed to amplify a 170 bp *narG* fragment from the targeted cluster using the Primer-Blast program providing by National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast). This program did not show any putative sequences deposited in the GenBank that could be amplified with the selected primers. The *narG* clone sequences grouped in the cluster III did not exhibit any mismatches with the above primer sequences. The primers were tested by real-time

Table 1
Locations and chemical properties of the river water at the sampling sites of Suquía River in the Province of Córdoba, Argentina.

Station	Description	Location by GPS	Season[a]	NO_3^- (mg/L)	NO_2^- (mg/L)	NH_4^+ (mg/L)	Water quality index[c]
1	La Calera	31°21′4.39″S	wet	2.2 ± 1.8	0.04 ± 0.01	<0.06	75
		64°20′59.38″O	dry	2.0 ± 0.02	0.11 ± 0.01	0.31 ± 0.01	74
2	Isla de los Patos	31°23′59.25″S	wet	4.09 ± 0.15	0.11 ± 0.01	0.85 ± 0.11	71
		64°12′14.96″O	dry	7.41 ± 5.63	0.12 ± 0.08	0.41 ± 0.38	62
3	Bajo Grande	31°25′46.15″S	wet	12.40 ± 0.41	1.75 ± 0.15	3.40 ± 0.59	53
		64° 2′39.82″O	dry	NS[b]	NS	NS	49
4	Corazón de María	31°26′49.21″S	wet	12.9 ± 0.41	0.50 ± 0.18	10.0 ± 1.88	48
		63°59′31.15″O	dry	16.3 ± 1.26	1.75 ± 0.21	13.5 ± 2.23	41
5	Capilla de los Remedios	31°26′5.26″S	wet	10.9 ± 1.29	0.32 ± 0.04	7.0 ± 0.33	50
		63°49′54.72″O	dry	27.8 ± 1.13	0.77 ± 0.02	16.5 ± 1.72	48
6	Río Primero	31°20′18.38″S	wet	17.6 ± 1.18	0.19 ± 0.03	0.36 ± 0.03	52
		63°36′34.32″O	dry	34.6 ± 1.58	0.72 ± 0.14	0.73 ± 0.27	51

[a] Wet season: October–March; dry season: April–September.
[b] NS: not sampled.
[c] Calculated as described in Pesce and Wunderlin (2000) for WQI_{obj}.

Please cite this article in press as: Reyna, L., et al., Identification and quantification of a novel nitrate-reducing community in sediments of..., Environ. Pollut. (2010), doi:10.1016/j.envpol.2009.12.014

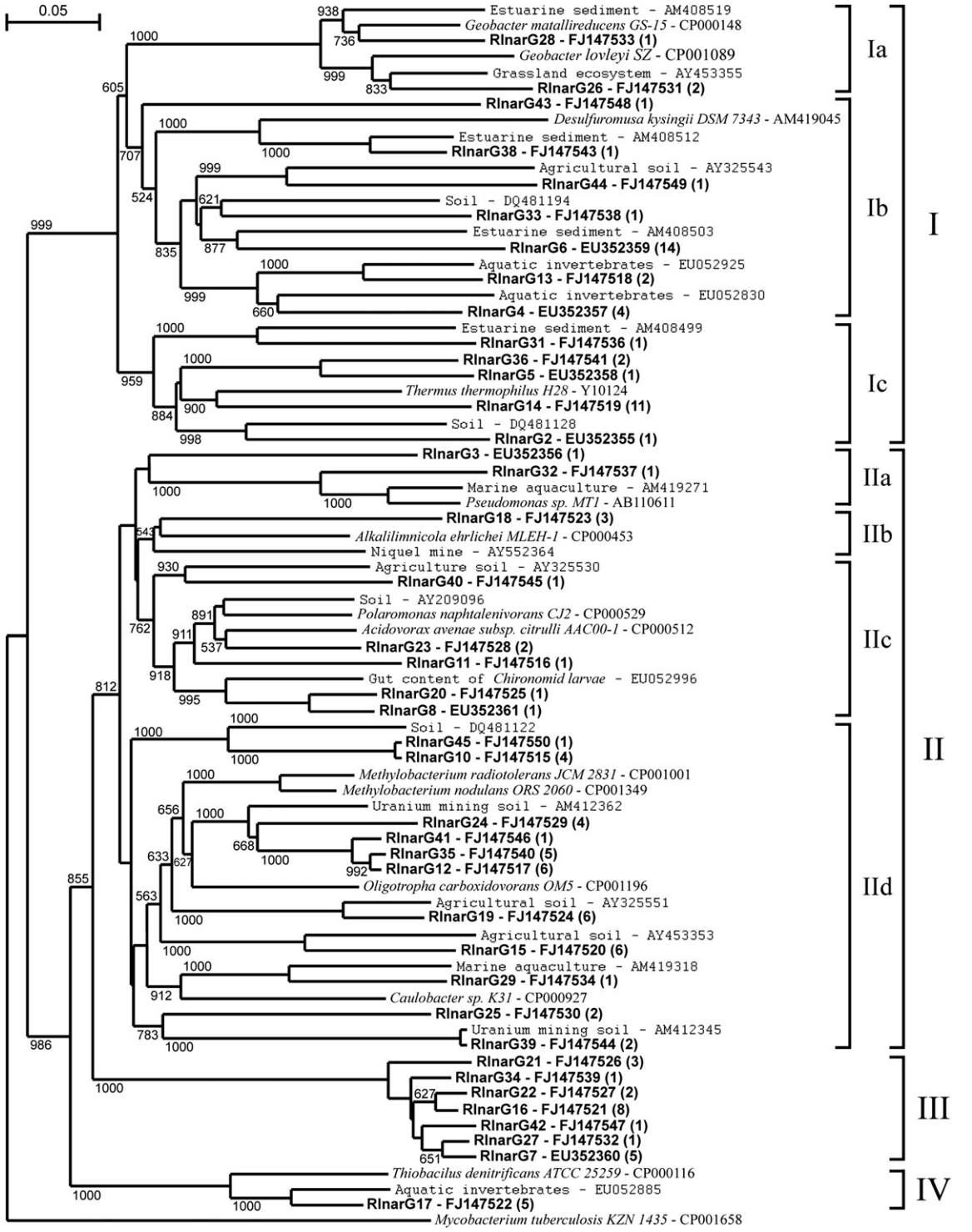

Fig. 2. Phylogenetic tree based on *narG* sequences cloned from Suquía River sediments of station 6 DNA (in boldface) and from cultured bacteria and other environmental clones. The phylogenetic tree was constructed using neighbor-joining analysis with 1000 bootstrap replicates (Saitou and Nei, 1987). The *narG* sequence from *Mycobacterium tuberculosis KZN 1435* (Accession number CP001658) was used as out-group. The percentage support values above 50% and the scale bar representing 5% sequence divergence are indicated. The accession numbers of the *narG* genes are as follows: *Acidovorax avenae subsp. citrulli AAC00-1*, CP000512; *Alkalilimnicola ehrlichei MLHE-1*, CP000453; *Caulobacter sp. K31*, CP000927; *Desulfuromusa kysingii DSM 7343*, AM419045; *Geobacter lovleyi SZ*, CP001089; *Geobacter metallireducens GS-15*, CP000148; *Methylobacterium nodulans ORS 2060*, CP0001349; *Methylobacterium radiotolerans JCM 2831*, CP001001; *Oligotropha carboxidovorans OM5*, CP0001196; *Polaromonas napthalenivorans CJ2*, CP000529; *Pseudomonas* sp. *MT-1*, AB110611; *Thermus thermophilus H28*, Y10124; *Thiobacillus denitrificans ATCC 25259*, CP000116. The numbers in parentheses indicate the quantity of clones related to the same sequence according to RFLP patterns.

PCR amplification of clones from the previously isolated collection belonging to the same and other *narG* cluster groups. DNA concentrations were those corresponding to *narG* copy numbers two logs above the real-time PCR detection limit. Primers were then used for real-time PCR amplification in the environmental samples.

2.6. Real-time PCR assay

Real-time PCRs were carried out in a ABI 7500 with Sequence Detection Software v1.4 (Applied Biosystems) in a reaction mixture containing 0.25 μM of each

primer for *narG* amplification, 12.5 μL of SYBR® Green PCR Master Mix, including AmpliTaq Gold® DNA Polymerase, optimized buffer components, dNTPs with dUTP, SYBR Green I dye, and Pasive Reference 1 (ROX dye) (Applied Biosystems), 5 μL of DNA diluted template corresponding to 0.4–7 ng of total DNA, and UltraPURE™ DNase/RNase-free Distilled Water (Gibco) to complete the 25 μL volume. The conditions for *narG* real-time PCR were: an initial cycle at 95 °C for 600 s (enzyme activation); 40 cycles of 95 °C for 15 s, 61 °C for 60 s, (extension and data acquisition step). An additional step from 60 °C to 95 °C (0.2 °C s^{-1}) was added to obtain a denaturation curve specific for each amplified sequence. The 16S rRNA fragment was amplified according to López-Gutierrez et al. (2004). Specificity was verified by melting-curve analysis and the presence of a band of the correct size in a 2% (wt/v) agarose gel stained with ethidium bromide.

2.7. Quantification of narG from sediment samples

narG copy numbers were quantified via comparison to standard curves. Two independent quantitative triplicates PCR assays were performed for each sediment sample. Standard curve was obtained by plotting threshold cycle (Ct) as a function of log of the target DNA copy number. For this purpose recombinant pGEM-T Easy Vector (Promega) plasmid containing RInarG21 sequence was linearized using 5 U of *Pst*I and incubating at 37 °C for 2 h (Promega). Tenfold serial dilutions of the linearized plasmid ranging from 10^2 to 10^7 *narG* copies were used as template, by triplicate, to determine the calibration curve. Three points of the standards were used as positive controls in each reaction. Automatic analysis settings were selected to determine Ct values and baseline settings. Standard curve slope, *y* intercept, and coefficient of determination (r^2) were calculated.

The detection limit of our assay was determined as the copy number of the less concentrated standard which was quantified with an SD of the Ct smaller than 0.2.

The presence of PCR inhibitors co-extracted with DNA was tested by doing serial dilutions of the DNA extracted from sediment samples which were quantified and compared. The absence of inhibitory substance in the presented data was also confirmed by the similar amplification efficiencies obtained with tenfold dilution of sediment DNA extracts (data not shown).

2.8. Calculation and statistical analysis

A one-way analysis of variance followed by Fisher's test (Infostat Software, http://www.infostat.com.ar) was performed to compare the *narG* abundance between the different sediment samples. A value of $p < 0.05$ was considered significant.

2.9. Nucleotide sequence accession numbers

Gene sequences from environmental samples have been deposited in the GenBank nucleotide sequence database under the following accession numbers: EU352354-EU352362 and FJ147515-FJ147550.

3. Results

3.1. Amplification of narG gene and RFLP analysis of clones

narG gene fragments (ca. 650 bp) were successfully amplified and PCR products of the predicted size were recovered from each sample. Initially, to study the *narG*-community composition in sediments of Suquía River a library corresponding to the highest freshwater nitrate levels (station 6, Table 1) was constructed. A total of 118 *narG* clones were randomly selected for RFLP analyses, resulting in the identification of 36 *Alu*I restriction fragment banding patterns and 8 clones without *Alu*I restriction enzyme sites. These last clones were analyzed with the *Msp*I restriction enzyme, resulting in 5 different *Msp*I restriction fragment banding patterns. The RFLP analysis showed two dominant *narG* groups comprised 21% of all *narG* clones (RInarG6 and RInarG14).

3.2. Sequence and phylogenetic analysis of narG fragments

narG gene fragments from 51 of the 118 screened clones were sequenced. The sequenced fragments represented all distinct *Alu*I RFLP patterns and also included clones with similar *Alu*I RFLP pattern. In addition, all the clones that had different *Msp*I RFLP patterns were sequenced. Comparison with the GenBank database by using BLASTN search revealed that all sequences showed homology to *narG* sequences from uncultured environmental clones and/or cultivated bacteria. Additionally, the deduced amino acid sequence within the amplified region was conserved in all sequenced *narG* clones. Multiple sequence alignment comparisons indicated ranges of 44.1–99.8% and 35.3–99.5% for nucleotide and amino acids identity levels, respectively.

To evaluate the phylogenetic diversity represented by the 41 RFLP patterns identified in the *narG* gene clone library, a phylogram was constructed from nucleotide sequences of *narG*, by a maximum likelihood method considering approximate lengths of 650 nucleotides. The phylogenetic analysis was done with the 41 different sequences from the library representing the 41 RFLP patterns and related ones obtained from the GenBank database (Fig. 2). The tree obtained can be divided into four major clusters based on the location of *narG* from known microorganisms (Fig. 2). The first cluster was divided in three sub-clusters; the subcluster Ia includes 3 clones encompassing *narG* sequences affiliated to *narG* from *Geobacter lovleyi SZ* (84% identity), *Geobacter metallirreducens GS-15* (87% identity) and *narG* sequences present in two uncultured environmental clones isolated from grassland ecosystem and estuarine lakes (Deiglmayr et al., 2004; Smith et al., 2007). The subcluster Ib comprises 24 clones encompassing *narG* sequences affiliated to *narG* from *Desulfuromusa kysingii DSM 7343* (75% identity) and *narG* sequences identified in uncultured bacteria present in estuarine sediments (73–91% identity) (Smith et al., 2007) and agricultural soils (76% identity) (Mounier et al., 2004). The subcluster Ic contains 16 clones encompassing *narG* sequences related to *narG* from the hyperthermophilic bacterium *Thermus thermophilus H28* (68–75% identity) and several *narG* sequences previously described in environmental uncultured bacteria (71–77% identity) (Smith et al., 2007). Cluster II was divided in four sub-clusters, being the RInarG32 sequence (1 clone) included in the subcluster IIa related to *narG* from *Pseudomonas* sp. *MT-1* and an environmental clone isolated from marine aquaculture biofilter (82% identity), while the subcluster IIb (3 clones) comprises *narG* sequence affiliated to the gammaproteobacterium *Akalilimnicola ehrlichei MLHE-1* (74% identity). The subcluster IIc contains 6 clones comprising *narG* sequences related to the corresponding ones from *Acidovorax avenae* subsp. *citrulli AACOO-1* (76–85% identity), from *Polaromonas naphtalenivorans CJ2* (79–80% identity), and other *narG* sequences from uncultured bacteria isolated from agricultural soil and gut content of *chironomid larvae* (Mounier et al., 2004; Chèneby et al., 2000; Stief et al., 2009). The subcluster IId comprises 38 clones with *narG* sequences associated to *narG* from the following alphaproteobacteria: *Methylobacterium radiotolerans JCM 2831* (76–82% identity), *Methylobacterium nodulans ORS 2060* (76–82% identity), *Oligotropha carboxidovorans OMS* (81% identity); *Caulobacter* sp. *K31* (75% identity) and several *narG* sequences identified in environmental uncultured bacteria (Deiglmayr et al., 2004; Héry et al., 2005; Mounier et al., 2004; Henry et al., 2008). Cluster III encompasses 21 clones including *narG* sequences non-related to *narG* from known microorganisms. This cluster comprises clones containing *narG* fragments with less than 69% similarity to *narG* sequences available in sequence databases, thus suggesting that they are indigenous of the Suquía River representing novel sequences of nitrate-reducing bacteria. Finally, cluster IV includes 5 clones that encode *narG* sequences that are related to *narG* from *Thiobacillus denitrificans ATCC 25259* (85% identity) and *narG* sequences described in environmental uncultured bacteria isolated in estuarine sediments (90% identity).

3.3. Quantification of narG gene in environmental samples

Since 18% of the all screened clones contain *narG* sequences with less than 69% similarity to *narG* sequences from both cultivated and

Please cite this article in press as: Reyna, L., et al., Identification and quantification of a novel nitrate-reducing community in sediments of..., Environ. Pollut. (2010), doi:10.1016/j.envpol.2009.12.014

environmental uncultured bacteria (cluster III), suggesting that they are indigenous of the Suquía River, we decided to evaluate by real-time PCR the presence of this novel group along the Suquía River basin and at different sampled times. For this purpose we designed a set of primers aligning the clone nucleotide sequences included in cluster III. The specificity of the *narG* primer set was verified experimentally by using phylogenetically diverse nitrate-reducing bacteria clones. Amplification resulted in the expected 170-bp fragment for all DNA plasmids corresponding to cluster III clones. In contrast, no products were amplified from clones grouped in the other clusters. Melting-curve analysis with target DNA from strains included in the cluster III or from environmental samples shows one distinctive Tm (Fig. 3A). Moreover, agarose gel electrophoresis showed the presence of only one band of the expected size (data not shown).

A plasmid containing RInarG21 sequence from cluster III were used to draw a standard curve relating Ct to the added mass of linearized plasmid DNA and the number of gene copies. Linear response was observed for 6 orders of magnitude, ranging from 10^2 to 10^7 *narG* gene copies ($r^2 = 0.995$) (Fig. 3B).

A detection limit was determined using a dilution series of RInarG21 plasmid and approximately 10–100 copies per assay were achieved.

To quantify the presence of this novel nitrate-reducing bacterial community in environmental samples, the *narG* and 16S rRNA gene fragments were evaluated in sediments of six monitoring sites along nitrate gradient and at different times. The *narG* copies revealed high variations between environments, with most numbers ranging from 2.82×10^2 to 5.0×10^4 copies per ng of DNA (Fig. 4). The higher copy numbers for *narG* gene were observed in the river sediment samples of station 3 and 4 corresponding to nitrate levels of 12.4 and 12.9 mg/L in the wet season, respectively, and 16.3 mg/L in the dry season for the station 4 (Table 1). Remarkably, these stations encompass the less water quality index values (Pesce and Wunderlin, 2000; Wunderlin et al., 2001 and Table 1). In contrast, in the less polluted area (station 1 and 2) corresponding the higher water quality index values and the lower nitrate concentrations (2.2 and 2.0 mg/L for station 1 and 4.09 and 7.41 mg/L for station 2 in the wet and dry seasons, respectively) the *narG* copy number of this novel nitrate-reducing bacteria group was lower than 1.0×10^2 copies per ng of DNA. In addition, significant changes in the community density were detected for the station 4, 5, and 6 at different times. Even though no straight correlation was established between these values and the nitrate concentration, the data could be directly associated with the river water quality index (Table 1). The copy number of 16S rRNA ranged from 2.07×10^5 to 5.39×10^6 copies per ng of DNA (Fig. 4). Analysis of the percentages of the specific *narG* fragments in proportion to 16S rRNA showed variable amounts, between 0.02% and 3.2%.

4. Discussion

In this study, the composition of nitrate-reducing bacterial communities was analyzed in sediment samples from the Suquía River basin (Córdoba, Argentina). This basin is characterized by a pollution gradient from its origin, through the city of Córdoba until the beginning of the low basin section, downstream from city sewage discharge pointed out by the objective Water Quality Index (Pesce and Wunderlin, 2000; Wunderlin et al., 2001). This study gives for the first time a unique insight into the structure of the nitrate-reducing community in the Suquía River basin by use of a molecular approach.

The *narG* nucleotide sequences identified showed similarity with those cultivated and environmental uncultured bacteria isolated from a variety of natural habitats and laboratory settings ranging from 67% to 85% and 68–91%, respectively (Chèneby et al., 2000; Dambreville et al., 2006; Mounier et al., 2004; Philippot et al., 2002; Smith et al., 2007; Héry et al., 2005; Stief et al., 2009). The use of *narG* is of special interest, because the phylogeny of the *narG* functional gene has been proposed to reflect the 16S rRNA phylogeny of the organisms from which the gene sequences were retrieved (Philippot et al., 2002) providing valuable information on the phylogenetic affiliation of the organisms in the environment. At this regard, a recent report have demonstrated that ninety percent of cultured bacterial nitrate reducers with a 16S rRNA gene similarity of ≥97% had a *narG* or *nosZ* similarity of ≥67% or ≥80%, respectively, suggesting that these percentages could be used as threshold similarity values (Palmer et al., 2009). Even though multiple *narG* copies are not always closely related, they established that the same genus generally form coherent clusters in phylogenetic trees suggesting that different environmental sequence clusters could provide evidence for new-genus level diversity. Furthermore, they concluded that recent horizontal gene transfer did not appear to have occurred for *narG* or *nosZ*.

However the primers used to construct the *narG* library were those designed to amplify the *narG* gene from *Proteobacteria*, gram-positive *Bacteria,* and *Archaea* (Philippot et al., 2002), the sequence analysis of the isolated *narG* clones showed that the most of the *narG* genes at the sampled site were represented by *Proteobacteria*, being alphaproteobacteria (33%), betaproteobacteria (9%),

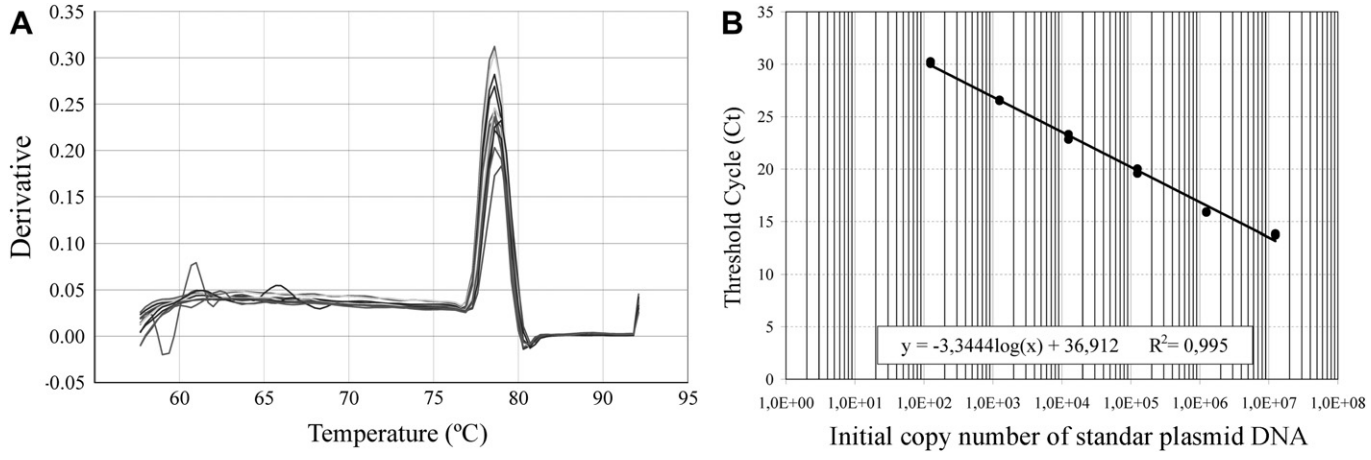

Fig. 3. A) Melting-curve profiles for the amplicons of RInarG21 plasmid dilutions obtained by narG primer set. B) Calibration curve plotting log starting narG copy numbers versus threshold cycle.

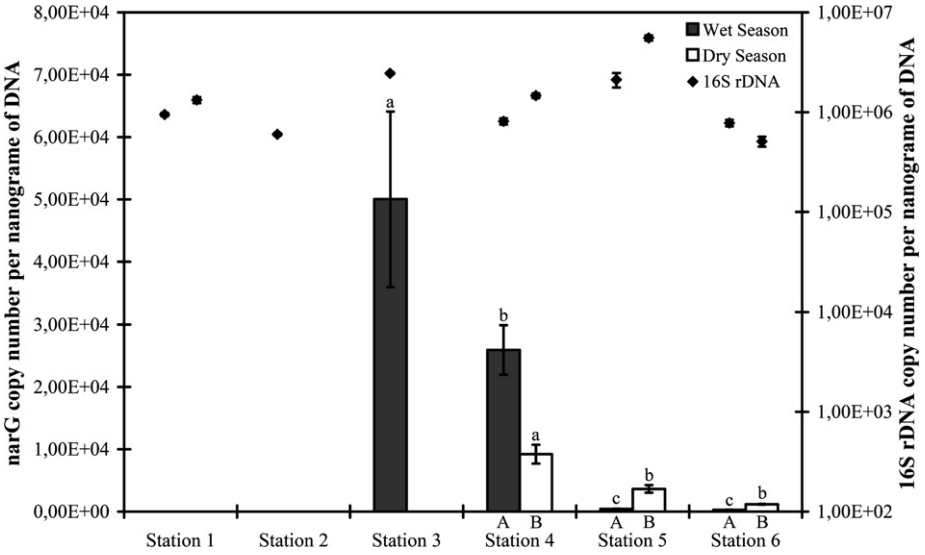

Fig. 4. Changes over time and at different spatial locations of *narG* copy numbers per ng of DNA along Suquía River basin. The *narG* copy number in the stations 1 and 2 were lower than 1.0×10^2 copies per ng of DNA. The copy number of 16S rRNA quantified in each monitoring stations was represented. Bars indicate mean ± SEM of two independent qPCR experiments performed in triplicates. Significantly different values ($P < 0.05$) between different sampled times in the same station are marked by capital letters (A and B) under the columns, and between different stations in the same sampled time are marked by lowercase letters (a to c) over the columns.

deltaproteobacteria (24%), gammaproteobacteria (3%), and *T. thermophilus H28* (13%). Regarding of *narG* fragment sequences affiliated to *narG* gene from *T. thermophilus H28* which is able to respire nitrate, due to the presence of a genetic element that can be transferred to aerobic strains by horizontal transfer (Ramírez-Arcos et al., 1998), and considering the relative low identity (68–75%) of these sequences, it is possible hypothesize that these clones were retrieved from unknown nitrate-reducing bacteria. Surprisingly, 18% of clones contain *narG* fragment genes with less than 69% similarity to *narG* sequences available in databases. Quantifications of these novel nitrate-reducing bacteria revealed high variations between the river sediment samples changing over spatial location and temporal variation ranging from less than 1.0×10^2 to 5.0×10^4 copies per ng DNA, which could be due to the selection of nitrate reductases in some habitats. Similar ranges were found when the *narG* primers used were designed to amplify a specific new group of nitrate reducers (López-Gutierrez et al., 2004; Bru et al., 2007). At this regard, Nijburg et al. (1997) analyzing the composition of the nitrate-reducing community in a culture-based study, suggested that a specific nitrate-reducing community is present in each environment. In addition, it was observed that the diversity of nitrate reducers and ammonia oxidizers bacterial communities were dependent on both management and plant species (Enwall et al., 2005; Patra et al., 2006; Rousel-Delif et al., 2005). On the other hand, a strong impact on the activity of the nitrate reducers and denitrifying communities and minor changes on their diversities were observed with mucilage or artificial root exudates addition (Henry et al., 2008; Mounier et al., 2004).

In summary, the data revealed an increase in the copy numbers per ng of DNA of these novel nitrate-reducing bacteria in correspondence with the lower river water quality index whereas the correlation with nitrate concentration was less evident. To evaluate the abundance of these nitrate-reducing bacteria relative to total bacteria, the percentages of these specific *narG* fragments in proportion to 16S rRNA were calculated. Analysis of these percentages in relation to 16S rRNA showed variable amounts, between 0.02% and 3.2%. These values are in agreement with those reported showing that the densities of denitrifier bacteria were 2 orders of magnitude lower than those of the total bacteria (López-Gutierrez et al., 2004; Henry et al., 2008; Bru et al., 2007).

In brief this study demonstrated the ability to quantify temporal and spatial variation in the abundance of this nitrate-reducing community in the Suquía River sediments. The competition with other nitrate-reducing communities may explain the increase in abundance of this *narG* community among several possibilities. This information could be extremely important both for revealing general mechanisms of adaptation to stress and for applied investigations regarding to the role of nitrate reducers in nitrogen turnover in nature. Currently, we are performing studies in order to determine whether different parameters such as the nitrate concentration, or the oxygen partial pressure, or carbon substrate, or a combination of them are important for the composition of this nitrate-reducing community.

5. Conclusions

In conclusion, our results indicate that the nitrate-reducing communities present in sediments of Suquía River are phylogenetically diverse. The presence of nitrate-reducing bacteria with novel *narG* sequences related to cluster III bacteria was quantified by qPCR. Results show a variable number of *narG* copies, ranging from less than 1.0×10^2 to 5.0×10^4 copies per ng DNA, in correspondence with the lower water quality index monitored along the basin at different times. Moreover, no straight correlation was established with the nitrate gradient. Thus, the nitrate-reducing bacteria biodiversity associated to cluster III is affected by the water quality index produced by anthropogenic activities. Further comprehensive study with regard to different physical and/or chemical parameters will help to find out potential impacts on this nitrate-reducing community.

Acknowledgments

S.G.-R. and D.A.W. are Career Investigators of CONICET. L.R. thanks FONCYT and CONICET for its fellowships. This work was granted by the Consejo Nacional de Investigaciones Científicas y Tecnológicas de Argentina (CONICET, PIP 6497), the Agencia Nacional de Promoción Ciencia y Técnica (ANPCYT, PICTR 327), and the Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba (SECyT-UNC).

Please cite this article in press as: Reyna, L., et al., Identification and quantification of a novel nitrate-reducing community in sediments of..., Environ. Pollut. (2010), doi:10.1016/j.envpol.2009.12.014

References

Alcántara-Hernández, R.J., Valenzuela-Encinas, C., Marsch, R., Dendooven, L., 2009. Respiratory and dissimilatory nitrate-reducing communities from an extreme saline alkaline soil of the former lake Texcoco (Mexico). Extremophiles 13, 169–178.

Bru, D., Sarr, A., Philippot, L., 2007. Relative abundances of proteobacterial membrane-bound and periplasmic nitrate reductases in selected environments. Appl. Environ. Microbiol. 73, 5971–5974.

Cabello, P., Roldán, M.D., Moreno-Vivián, C., 2004. Nitrate reduction and the nitrogen cycle in archaea. Microbiology 150, 3527–3546.

Cazenave, J., Wunderlin, D.A., Hued, A.C., Bistoni, M.A., 2005. Haematological characterization of a neotropical fish, *Corydoras paleatus* (Pisces, Callichthyidae), captured from pristine and polluted water. Hydrobiologia 537, 25–33.

Chèneby, D., Philippot, L., Hartmann, A., Hènault, C.F., Germon, J.C., 2000. 16S rRNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. FEMS Microbiol. Ecol. 34, 121–128.

Conrad, R., 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). Microbiol. Rev. 60, 609–640.

Dambreville, C., Hallet, S., Nguyen, C., Morvan, T., Germon, J.C., Philippot, L., 2006. Structure and activity of the denitrifying community in a maize-cropped field fertilized with composted pig manure or ammonium nitrate. FEMS Microbiol. Ecol. 56, 119–131.

Deiglmayr, K., Philippot, L., Hartwig, U.A., Kandeler, E., 2004. Structure and activity of the nitrate-reducing community in the rhizosphere of Lolium perenne and Trifolium repens under long-term elevated atmospheric pCO. FEMS Microbiol. Ecol. 49, 445–454.

Deiglmayr, K., Philippot, L., Tscherko, D., Kandeler, E., 2006. Microbial succession of nitrate-reducing bacteria in the rhizosphere of *Poa alpina* across a glacier foreland in the Central Alps. Environ. Microbiol. 8, 1600–1612.

Delorme, S., Philippot, L., Edel-Hermann, V., Deulvot, C., Mougel, C., Lemanceau, P., 2003. Comparative genetic diversity of the *narG, nosZ*, and 16S rRNA genes in fluorescent pseudomonads. Appl. Environ. Microbiol. 69, 1004–1012.

Enwall, K., Philippot, L., Hallin, S., 2005. Activity and composition of the bacterial community respond differently to long-term fertilization. Appl. Environ. Microbiol. 71, 8335–8343.

González, P.J., Correia, C., Moura, I., Brondino, C.D., Moura, J.J.G., 2006. Bacterial nitrate reductases: molecular and biological aspects of nitrate reduction. J. Inorg. Biochem. 100, 1015–1023.

Gregory, L.G., Bond, P.L., Richardson, D.J., Spiro, S., 2003. Characterization of a nitrate-respiring bacterial community using the nitrate reductase gene (*narG*) as a functional marker. Microbiology 149, 229–237.

Henry, S., Texier, S., Hallet, S., Bru, D., Dambreville, C., Chèneby, D., Bizouard, F., Germon, J.C., Philippot, L., 2008. Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: insight into the role of root exudates. Environ. Microbiol. 11, 3082–3092.

Héry, M., Philippot, L., Mériaux, E., Poly, F., Le Roux, X., Navarro, E., 2005. Nickel mine spoils revegetation attempts: effect of pioneer plants on two functional bacterial communities involved in the N-cycle. Environ. Microbiol. 4, 486–498.

Hued, A.C., Bistoni, M.A., 2005. Development and validation of a biotic index for evaluation of environmental quality in the central region of Argentina. Hydrobiologia 543, 279–298.

Hued, A.C., Caruso, M.N., Wunderlin, D.A., Bistoni, M.A., 2006. Field and *in vitro* evaluation of ammonia toxicity on native fish species of the central region of Argentina. Bull. Environ. Contam. Toxicol. 76, 984–991.

Kandeler, E., Deiglmayr, K., Tscherko, D., Philippot, L., 2006. Quantification of functional genes *narG, nirK* and *nosZ* of denitrifying bacteria across a glacier foreland by real time PCR. Appl. Environ. Microbiol. 72, 5957–5962.

López-Gutierrez, J.C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J. Microbiol. Methods 57, 309–407.

Moreno-Vivian, C., Cabello, P., Martinez-Luque, M., Blasco, R., Castillo, F., 1999. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J. Bacteriol. 181, 6573–6584.

Morozkina, E.V., Zvyagilskaya, R.A., 2007. Nitrate reductases: structure, functions, and effect of stress factors. Biochemistry (Mosc) 72, 1151–1160.

Mounier, E., Hallet, S., Chèneby, D., Benizri, E., Gruet, Y., Nguyen, C., Piutti, S., Robin, C., Slezack-Deschaumes, S., Martin-Laurent, F., Germon, J.C., Philippot, L., 2004. Influence of maize mucilage on the diversity and activity of the denitrifying community. Environ. Microbiol. 6, 301–312.

Nijburg, J.W., Coolen, M., Gerards, S., Gunnewiek, P., Laanbroek, H.J., 1997. Effects of nitrate availability and the presence of glyceria maxima on the composition and activity of the dissimilatory nitrate-reducing bacterial community. Appl. Environ. Microbiol. 63, 931–937.

Nimptsch, J., Wunderlin, D.A., Dollan, A., Pflugmacher, S., 2005. Antioxidant and biotransformation enzymes in *Myriophyllum quitense* as biomarkers of heavy metal exposure and eutrophication in Suquía River basin (Córdoba, Argentina). Chemosphere 61, 147–157.

Nogales, B., Timmis, K.N., Nedwell, D.B., Osborn, A.M., 2002. Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. Appl. Environ. Microbiol. 68, 5017–5025.

Palmer, K., Drake, H.L., Horn, M.A., 2009. Genome-derived criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units of nitrate reducers. Appl. Environ. Microbio 75, 5170–5174.

Patra, A.K., Abbadie, L., Clays-Josserand, A., Degrange, V., Grayston, S.J., Guillaumaud, N., Loiseau, P., Louault, F., Mahmood, S., Nazaret, S., Philippot, L., Poly, F., Prosser, J.I., Le Roux, X., 2006. Effects of management regime and plant species on the enzyme activity and genetic structure of N-fixing, denitrifying and nitrifying bacterial communities in grassland soils. Environ. Microbiol. 8, 1005–1016.

Perriere, G., Gouy, M., 1996. WWW-Query: an on-line retrieval system for biological sequence banks. Biochimie 78, 364–369.

Pesce, S.F., Wunderlin, D.A., 2000. Use of water quality indices to verify the impact of Córdoba city (Argentina) on Suquía River. Water Res. 34, 2915–2926.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., Germon, J., 2002. Molecular analysis of the nitrate reducing community from unplanted and maize-planted soils. Appl. Environ. Microbiol. 68, 6121–6128.

Ramírez-Arcos, S., Fernández-Herrero, L.A., Berenguer, J., 1998. A thermophilic nitrate reductase is responsible for the strain specific anaerobic growth of Thermus thermophilus HB8. Biochim. Biophys. Acta 1396, 215–227.

Rousel-Delif, L., Tarnawski, S., Hamelin, J., Philippot, L., Aragno, M., Fromin, N., 2005. Frequency and diversity of nitrate reductase genes among nitrate-dissimilating Pseudomonas in the rhizosphere of perennial grasses grown in field conditions. Microb. Ecol. 49, 63–72.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Smith, C.J., Nedwell, D.B., Dong, L.F., 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.

Stief, P., Poulsen, M., Nielsen, L.P., Brix, H., Schramn, A., 2009. Nitrous oxide emission by aquatic macrofauna. Proc. Natl. Acad. Sci. U S A 106, 4296–42300.

Takaya, N., 2009. Response to hypoxia, reduction of electron acceptors, and subsequent survival by filamentous fungi. Biosci. Biotechnol. Biochem. 1, 1–8.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Wunderlin, D.A., Díaz, M.P., Amé, M.V., Pesce, S.F., Hued, A.C., Bistoni, M.A., 2001. Pattern recognition techniques for the evaluation of spatial and temporal variations in water quality. A case study: Suquía River Basin (Córdoba – Argentina). Water Res. 35, 2881–2894.

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

Please cite this article in press as: Reyna, L., et al., Identification and quantification of a novel nitrate-reducing community in sediments of..., Environ. Pollut. (2010), doi:10.1016/j.envpol.2009.12.014