Appl Microbiol Biotechnol (2006) 72: 393–400
DOI 10.1007/s00253-005-0272-7

ENVIRONMENTAL BIOTECHNOLOGY

B. Gómez-Villalba · C. Calvo · R. Vilchez · J. González-López · B. Rodelas

TGGE analysis of the diversity of ammonia-oxidizing and denitrifying bacteria in submerged filter biofilms for the treatment of urban wastewater

Received: 4 July 2005 / Revised: 21 November 2005 / Accepted: 27 November 2005 / Published online: 4 January 2006

Abstract The spatial and temporal diversity of the bacterial community-forming biofilms in a pilot-scale submerged biofilter used for the treatment of urban wastewater was analyzed by a temperature-gradient gel electrophoresis (TGGE) approach. TGGE profiles based on partial sequence of the *16S rRNA* gene showed that the community composition of the biofilms remained fairly stable along the column system and during the whole time of operation of the biofilter (more than 1 year). Community-profiling based on the amplification and separation of partial ammonia monooxygenase (*amoA*) and nitrous oxide reductase (*nosZ*) genes demonstrated that ammonia-oxidizing and denitrifying bacteria coexisted in both the anoxic and the aerated parts of the system. Several *amoA* and *nosZ* bands separated by TGGE were reamplified and sequenced, in order to further analyze the composition of these microbial communities in the biofilm. Phylogeny inferred from *amoA*/AmoA revealed the prevalence of *Nitrosomonas* species with five sequences affiliated to *Nitrosomonas oligotropha*, six sequences affiliated to *Nitrosomonas europaea*, and three sequences that showed only 75.7–76.1% identity of the DNA sequence with the closest described species (*Nitrosomonas nitrosa*). According to literature, this low identity value is indicative of previously undiscovered species. Eighteen new partial *nosZ* sequences were obtained which were mostly related to *nosZ* of gamma-proteobacteria (*Pseudomonas*) or clustered in the periphery of previously known denitrifying alpha-proteobacteria (*Bradyrhizobium* and *Azospirillum*).

B. Gómez-Villalba · C. Calvo · R. Vilchez · J. González-López (✉) · B. Rodelas
Instituto del Agua, Universidad de Granada,
18071 Granada, Spain
e-mail: jgl@ugr.es
Tel.: +34-958-244170
Fax: +34-958-243094

C. Calvo · J. González-López · B. Rodelas
Departamento de Microbiología, Facultad de Farmacia,
Universidad de Granada,
18071 Granada, Spain

Introduction

Urban wastewater (UWW) treatment is one of the major biotechnological processes used worldwide (Wagner et al. 2002). In countries of the European Union (EU), these effluents are regulated by the Central and Eastern Europe 271/91 directive. Fixed film technology is broadly used for the removal of organic matter and nitrogen (N) from UWW through the biological process of nitrification-denitrification (Gálvez et al. 2003; Hem et al. 1994; Lynga and Balmer 1992; Sakano et al. 2002). Among other advantages compared to the more generally applied activate sludge technology, submerged filters are simple to control and maintain, reduce room usage, lower costs, and minimize odors and noise (Pujol et al. 1994).

The physiology and biochemistry of both nitrifying and denitrifying bacteria are extensively known, however, the information about the ecology of these organisms is still scarce (Bothe et al. 2000). The introduction of molecular techniques in microbial ecology significantly implemented the knowledge of these organisms in their habitats as it enabled the study of the nonculturable bacteria. Muyzer et al. (1993) introduced the denaturing gradient gel electrophoresis (DGGE) technique in the field of microbial ecology. This method and its homologous temperature-gradient gel electrophoresis (TGGE) allow the separation of DNA fragments of similar size depending on their nucleotide sequence. DGGE was widely applied to the study of the population dynamics of microorganisms in natural habitats (Muyzer 1999; Muyzer and Smalla 1998) and in recent years it has considerably helped to broaden the knowledge of the microbial communities of biological systems for the depuration of UWW, particularly the ammonia oxidizers (for a review see Bothe et al. 2000; Wagner et al. 2002).

In this study, we used a pilot-scale UWW treatment plant consisting of two submerged filters working in predenitrification mode. The first one worked under anoxic conditions, keeping the denitrifying activity by the use of inlet UWW as carbon source, while the second one worked aerobically oxidizing organic nitrogen and ammonia to

nitrate. Biological treatment of UWW by the submerged filter technology needs to be performed under the predenitrification mode, in order to be economically advantageous. This type of system was previously applied with success in our laboratory under different working conditions for the removal of suspended solids, organic matter, and total N from UWW (Calvo et al. 2001; Gálvez et al. 2003). We explored in this work the potential of the TGGE approach on the monitoring of the spatial and temporal variations of the microbial diversity along the described UWW treatment system. For this purpose, TGGE was used to separate DNA fragments after amplification from total DNA extracted from the biofilms by primers targeting the V3 hypervariable region of the *16S rRNA* gene (Muyzer et al. 1993). In addition, the structure of the nitrifying and denitrifying microbial communities was further studied by the amplification and separation of genes encoding for the functional proteins ammonia monooxygenase (*amoA*) and nitrous oxide reductase (*nosZ*) after the subsequent identification of the bands by DNA sequencing. Characterization of specific bacterial members in the bioremediating beta subclass ammonia-oxidizing microbial community was achieved by this approach as phylogeny based on *amoA*/AmoA provides highly similar orders of taxa as phylogeny based on 16S rRNA (Purkhold et al. 2000). The *nosZ* gene was chosen as a marker in this work because its high diversity in natural habitats was consistently reported in recent literature (Scala and Kerkhof 1998, 1999; Rösch et al. 2002; Throbäck et al. 2004).

Materials and methods

Description of the pilot-scale UWW treatment plant

The pilot-scale plant used for these experiments is depicted in Fig. 1 and was based on a design already used successfully for UWW treatment in previous studies (Gálvez et al. 2003). It consisted of two methacrylate cylindrical columns (each 2 m high and 6 cm in inner diameter) both packed with clayey schists about 5–7 cm in size, 1.75 g/cm^3 in density, and up to 1 m in height. The columns are connected with a valve that allows a separated cleaning of the biofilters. To avoid filter clogging, cleaning cycles of the biofilters were carried out every 3 days. Hydraulic charge was kept at 0.354 m^3 m^{-2} h^{-1} and the nitrifying column was supplied with an airflow of 4.2 m^3 m^{-2} h^{-1}. Water flow was regulated by a peristaltic Watson Marlow 505S pump. The system was operated on a predenitrification mode with a recycle rate of 1,000 ml/h. The biofilters operate downflow (denitrifying column, anoxic) and upflow (nitrifying column, oxic) fed with UWW coming from primary treatment of the UWW treatment plant "EDAR Churriana" (EMASAGRA, Granada, Spain). The average composition of wastewater was determined by standard methods (APHA 1995) and was composed of the following: biochemical oxygen demand (5 days), 135 mg/l; chemical oxygen demand (COD), 450 mg/l; total N, 50 mg/l; and suspended solids, 120 mg/l. Under these working conditions, the removal of organic matter reached 75% and the COD in effluent water was never more than 125 mg/l, as required by the EU Directive 271/91, while a rate of 60% removal of total N was achieved. This level of N elimination is close to the theoretical maximum that can be achieved for the low COD/N ratio of the influent water (Van Loosdrecht and Jetten 1998).

Biofilm recovery and DNA extraction

Total DNA was periodically extracted from biofilm samples recovered from four locations of the reactor (sampling points 25DN, 75DN, 25N, and 75N as depicted in Fig. 1). Biofilm recovery was achieved by the following method: 5–50 g of clayey schists with biofilm adhered were extracted from each sampling point (see Fig. 1) and placed in flasks with 50 ml of sterile saline (0.9% NaCl). These suspensions were sonicated for 1 min and then placed in an orbital shaker at 155 rpm for 1 h. The process was repeated twice. Finally, 100 ml of saline with suspended material from the biofilm was centrifuged at 3,500 rpm for 30 min. The pellets were immediately used for DNA extraction, following a method based on the one previously described by Watanabe et al. (1998). Pellets were suspended briefly in 1 ml of buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.35 M sucrose, and 20 mg of lysozymes per milliliter] and incubated at 37°C for 10 min. After incubation, 1.5 ml of lysis buffer [100 mM Tris–HCl (pH 8.0), 0.3 M NaCl, 20 mM EDTA, 2% (wt/vol) sodium dodecyl sulfate, 4 μg/ml proteinase K, and 2% (wt/vol) 2-mercaptoethanol] was added and the samples were incubated at 37°C for 1 h. Lysates were extracted twice with 1 vol of phenol–chloroform solution (1/1 vol/vol), incubated for 10 min with 125 μg/ml RNAase, and extracted once with 1 vol of chloroform. Aliquots of 800 μl were precipitated with 1 vol of 2-propanol (30-min incubation at −20°C followed by centrifugation for 20 min at 13,000 rpm). The pellets were washed with 500 μl of 70% ethanol solution before being resuspended in 50 μl of Tris EDTA buffer and was left to rehydrate at 4°C overnight. DNA was further purified by a new phenol–chloroform extraction and reprecipitation the following day before being stored at −20°C.

PCR

Three genes were used as target for the study of bacterial diversity: the *16S rRNA* gene, the *amoA* gene for beta subclass ammonia-oxidizing bacteria, and the *nosZ* gene for denitrifying bacteria. Fragments of the three genes of a size adequate for TGGE separation (ca. 200 bp for *16S rRNA*, 475 bp for *amoA*, and 250 bp for *nosZ*) were amplified by polymerase chain reaction (PCR) using nested or seminested approaches as these strategies were previously reported to avoid unspecific priming, improve amplification of species present in low numbers, and yield richer DGGE band patterns (Nicolaisen and Ramsing 2002; Ward and O'Mullan 2002). The primers used were previously

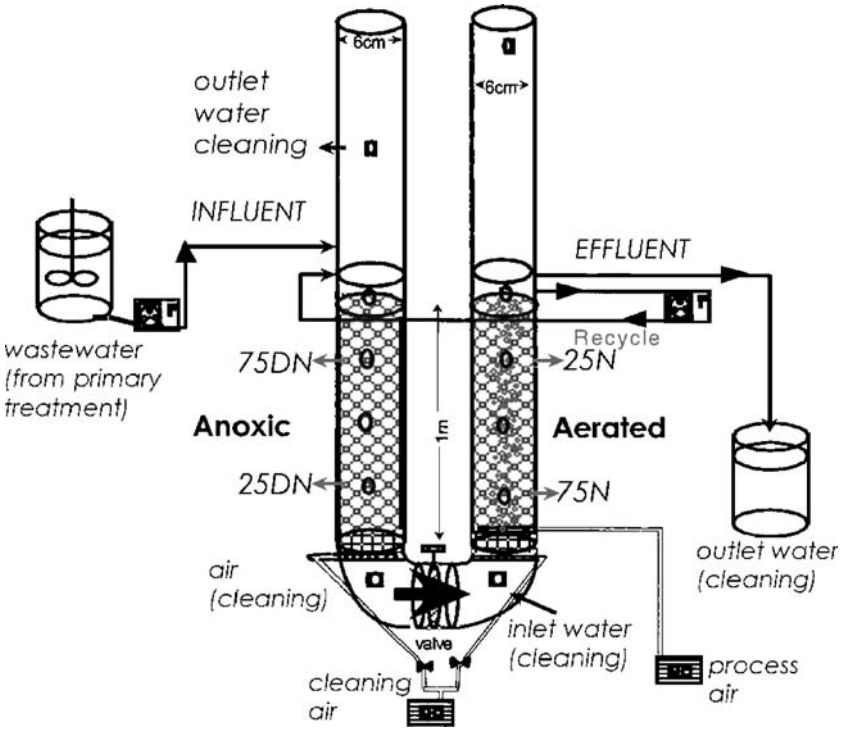


Fig. 1 Diagram of the pilot plant used in the study showing the disposition of anoxic and aerated columns and the sampling ports at different locations in the plant (25D, 75D, 25N, and 75N)

described in literature and their references are listed in Table 1. All primers were purified by high-performance liquid chromatography and purchased from Sigma-Aldrich. One microliter (2–5 ng) of DNA was used as template for PCR and subsequently, 1 μl of the first PCR product was used as template for the nested PCR. Conditions for each of the PCR reactions are summarized in Table 1. Final PCR products were cleaned and/or concentrated (when required) using Microcon-YM cartridges. Two to 5 μl were loaded in each well for TGGE.

TGGE and DNA sequencing

TGGE was done on a TGGE Maxi system (Whatman Biometra). Denaturing gels (6% polyacrylamide gel electrophoresis with 20% deionized formamide, 2% glycerol, and 8 M urea) were made and run with 1×Tris–acetate–EDTA buffer. Temperature gradients were optimized for efficient separation of bands and were as follows: 43 to 63°C for the *16S rRNA*, 38 to 52°C for the *amoA*, and 47.5 to 54.5°C for the *nosZ* gene. The gels were run at 115 v for 18 h except the gels for *nosZ*, which were run for 24 h. Gel bands were visualized either by staining with SYBR-green I (Sigma-Aldrich) for 20 min or by silver staining using the Gelcode silver staining kit (Pierce) following the manufacturer's indications except for the omission of the stabilization step. SYBR-green I stained gels were analyzed with a Gel Doc 2000 System (BioRad) and silver-stained gels were photographed with a Canon digital camera.

Portions of individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 μl of sterile PCR water, and directly used for reamplification with the appropriate primers. PCR products were purified by gel running and extraction with the QIAEX II kit (Qiagen). The DNA recovered was used for automated sequencing in an Applied Biosystems 3100 capillary automatic sequencer using the appropriate primers.

Biocomputing and phylogenetic analysis

DNA sequences were analyzed using the biocomputing tools provided online by the European Bioinformatics Institute (http://www.ebi.ac.uk). The BLASTn, BLASTx (Altschul et al. 1997), and FASTA (Pearson and Lipman 1988) programs were used for preliminary sequence similarity analysis. Phylogenic inference from *amoA*/AmoA and *nosZ*/NosZ was calculated using the CLUSTALX V.1.8 software (Jeanmougin et al. 1998) for the aligning of sequences, calculation of distance matrixes, and construction of bootstrapped neighbor-joining trees (Felsenstein 1985; Saitou and Nei 1987). Gaps were excluded from the analysis and the Kimura correction was applied (Kimura 1983). Bootstrap values below 50% are not shown in trees.

Results

Bacterial community-profiling by TGGE

TGGE profiles which were based on the V3 region of the *16S rRNA* gene were produced from samples taken during three consecutive weeks and after 1 year of plant operation. These profiles reflected a high uniformity of the populations especially in the anoxic biofilm while the aerated column

Table 1 PCR conditions applied in each experiment described in the "Material and methods"

Amplification	PCR conditions	References
16S rDNA		
First PCR-primers fD1 and rD1 (Weisburg et al. 1991)	Initial denaturation: 95°C, 7' 25 cycles: 94°C, 1' 10"; 56°C, 40"; 72°C, 2' Final extension: 72°C, 6' 10" Initial denaturation: 94°C, 7'	Modified from Vinuesa et al. 1998
Nested PCR-primers GC-P1 and P2 (Muyzer et al. 1993)	20 cycles: 94°C, 1' 10"; 65°C, 1', scaling down 1° each two cycles until reaching 55°C, 72°C, 2' Ten cycles: 94°C, 1' 10"; 55°C, 1'; 72°C, 2' Final extension: 72°C, 10'	Modified from Muyzer et al. 1993
amoA		
First PCR-primers AmoA-1F and AmoA-2R-TC (Nicolaisen and Ramsing 2002)	Initial denaturation: 92°C, 7' 35 cycles: 92°C, 30"; 57°C, 30"; 72°C, 45", increasing 1" per each additional cycle Final extension: 72°C, 5'	Modified from Nicolaisen and Ramsing 2002
Semi-nested PCR-primers AmoA-1F-Clamp and AmoA-2R-TC (Nicolaisen and Ramsing 2002)	Same as above	
nosZ		
First PCR-primers Nos1527f and Nos 1773R (Scala and Kerkhof 1998)	Initial denaturation: 94°C, 7' 20 cycles: 94°C, 1'; 64°C, 1' scaling down 1° each two cycles until reaching 54°C; 72°C, 2' Ten cycles: 94°C, 1'; 54°C, 1'; 72°C, 2' Final extension: 72°C, 10'	This study
Semi-nested PCR-primers GC-Nos1527f [a] and Nos 1773R (Scala and Kerkhof 1998)	Same as above	

[a] A 5' *GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG* 3' clamp was added to primer Nos1527F to allow TGGE separation

showed significant variation of the band patterns in the samples taken with a 1-year interval (data not shown). Samples taken at different locations of the column system (25DN, 75DN, 25N, or 75N) also reflected a spatial variation only in the aerated part of the system. As expected, the profiles obtained had a high complexity but it was possible to reamplify and sequence eight dominant bands whose affiliations are shown in Table 2. It is remarkable that the

Table 2 Comparison of the 16S rDNA sequences of major bands isolated from TGGE gels (ca. 160 bp) and sequences of the European Molecular Biology Laboratory database

Band #	Closest taxonomic affiliation (Class)	Most similar organisms	Access #	% identity
1	Beta-proteobacteria	*Ralstonia* sp. strain MSB2080	AY275490	91
		Burkholderia sp. strain Yws-12	AJ704385	90
2	Beta-proteobacteria	*Aquaspirillum* sp. strain TG27	AF384190	95
		Delftia tsuruhatensis strain	AJ852059	94
3	Beta-proteobacteria	*Aquaspirillum* sp. strain TG27	AF384190	100
		Variovorax paradoxus	AF508103	99
4	Alpha-proteobacteria	*Albidovulum inexpectatum*	AF465833	96
		Rhodobacter sphaeroides	AJ415571	95
5	Beta-proteobacteria	*Aquaspirillum delicatum*	AF078756	96
		Variovorax sp. WDL1	AF538929	96
6	Acidobacteria	*Desulfovibrio* sp. strain ABHU1SBfatS	AF056089	84
		Desulfovibrio fairfieldensis	U42221	83
7	*Nitrospira*	*Nitrospira* sp. strain RC14	NSPY14637	93
		Nitrospira moscoviensis	AF155154	93
8	Beta-proteobacteria	Uncultured organism clone M8907A10	AY898045	93
		Acidovorax delafieldii	AJ518818	93

sequence of a band which predominated in samples from the aerated biofilm, but not from the anoxic biofilm, was found related to the nitrite oxidizer genus *Nitrospira*, confirming a major presence of these bacteria in the aerated part of the biofilm and suggesting that these group of organisms are the most likely candidates responsible for the oxidation of nitrite to nitrate in the system used for this study.

Composition of microbial communities of functional microbial groups—phylogeny inferred from NosZ and AmoA

TGGE profiles of *amoA* and *nosZ* genes showed the presence of ammonia-oxidizing and denitrifying bacteria in both the anoxic and aerobic filters. The community profiles

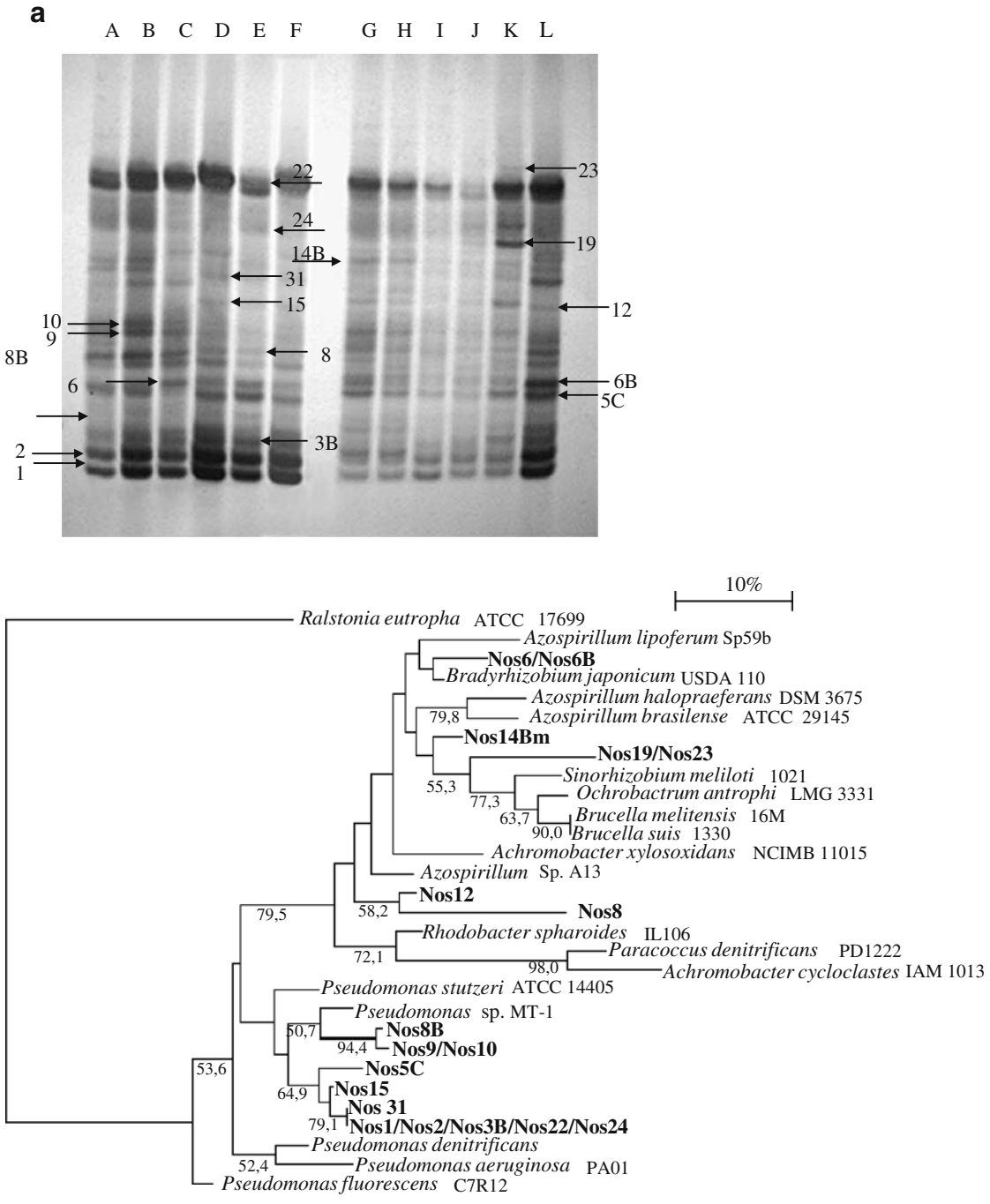
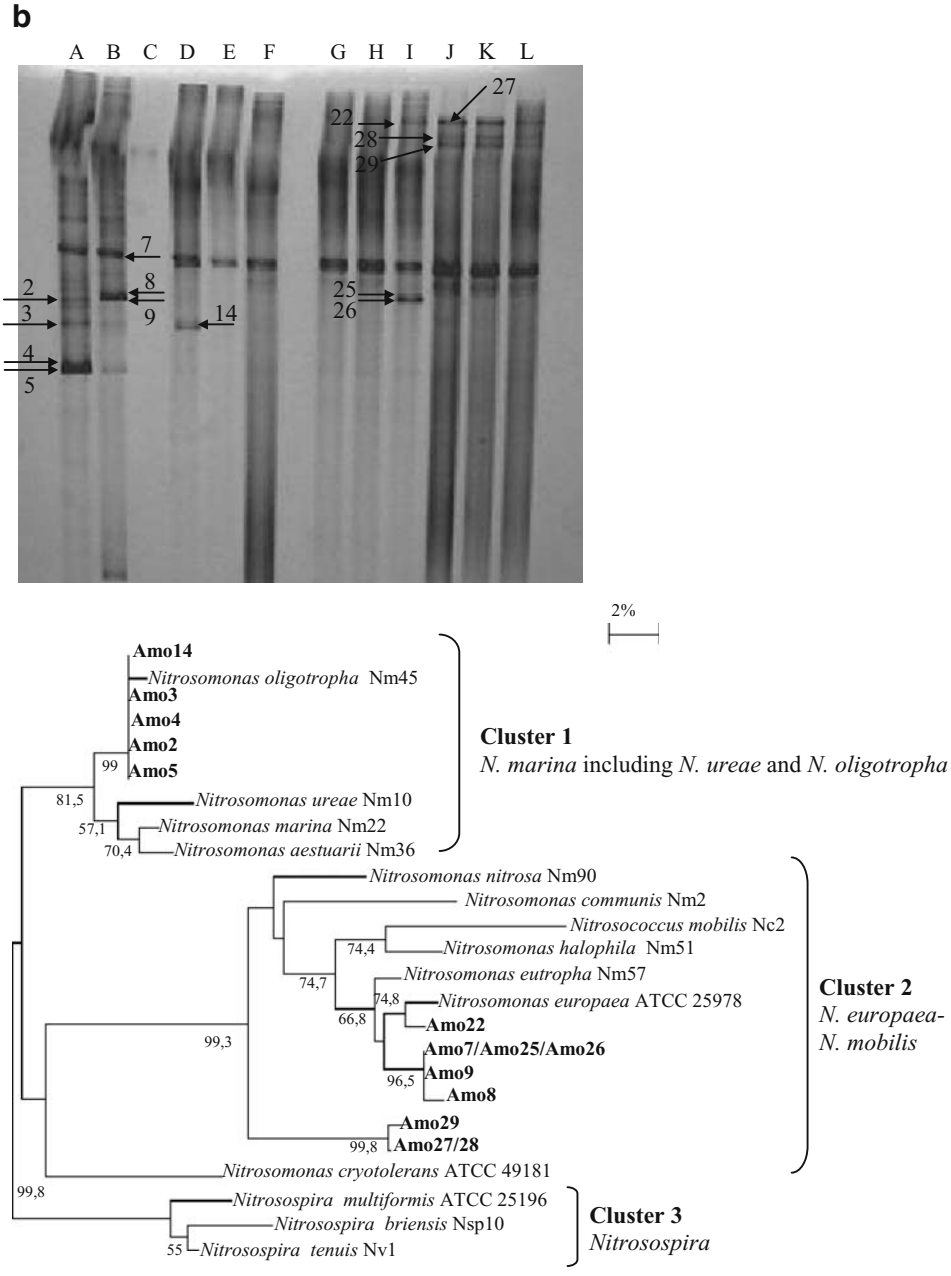


Fig. 2 TGGE diversity profiles and phylogenetic neighbor-joining trees based on partial NosZ (**a**) and AmoA (**b**) proteins (150 and 70 AA-aligned, respectively). *Lanes A*, *B*, and *C*: samples taken from the anoxic column during three consecutive weeks. *Lanes G*, *H*, and *I*: samples taken from the aerated column during three consecutive weeks. *Lanes D* and *J*: samples taken after 1 year of plant functioning (anoxic and aerated columns, respectively). *Lanes E*, *F*, *K*, and *L*: samples taken from 25DN, 75DN, 25N, and 75N points, respectively, after 1 year of plant functioning. Bands numbered pointed with *arrows* in each gel (Amo or Nos) correspond to bands numbered in each phylogenetic tree

Fig. 2 (continued)



of denitrifying bacteria remained very stable over space and time (Fig. 2a), while profiles of ammonia oxidizers greatly varied at different sampling times and locations in the reactor (Fig. 2b). Several bands separated by TGGE were reamplified and sequenced to analyze the composition of these microbial communities in the biofilm.

A total of 43 different bands were extracted from *nosZ* TGGE gels from both the anoxic and the aerated parts of the submerged filter biofilm, although only 18 partial *nosZ* sequences were successfully read in their entirety, the rest either did not amplify successfully or showed the presence of overlapped sequences. Several individual bands yielded identical DNA sequences (Nos19 and Nos23; Nos6 and Nos6B; Nos9 and Nos10; and Nos1, Nos2, Nos3B, Nos22, and Nos24). These high occurrence of redundancies were most probably due to PCR artifacts (heteroduplexes), favored by the combination of a mixed template and the use of degenerated primers for amplification. None of the 11 deduced NosZ sequences showed a 100% identity with the sequences on databases, but most of them were related to those of gamma-proteobacteria (genus *Pseudomonas*), while the rest clustered in the periphery of previously known denitrifying alpha-proteobacteria (*Bradyrhizobium* and *Azospirillum*) (Fig. 2a).

A total of 21 bands were extracted from the *amoA* gels, where 14 of them were successfully reamplified and fully sequenced. Again, some bands physically separated on the gel yielded identical DNA sequences, but with a much lower incidence compared to the *nosZ* gels (Amo27 and Amo28; Amo7, Amo25, and Amo26). Several bands

have different DNA sequences but translated into identical protein sequences (i.e., Amo2, Amo3, Amo4, and Amo5 bands).

Phylogeny inferred from the partial *amoA*/AmoA sequences revealed the prevalence of *Nitrosomonas* species in the UWW submerged filter treatment system with five band sequences (Amo2, Amo3, Amo4, Amo5, and Amo14) affiliated to *Nitrosomonas oligotropha*, six band sequences (Amo7/Amo25/Amo26, Amo8/Amo9, and Amo22) affiliated to *Nitrosomonas europaea*, and three band sequences (Amo27, Amo28, and Amo29) that showed only 75.7–76.1% similarity of the DNA sequence with the closest described species (*Nitrosomonas nitrosa*). However, the Amo27/Amo28 and Amo29 protein sequences showed a similarity of 100 and 99%, respectively, which are identical to the sequence of AmoA of unidentified bacteria GLII-20 (accession No. AAG39794.1) that was also isolated from a nitrifying wastewater treatment plant (Purkhold et al. 2000) and to sequence ST-A-mRNA-1 (accession No. BAD12250.1) that was isolated by DGGE from mRNA extracted from a septic tank (Ebie et al. 2004). Another two sequences also isolated in the aforesaid study (BAD 12251.1 and BAD12252.1) gave high identity values with sequences Amo27/Amo28 and Amo29. According to Purkhold et al. (2000), the low identity values with any previously known *Nitrosomonas* indicate that this branch may represent a new species.

Discussion

Research on the use of biofilters for UWW management has traditionally focused on changes on water parameters, mostly organic matter disappearance and N elimination. However, the study of the bacterial ecology of these systems is needed to get a more detailed picture of the interaction between its biology and chemistry as a way to improve the necessary knowledge to progress in the design and management of treatment plants.

In this study, we appraised the use of the TGGE approach for the profiling and characterization of the bacterial communities in a UWW treatment plant based on submerged biofilters whose efficiency for the removal of COD, total N, and pathogenic bacteria was proven in previous studies (Calvo et al. 2001; Gálvez et al. 2003). When the method was founded on the amplification of the hypervariable region V3 of *16S rRNA* gene, this approach has proven to be a suitable tool for the spatial and temporal monitoring of the community profiles of the fixed biofilm used in the study. Community structure monitoring is particularly required by UWW plants expected to suffer shock loads of pollutants, which often cause the breakdown of the community structure of the biological systems and the loss of its normal mineralization activity (Eichner et al. 1999; Watanabe et al. 1999). The results reported here support the value of TGGE as a method that could be applied in future research to follow the effects of relevant external factors, such as fluctuations on inlet water composition, on the community structure of the UWW submerged filter system used in this study.

The wide distribution of the denitrification activity within phylogenetically distant bacterial species makes the design of universal primers suitable for molecular studies of this functional microbial group unfeasible. The degenerated primers used in this study were previously designed by Scala and Kerkhof (1998) who suggested their potential utility for future DGGE/TGGE studies. We encountered several difficulties in the use of these degenerated primers for TGGE-based diversity studies. The approach yielded a number of new sequences of the *nosZ* gene, but the frequent finding of redundant sequences pointed out that the band patterns can heavily overestimate the diversity of *nosZ* copies in the analyzed samples, hence, such patterns must be interpreted cautiously in terms of evaluation of diversity. The occurrence of *nosZ* copies phylogenetically close to those of well-known denitrifyers was also found in a previous study using an equivalent fixed biofilm based on the denitrification-nitrification process (Sakano et al. 2002). However, copies of *nosZ* recovered from natural environmental samples are often unrelated to any previously known sequences (Scala and Kerkhof 1999).

Based on the *amoA* sequences obtained from reamplified TGGE bands, we identified fairly diverse ammonia-oxidizing populations in the biofilm of the UWW system, although all partial sequences were affiliated to *Nitrosomonas*-like *amoA*, and no *Nitrosospira*-related sequences were found. The predominance of ammonia oxidizers of the genus *Nitrosomonas* over *Nitrosospira* was commonly reported in previous studies of the ammonia oxidizer communities on UWW plants based on the activated sludge technology (Purkhold et al. 2000).

TGGE coupled to reamplification and sequencing of bands allowed the temporal and spatial monitoring of the community structure of the UWW plant used in this study, and provided characterization of the predominant populations of ammonia-oxidizing and denitrifying communities of the fixed biofilm involved in the process. The TGGE approach offers advantages over cloning methods, which require a greater amount of time and money, providing an outcome of equivalent validity on the characterization of the populations. The major disadvantage of TGGE is the limitation of the size of the DNA fragments and the number of bands that can be resolved on the surface of the gel. A key drawback met in this particular study were the artifacts due to mixed template PCR reactions, especially when the use of degenerated primers could not be avoided (*nosZ*–based studies).

Acknowledgements This research was supported by Programa Nacional de I+D, Ministerio de Ciencia y Tecnología (MCYT), Spain (AMB99-0666-C02-01). Work by B. Gómez-Villalba was supported by an FPI Ph.D. grant (MCYT, Spain). Work by B. Rodelas was funded by Programa Ramón y Cajal (MCYT, Spain).

References

Altschul SF, Madden TL, Schaeffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

APHA (1995) Standard methods for the examination of water and wastewater, 19th edn. American Public Health Association, Washington DC

Bothe H, Günter J, Schloter M, Ward BB, Wizel KP (2000) Molecular analysis of ammonia oxidation and denitrification in natural environments. FEMS Microbiol Rev 24:673–690

Calvo C, Gálvez JM, Gómez-Villalba B, Toledo FL, Rodelas B, González-López J (2001) Behaviour of a nitrifying and denitrifying submerged filter in the treatment of urban wastewater. In: Current studies of biotechnology, vol 2. Croatian Society for Biotechnology, p 175–182

Ebie Y, Noda N, Miura H, Matsumura M, Tsuneda S, Hirata A, Inamori Y (2004) Comparative analysis of genetic diversity and expression of *amoA* in wastewater treatment processes. Appl Microbiol Biotechnol 64:740–744

Eichner CA, Erb RW, Timmis KN, Wagner-Dobler I (1999) Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. Appl Environ Microbiol 65:102–109

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Gálvez JM, Gomez MA, Hontoria E, González-López J (2003) Influence of hydraulic loading and air flowrate on urban wastewater nitrogen removal with a submerged fixed-film reactor. J Hazard Mater 101:219–229

Hem LJ, Rusten B, Odegarard H (1994) Nitrification in a moving bed biofilm reactor. Water Sci Technol 28:1425–1433

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends Biochem Sci 23:403–405

Kimura M (1983) The neutral theory of molecular evolution. Cambridge Univ. Press, Cambridge, U.K., p 75

Lynga A, Balmer P (1992) Denitrification in a non-nitrifying activated sludge system. Water Sci Technol 26:1097–1104

Muyzer G (1999) DGGE/TGGE, a method for identifying genes from natural ecosystems. Curr Opin Microbiol 2:317–322

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73:127–141

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16s RNA. Appl Environ Microbiol 59:695–700

Nicolaisen MH, Ramsing NB (2002) Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J Microbiol Methods 50:189–203

Pearson W, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448

Pujol R, Hamon H, Kandel X, Lemel H (1994) Biofilters, flexible, reliable biological reactors. Water Sci Technol 29:33–38

Purkhold U, Pommerening-Roser A, Juretschko S, Schmid M, Koops H, Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and AmoA sequence analysis: implications for molecular diversity surveys. Appl Environ Microbiol 66:5386–5382

Rösch C, Mergel A, Bothe H (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl Environ Microbiol 68:3818–3829

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

Sakano Y, Pickering K, Ström P, Kerkhof LJ (2002) Spatial distribution of total, ammonia-oxidizing, and denitrifying bacteria in biological wastewater treatment reactors for bioregenerative life support. Appl Environ Microbiol 68:2285–2293

Scala DJ, Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol Lett 162:61–68

Scala DJ, Kerkhof LJ (1999) Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl Environ Microbiol 65:1681–1687

Throbäck IN, Enwall K, Jarvis A, Hallin S (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol 49:401–417

Van Loosdrecht MC, Jetten M (1998) Microbiological conversion in nitrogen removal. Water Sci Technol 38:1–7

Vinuesa P, Rademaker JLW, de Bruijn FJ, Werner D (1998) Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. Appl Environ Microbiol 64:2096–2104

Wagner M, Loy AL, Nogueira R, Purkhold U, Lee N, Daims H (2002) Microbial community composition and function in wastewater treatment plants. Antonie van Leeuwenhoek 81: 665–680

Ward BB, O'Mullan GDO (2002) Worldwide distribution of *Nitrosococcus oceani*, a marine ammonia-oxidizing γ-proteobacterium, detected by PCR and sequencing of 16S rRNA and *amoA* genes. Appl Environ Microbiol 68:4153–4157

Watanabe K, Yamamoto S, Hino S, Harayama S (1998) Population dynamics of phenol degrading bacteria in activated sludge determined by *gyrB*-targeted quantitative PCR. Appl Environ Microbiol 64:1203–1209

Watanabe K, Teramoto M, Harayama S (1999) An outbreak of nonflocculating catabolic populations caused the breakdown of a phenol-digesting activated-sludge process. Appl Environ Microbiol 65:2813–2819

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703