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Recovery of a *Nitrosomonas*-like 16S rDNA Sequence Group from Freshwater Habitats*

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Summary

In order to study the diversity of ammonia-oxidising bacteria in freshwater habitats, including sediments, a molecular approach focused on the sequencing of 16S rDNA was adopted. 16S rDNA sequences showing affinity with the β-subgroup of ammonia-oxidising bacteria were recovered by specific PCR of directly isolated DNA from freshwater samples, and samples from brackish water and *Glyceria maxima* rhizosphere were included in the analysis for comparison. The ammonia oxidiser-like sequences recovered from several locations, which exhibit differences in the composition of their total microbial communities as indicated by denaturing gradient gel electrophoresis, formed a strong monophyletic cluster including *Nitrosomonas ureae*. This is the first report presenting sequences from an apparently dominant group of *Nitrosomonas*-like organisms among the β-subdivision of ammonia-oxidising bacteria in freshwater environments. This group of sequences extends the known diversity within the β-subgroup of ammonia-oxidisers. The new sequences related to *Nitrosomonas ureae* do not match with some published primers and probes designed for the detection of *Nitrosomonas* species, which may explain why these sequences have not previously been detected in freshwater habitats. The sequence diversity detected within this group of sequences was minimal across the environments examined, and no patterns of distribution were indicated with respect to environmental factors such as sediment depth or location.

Key words: ammonia oxidation – β-proteobacteria, PCR – DGGE – *Nitrosomonas ureae*, nitrification – DNA isolation.

Introduction

Chemolithotrophic ammonia-oxidising bacteria, which carry out the conversion of ammonia to nitrite, are critical to the global cycling of nitrogen (*Prosser*, 1989). These organisms have been shown to comprise two monophyletic groups within the Proteobacteria, based upon 16S rDNA sequence analysis (HEAD et al., 1993; TESKE et al., 1994; WOESE et al., 1984 & 1985). One group, within the γ-subdivision of the proteobacteria, contains marine strains belonging to the genus *Nitrosococcus*. The other group, within the β-subdivision of the Proteobacteria, contains the genera *Nitrosomonas* and *Nitrosospira*, the latter encompassing the previous genus designations *Nitrosolobus* and *Nitrosovibrio* (HEAD et al., 1993). The β-subdivision ammonia-oxidising bacteria have been the subject of intense research in recent years and have been displayed as being responsible for the majority of the ammonia oxidation in a variety of habitats (BELSER, 1979; HALL, 1986; LAANBROEK and WOLDENDORP, 1995).

Ammonia-oxidising bacteria are notoriously refractory to traditional laboratory culture techniques, and their slow growth rates and low maximum growth yields have complicated the study of their ecological importance and *in situ* diversity. Furthermore, the use of lipid profiles has failed to provide satisfactory discriminatory capabilities for the genera which have been examined (BLUMER et al, 1969). However, the monophyletic nature of the β-proteobacterial ammonia oxidisers has recently facilitated the use of molecular biological strategies targeting the 16S rRNA gene to study the distribution and diversity of these organisms in various environments, including freshwater habitats (WARD et al., 1997; HIORNS et al., 1995). The recovery of 16S rDNA sequence information directly from the environment after PCR amplification using primers designed to target the full breadth of the

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β-subdivision ammonia oxidiser clade has revealed that the total diversity of recoverable sequences which show affinity with the β-subdivision ammonia-oxidising bacteria exceeds the diversity of culture collection isolates (MCCAIG et al., 1994; STEPHEN et al., 1996). Currently available sequence information identifies at least seven phylogenetically supported clusters within the β-subgroup ammonia oxidisers, four within the genus *Nitrosospira* and three designated within the genus *Nitrosomonas* (Figure 1; STEPHEN et al., 1996). It has also been shown that the presence of these different sequence clusters can be coupled with environmental gradients such as pH and ammonia concentration. (KOWALCHUK et al., 1997; STEPHEN and MCCAIG, personal communication).

The preservation and monitoring of freshwater lake and river systems are of both economic and environmental importance (*Laanbroek* et al. 1996). The increased ammonia deposition in recent decades due to intense agricultural runoff and industrial input has elevated nitrogen levels in many freshwater systems (VAN BREEMEN and VAN DIJK, 1988). Nitrification in such habitats can lead to nitrate contamination in sources of drinking water as well as habitat acidification and eutrophication (HALL, 1986; PROSSER, 1989). However, relatively little is known about the diversity and distribution of ammonia-oxidising bacteria in such systems. It was therefore our goal to use molecular techniques targeting the 16S-rDNA to describe the sequence diversity of the β-subgroup ammonia oxidisers inhabiting a variety of freshwater pelagic and sediment environments.

Materials and Methods

Sample sites and sampling procedure. The studied sample sites are listed in Table 1. Water column samples were taken from the Lake IJsselmeer at Medemblik (5°34′E, 52°50′N), the river IJssel (5°50′E, 52°35′N), and the Westerscheldt estuary (4°20′E, 51°12″N). Lake IJsselmeer was formed by the closing off of the saltwater Zuiderzee by a dam in 1933 and is the largest freshwater lake in the Netherlands (1190 km^2). The river IJssel splits off from the river Rhine and feeds into Lake IJsselmeer. The Westerscheldt estuary system is described elsewhere (GOOSEN et al., 1995) and was used as a brackish water reference. Water samples were taken by filtering 50ml of a mixed sample, pooled from different depths, over a polycarbonate filter (25 mm diameter 0.2 μm cut-off, BA83 Schleicher and Schuell, Dassel, Germany).

Multiple sediment samples were taken from Lake Gooimeer (5°10′E, 52°18′N, non-clayey moderately coarse sand, 26 km^2 surface area) and Lake Veluwemeer (5°38′E, 52°22′N, non-clayey medium fine sand, 32 km^2 surface area). These two lakes, as well as Lake Drontermeer (see below), are man-made lakes used for the water management of land areas reclaimed from the southern part of Lake IJsselmeer. Lake Reeuwijk (4°45′E, 52°02′N, non-clayey coarse sand, 9 km^2 surface area) is also an artificial lake, formed by the flooding of a former sand winning area. Sediment sampling was performed with plastic core samplers with a diameter of 5 cm. The sediment in each core was pushed upwards with a plunger allowing 5 mm-thick layers to be sliced off with a sterile razor blade. pH measurements were determined at the sampling site. Lake Drontermeer rhizosphere sediments (5°50′E, 52°28′N, non-clayey medium fine sand, 6 km^2 surface area) were sampled according to BODELIER et al. (1996).

DNA extraction. DNA was extracted and purified from water and sediment samples using a modification of the method described by KOWALCHUK et al. (1997) and STEPHEN et al. (1996). 0.5 g sediment sample or a polycarbonate filter containing filtered water samples was added to a 2-ml screw-cap tube containing 0.5 ml TE (Tris 10 mM/EDTA 1mM pH 7.6) buffer, 0.5 ml TE saturated phenol (Gibco Laboratories, Detroit, MI, USA) and 0.5 g 0.1 mm diameter acid-washed Zirconium beads (Biospec Products, Bartlesville, OK, USA). The tubes were shaken at 5K rpm for four times 30 seconds in a Mini Beadbeater (Biospec Products) and chilled on ice between shaking periods. After centrifugation for 3 minutes at 5K × *g*, 0.5 ml of the aqueous layer was removed and extracted once with 0.5 ml phenol/chloroform/isoamylalcohol 25:24:1 (Gibco) and one time with chloroform/isoamylalcohol 24:1. 0.4 ml of remaining aqueous layer was recovered for subsequent DNA precipitation with 0.1 volume 3M sodium acetate pH 5.2 and 2 volumes ethanol for 3 hours at –20 °C. DNA was pelleted at 13K × *g* for 20 minutes and was washed once with 70% ice cold ethanol. After air drying, the pellet was resuspended in 40 μl TE. Polyvinylpolypirrolidone (PVPP; Sigma, St. Louis, MO, USA) gel purification was performed as described by KOWALCHUK et al. (1997). DNA was resuspended in a final volume of 30 μl water after recovery from the gel matrix and stored at –20 °C until use.

PCR and DGGE profiling of environmental samples. 0.5 μl of each DNA isolation was used as template in 50 μl total volume reactions with the 357f and 535r primers, which are designed to amplify eubacterial 16S rDNA (Figure 2; MUYZER et al., 1993). The final reaction mix contained 0.5 pM of each primer, 2 μl 10 mg/ml bovine serum albumine (New England Biolabs, Beverly, MA, USA), 2.5 units Expand High-Fidelity DNA polymerase (Boehringer Mannheim, Germany), and the buffer conditions recommended by the manufacturer. PCR was performed in a PE480 thermocycler (Perkin Elmer, Foster City, CA, USA) using the program described by MUYZER et al. (1993), and the PCR product of approximately 180 bp was examined on a 2% agarose 0.5 × TBE (1 × TBE = 90 mM Tris-Borate, 2 mM EDTA, pH8.3) gel and stained with ethidium bromide to confirm product integrity and to estimate DNA concentration. For each sample, approximately 1 μg PCR product was analysed by denaturing gradient gel electrophoresis (DGGE) using a Biorad Protean II electrophoresis unit as described by ZWART et al. (1997). The DGGE gel was recorded with a CCD camera system (The Imager, Appligene, Illkirch, France). Processing of the image was performed with the software program Aldus Photostyler 2.0 (Aldus corporation, Seattle, WA, USA). Brightness and contrast were adjusted for the rhizosphere sample lane, all images were sharpened once.

Construction of 16S rDNA gene libraries. Partial 16S rDNA libraries were constructed by cloning PCR products recovered after direct amplification with the βAMOf / βAMOr primer set (Figure 2; MCCAIG et al., 1994). This primer set is known to be semi-specific for β-subgroup ammonia-oxidising bacteria as it also amplifies 16S rRNA genes from other β-subgroup proteobacteria (STEPHEN et al., 1996). However, the regions targeted by these primers are suitable for the detection of all described members of β-subgroup ammonia-oxidising bacteria (POMMERENING-RÖSER et al., 1996). PCR was performed by using 0.5 μl DNA extract as template in a 50 μl total volume reaction with 2.5 units Expand H-F DNA polymerase (Boehringer) using the manufacturer's recommended buffer conditions. All custom primers used in this study were synthesised by Isogen Bioscience, BV (Maarssen, the Netherlands). The βAMOr primer was added to a concentration of 1 pM, and twice as much of the βAMOf primer, 2 pM, was added to com-

pensate for its ambiguous base positions. After an initial 5 min denaturation step at 94 °C, amplification was performed in an OmniGene thermocycler (Hybaid, Middlesex, UK) with the thermocycling program: 30 × (92° 1 min, 59° 1 min; 68° 1 min 30 sec) followed by 5 min at 68° C. Of the PCR product 25 µl was loaded onto a 1% agarose 0.5 × TBE gel. After electrophoresis and ethidium bromide staining, the expected 1180 bp band was excised and DNA isolated using QIAquick spin columns (Qiagen, Hilden, Germany). Ligation of the 1180 bp product in pGEM-T vector (Promega) and transformation by heat shock in Epicurian Coli XL1-Blue MRF' supercompetent *E.coli* cells (Stratagene, La Jolla, CA, USA) were executed according to the manufacturers' protocols. Plasmids were isolated, after overnight culturing of the colonies in liquid LB medium, using the High Pure Plasmid Isolation Kit (Boehringer).

Screening of 16S rDNA libraries. The number of clones examined per sample site, and the primer combinations used to screen them are summarised in Table 1. Primer positions are shown in Figure 2. Colony screening with the CTO189f / CTO654r and the 357f-535r primer set was as described by KOWALCHUK et al. (1997) and ZWART et al. (1997), respectively PCR screening was performed using a toothpick to take a small portion of a colony as template DNA. Each 25 µl reaction contained 1.25 units Taq DNA polymerase (Boehringer), 1.5 mM

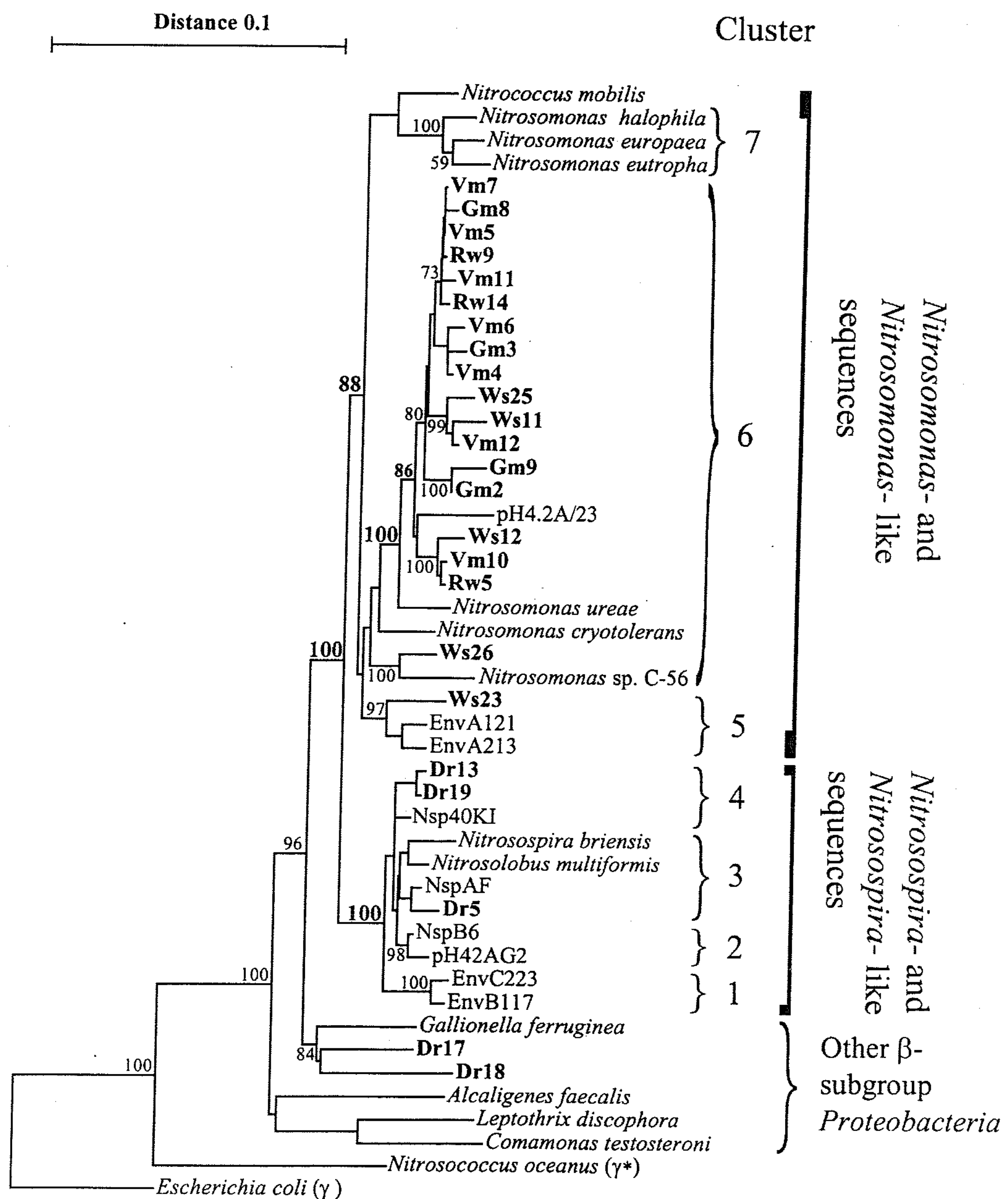

Fig. 1. Neighbor-joining tree of β-subgroup proteobacteria 16S rDNA sequences: Phylogenetic tree including freshwater sediment clones recovered during this study as well as representatives of the seven recognised sequence clusters of the β-subgroup ammonia-oxidising bacteria (STEPHEN et al., 1996). Sequence abbreviations are given in Table 1, and phylogenetic analysis were done for 1111 informative positions of 16S rDNA as described in the text. Only significant bootstrap values relevant to the discussion are shown.

MgCl2, 10mM Tris/HCL pH 8.3, 50 mM KCl, 0.01% w/v gelatine, 200 μM of each deoxynucleotide, and CTO primers were added as described by *Kowalchuk* et al. (1997). Amplification was performed using a thermocycling regime of 28 times (95°, 1 min; 57°, 1 min; 72°, 45 sec + 1 sec / cycle). Of each reaction 5 μl was analysed by DGGE followed by ethidium bromide staining as described by KOWALCHUK et al. (1997). After DGGE analysis of the PCR products from positive CTO primer reactions (results not shown), a single representative of every observed DGGE "type" within each clone library was selected for sequence analysis. Clones from the pelagic and rhizosphere samples were also screened with bacterial primers (357f / 518r) and sequenced to test the inclusiveness of the CTO189f / CTO654r primer set in the screening for β-subgroup ammonia oxidiser-like 16S rDNA clones.

Nucleotide sequence determination and phylogenetic analysis. Double-stranded cycle sequencing reactions were performed with the Thermosequenase kit (Amersham, Little Chalfont, UK) according to the manufacturers recommendations using Texas Red- labelled primers. Sequencing primers included the vector-specific primers Sp6 and T7 and the bacterial-specific 16S rDNA primers 357f and 1053r (EDWARDS et al., 1989). Medemblik (Lake IJsselmeer) and river IJssel water column DNA inserts were only partially sequenced (450 bp). Sequencing reactions were run and analysed on a Vistra DNA sequencer 725 (Amersham). Sequence data was edited and assembled with the Sequencer 3.0 (Gene Codes Corporation, Ann Arbor, MI, USA) software package and format conversions were carried out in Seqapp 1.9a169 (GILBERT, 1993). 16S rDNA sequence alignments of 1165 bp nucleotide positions were carried out with the addition of sequences retrieved from the Ribosomal Database Project (RDP; MAIDAK et al., 1997) and were performed in the Dedicated Comparative Sequence Editor program (*De Rijk* and DE WACHTER, 1993) using recognised 16S rRNA secondary structures (VAN DE PEER et al., 1997). Phylogenetic trees were constructed with the Treecon program (VAN DE PEER and DE WACHTER, 1994) using neighbor-joining analysis with JUKES and CANTOR (1969) correction. Gaps were not taken into account in the analysis, and bootstrap analysis was based upon 100 replicates. Parsimony analysis was performed using the PHYLIP 3.5 package written by Felsenstein (1993, University of Washington, USA). Previously determined ammonia-oxidising bacterial and ammonia oxidiser-like sequences used in the analysis are listed in Table 2. Partial 16S rDNA sequences determined in this study have been deposited into the EMBL sequence databank and their accession numbers are given in Table 1.

Results

Comparison of bacterial communities by DGGE

To compare the total bacterial communities of the pelagic, sediment and rhizosphere samples tested, DGGE was performed on PCR products obtained after direct amplification with the 357f-535r primer combination (MUYZER et al., 1993). The recovered DGGE profiles are presented in Figure 3. In general, each location appeared to contain a different microbial community as evidenced by the differences in DGGE banding patterns. In contrast, the banding patterns from the different sediment layers from a single location appeared to be nearly identical. The DNA from the gel shown in Figure 3 was also blotted for subsequent hybridisation with oligonucleotide probes specific for β-subdivision ammonia-oxidising bacteria as described by BRUNS et al. (1996), but no detectable signal was observed (not shown). There were no further attempts made to phylogenetically characterise the DGGE bands coming from the bacterial-specific PCR.

Recovery of ammonia oxidiser-like 16S rDNA from freshwater pelagic and sediment samples

The βAMOf-βAMOr primer set, used to create the clone libraries in this study, are known to be semi-specific for the amplification β-subgroup ammonia oxidiser 16S rDNA (STEPHEN et al., 1996). These primers, which target approximately 1160 bp of the 16S rRNA gene, also amplify 16S rDNA from a number of closely related β-proteobacteria, and the proportion of ammonia-oxidising bacteria like sequences recovered with this primer set can vary greatly between different sample types. The

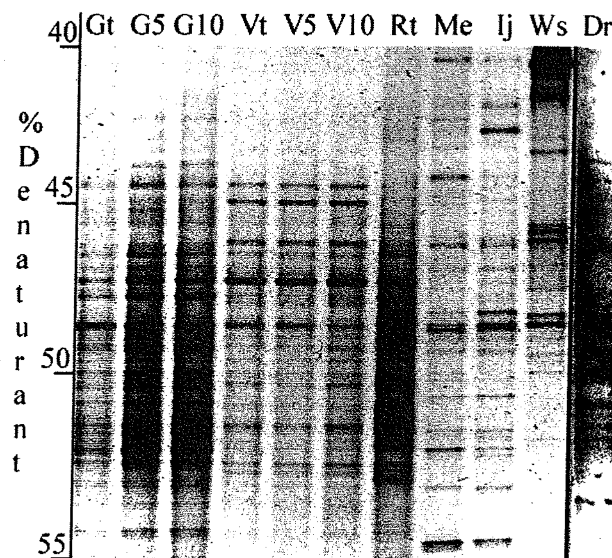

Fig. 3. DGGE profiles of sample sites using bacterial primers: The DGGE banding patterns of PCR products produced by amplification with the 357f / 535r primer set (MUYZER et al., 1993) are shown for the various sample sites investigated. Site abbreviations are given in Table 1.

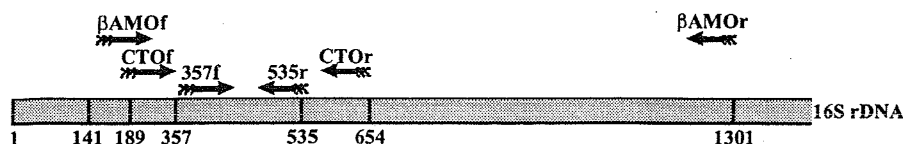

Fig. 2. Positions of 16S rDNA directed primers used in this study.

CTO primers, whose sequences are based upon both sequences from pure cultures and environmental clones, allow for the efficient PCR screening of clone libraries for sequences showing affinity to the β-subgroup ammonia oxidisers (KOWALCHUK et al., 1997). The clone libraries tested revealed between 0 and 80% positive amplification with the CTO primers (Table 1). The PCR products from all positive inserts were also subjected to DGGE (not shown), revealing that many of the generated clones were indistinguishable by DGGE. To avoid repeated sequencing of identical clones, only unique DGGE types within each clone library were sequenced. The freshwater pelagic and rhizosphere samples were additionally tested with the bacterial primers, 357f and 535r, to show that the vast majority of clones did in fact contain 16S rDNA sequences (Table 1).

Phylogenetic analysis of cloned 16S rDNA sequences

All clones which gave a positive reaction with the CTO primers contain sequences which fall within the β-subgroup ammonia oxidiser clade (Figure 1). Conversely, all sequenced clones which failed to produce PCR product with the CTO primer pair grouped outside of this monophyletic group, accept for the Westerscheldt brackish water clone Ws23, which showed affinity with *Nitrosomonas* cluster 5 (Figure 1 and results not shown). These results are in good agreement with previous results which show that the CTO primers are effective in screening clone libraries (KOWALCHUK et al., 1997), although their specificity to the β-subgroup ammonia oxidisers is not absolute (this study; Kowalchuk, unpublished). The fact that some clones produced by the βAMOf-βAMOr primer set fell outside the β-subgroup ammonia oxidiser clade is in agreement with previous results that show that these primers also target other members of the β-subclass of the Proteobacteria (STEPHEN et al., 1996).

All sediment clones that gave positive PCR results with the CTO primer screening formed a strongly supported monophyletic group based upon neighbor-joining analysis of 1111 bp of 16S rDNA sequence (Figure 1). This group fell within the *Nitrosomonas* cluster 6 as de-

Table 1. Summary of sample sites and clone recovery.

Code	Sample site	number of clones[a]	Screening with CTO primers[b]	Sequenced clones and EMBL accession numbers
Sediment samples				
	Lake Gooimeer			
Gt	top layer, pH8.5	68	0 of 20	–
G5	–5 mm layer, pH 8.5	19	2 of 19	Gm8 AJ003749
G10	–10 mm layer, pH 8.7	>400	7 of 10	Gm2, Gm3, Gm9 AJ003750 to AJ003752
Gtw	top layer in wintertime, pH 8.2	35	6 of 10	Gm115, Gm312, Gm112 AJ003753 to AJ003755
	Lake Veluwemeer			
Vt	top layer, pH 8.2	160	4 of 10	Vm4, Vm5, Vm6 AJ003756 to AJ003758
V5	–5 mm layer, pH 8.2	90	6 of 10	Vm7, Vm10 AJ003759 to AJ003760
V10	–10 mm layer, pH 8.0	>400	8 of 10	Vm11, Vm12 AJ003761 to AJ003762
	Lake Reeuwijk			
Rt	toplayer, pH 8.0	70	5 of 20	Rw5, Rw9, Rw14 AJ003763 to AJ003765
Pelagic samples				
Ij	River IJsel, pH 8.2, $[O_2]$ 12.1 mg/l	13	2 of 13 / 13 of 13[c]	Ij6, Ij8[c†], Ij13 AJ003766 to AJ003768
Me	Lake IJselmeer, pH 8.5, $[O_2]$ 10.2 mg/l	107	4 of 15 / 15 of 15[c]	Me2, Me9, Me11[c†], Me15[c†] AJ003769 to AJ003772
Ws	Westerscheldt, pH 7.4, $[O_2]$ 5 mg/l, salt conc. 11.5 g/l	150	7 of 8 / 19 of 20[c]	Ws 11, 12, 23[c], 25, 26 AJ003773 to AJ00377
Emergent macrophyte rhizosphere sample				
Dr	Lake Drontermeer rhizosphere, pH 7.6	180	15 of 25 / 23 of 25[c]	Dr5, Dr13, Dr17[c†], Dr18[c†], Dr19 AJ003778 to AJ003782

[a] Generated with the βAMOf-βAMOr primer set (MCCAIG et al., 1994)
[b] Screened with the CTOf-CTOr primer set (KOWALCHUK et al., 1997a)
[c] Screened with 357f-535r primer set (MUYZER et al., 1993)
[†] Non ammonia oxidiser-like sequence

fined by Stephen et al. (1996) and showed the closest affinity with the culture strain *Nitrosomonas ureae*. Similarity comparisons between these clones and *Nitrosomonas ureae* revealed between 94.9 and 96.3% identity with this species over the analysed region, and clones within this group were between 94.3 and 100.0% identical with each other. The three *Nitrosomonas*-like clones isolated from soil by Stephen et al. (1996; only

Table 2. Summary of database sequences used for phylogenetic tree construction: The strain or clone designations for the β-subgroup ammonia oxidiser 16S rDNA sequences used in the construction of the phylogenetic tree in Figure 1 are given.

Species / environmental clones	Origin	Sequence author	Accession number
Nitrosospira briensis C-128 (Nsp4)	soil	Head et al., 1993	M96396
Nitrosomonas europaea C-31 (Nm50)	soil, USA	Head et al., 1993	M96399
Nitrosomonas sp. C-56 (Nm63)	sea water, Gulf of Maine	Head et al., 1993	M96400
Nitrosolobus multiformis C-71 (NL13)	soil	Head et al., 1993	M96401
Nitrosomonas eutropha C-91 (Nm57)	sewage, Chicago	Head et al., 1993	M96402
Nitrosococcus mobilis (Nc2)	brackish water	Head et al., 1993	M96403
Nitrosospira sp. 40KI	garden soil, Norway	Utåker et al., 1996	X84656
Nitrosospira sp. B6	sewage treatment column, Norway	Utåker et al., 1996	X84657
Nitrosospira sp. AF	sandy loam, Zambia	Utåker et al., 1996	X84658
Nitrosospira sp. AV	sea water, Scotland	McCaig et al., 1994	Y10127
Nitrosomonas cryotolerans (Nm55)	sea water, Kasitsna Bay	Pommerening-Röser et al., 1996	Z46984
Nitrosomonas halophila (Nm1)	sea water, North Sea	Pommerening-Röser et al., 1996	Z46987
Nitrosomonas ureae (Nm10)	soil, Sardinia	Pommerening-Röser et al., 1996	Z46993
pH4.2A/23 and pH4.2A/G2 clones	agricultural soil, Scotland	Stephen et al., 1996Z69151,	Z69164
EnvA1-21 and EnvA2-13 clones	marine sediment, polluted, Scotland	Stephen et al., 1996	Z69091, Z69097
EnvB1-17 and EnvC2-23 clones	marine sediment, Scotland	Stephen et al., 1996	Z69104, Z69125

Table 3. Oligonucleotide probe compatibility with fresh water *Nitrosomonas*-like 16S rDNA sequences: The target sites for previously published primers and probes used for the detection of *Nitrosomonas*-like sequences are given with reference to their compatibility with the freshwater and sediment sequences recovered in this study.

Primer / probe[a]	Sequence and compatibility with fresh-water and sediment cluster 6 sequences[b]	primer / probe target	Authors
AAO-258	GGTAAaGGCtTACCAAGGC	non-marine β ammonia oxidisers	Hiorns et al ,1995
RNM-1007	tcTaaTggAGAcATaAgAGTaCCCG	non-marine *Nitrosomonas*	Hiorns et al., 1995
Nm75[c]	*cggcagcgggggcttcggcc*	*Nitrosomonas* genus	Hiorns et al., 1995
NEU (653-670)	TAGAGTGcaGCAGAGGGG	subset of *Nitrosomonas* cluster[d]	Wagner et al., 1995
NitA (137-159)	CTTAAGTGGGGaATAACGCATCG	all β ammonia oxidisers	Voytek and Ward, 1995
NitB (1214-1234)	GGGTAGGGCTTCACACGTAA	all β ammonia oxidisers	Voytek and Ward, 1995
NitD (455-479)	tAGTCGgaAAGAAA-GaGTTgcaA	*N. europaea* and close relatives	Ward et al., 1997
NitF (846-865)	AcGgacTGGTAACGTAGCT	subset of β ammonia oxidisers[e]	Ward et al., 1997
Nsm156	ATCGAAAGATGTGCTAATA	*Nitrosomonas* genus	Mobarry et al., 1996
Nso190	GGAGaAAAGcAGGGGATCG	all β ammonia oxidisers	Mobarry et al., 1996
Nso1225	CGCGATTGTATTACGTGTGA	all β ammonia oxidisers	Mobarry et al., 1996
NITROSO4E (639-658)	GAAACTACAaRgCTAGAGTG	all β ammonia oxidisers	Hovanec et al., 1996
NSM1B (479-495)	ATGACGGTAcCGACAGA	subset of *Nitrosomonas* cluster[d]	Hovanec et al., 1996
CTO189f	GGAGRAAAGYAGGGGATGC	all β ammonia oxidisers	Kowalchuk et al., 1997a
CTO654r	GCGTTTGAAACTACAaRgCTAG	all β ammonia oxidisers	Kowalchuk et al., 1997a
Nm0 (148-165)	ATAACGCATCGAAAGATG	*Nitrosomonas* genus	Pommerening-Röser et al., 1996

[a] base positions as given with respect to *E. coli* (Brosius et al., 1989).
[b] bases given in upper-case letters represent identity to all recovered *Nitrosomonas* cluster 6 clones. Bases given in lower-case bold type denote mismatches with all recovered *Nitrosomonas* cluster 6 clones, and plain lower-case letters give positions where mismatches occur with only some for of the recovered clones. Underlined sequences overlap the region targeted by the βAMOf primer.
[c] Not included in the amplified 1100bp region.
[d] *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosococcus mobilis*, and close relatives. [e] All β-subgroup ammonia-oxidising bacteria except *Nitrosomonas* sp. C-56.

pH4.2A/23 shown in Figure 1) also fall within this group. The clones from the Lake Drontermeer rhizosphere sample, which showed affinity with the β-subgroup ammonia oxidiser clade, grouped with *Nitrosospira* clusters 3 and 4. Of the Westerscheldt brackish water clones that reacted positively with the CTO primers, two grouped within the clade formed by the water and sediment clones originating from freshwater habitats, while a third showed greatest affinity with a marine *Nitrosomonas* sp. C-56.

Phylogenetic analysis of 427 base positions (not shown), which also included the partially sequenced clones from Lake IJsselmeer (Medemblik), the river IJssel, and Lake Gooimeer winter samples revealed that all ammonia oxidiser-like sequences from both the freshwater and the sediment samples fell within a strongly supported group with *Nitrosomonas* cluster 6. Tree topology was similar to that revealed using the full-length sequences although bootstrap values were lower. Comparison of the two trees as well as separate analysis of the 3' downstream half of the 1165 bp alignment did not reveal any evidence for the recovery of chimeric sequences, although such sequences would be difficult to detect for nearly identical 16S rRNA genes (LARSEN, 1997). Parsimony analyses revealed similar tree configurations with identical sequence clustering and similar bootstrap values with only minor differences in the branching orders for closely related sequences.

Table 4. Alignment of potential probe for the detection of non-marine *Nitrosomonas* cluster 6 with other ammonia oxidiser sequences: A potential probe or specific primer site for the targeting of the monophyletic *Nitrosomonas*-like group of sequences formed by freshwater, sediment, and soil (*Stephen* et al., 1996) clones is presented.

Strain or clone	Sequence (positions 205-222)[a]
S-*-Nsm6a-0205-a-S-17	A T C G A A A G A C C T T A T G C
freshwater *Nitrosomonas* clones	. . . .
Nitrosomonas ureae (Nm10)	. . . .
terrestrial *Nitrosomonas* clone pH4.2A/23	. . . .
Ijs13[b]	. . . . G . . . .
Gm8[b]	. . . . . G . . . .
Vm6[b]	. . . . . GT . . . .
Nitrosomonas communis (Nm2)	. . . . G . . .
Nitrosococcus mobilis (Nc2)	. . . . G C . .
Zooglea ramigera	. C . . C . . . .
Nitrosomonas sp. C-56 (Nm63)	. . . . T . . . . G C . .
Nitrosomonas europaea C-31 (Nm50)	. . . . C . . . . G C . .
Nitrosomonas eutropha C-91 (Nm57)	. . . . T . . . . G C . .
Ws23	. . . T T . G . . . . G . . .
Nitrosospira briensis C-128 (Nsp4)	. . . . C . . . . G C . .
Nitrosolobus multiformis C-71 (Nl13)	. . . . C . . . . G C . .
Nitrosococcus oceanus	. C . . . . . G . . . . C G C . .
Gallionella ferruginea	. . . . C . . . . C T C .

[a] Sequence of target site. Positions identical to the probe target sequence are represented by a period.

[b] Freshwater or sediment clones that contain mismatch(es) with the proposed probe binding site.

Potential probe and primer detection of ammonia-oxidising bacteria from freshwater and sediment

Table 3 contains a list of target sites for previously published 16S rRNA probes and primers for the detection of β-subgroup ammonia-oxidising bacteria and of the genus *Nitrosomonas* including their similarity with the freshwater and sediment 16S rDNA clones recovered in this study. While some primer and probe sites appear to match well with recovered *Nitrosomonas* cluster 6 clones, other primers and probes show multiple mismatches with this group. The CTO reverse primer contains a one bp discrepancy, a level of difference which is tolerated by the PCR conditions used for clone screening and amplification from environmental samples (this study; KOWALCHUK et al., 1997). Oligonucleotides AAO-258 (HIORNS et al., 1995) and NitF (WARD et al., 1997) which were designed to detect all non-marine β-subgroup ammonia-oxidising bacteria contain one mismatch and three mismatches, respectively. Oligonucleotides NitA (VOYTEK and WARD, 1995) and NITROSO4E (HOVANEC et al., 1996) designed for general β-subgroup ammonia oxidiser detection also contain a single mismatch.

Oligonucleotide RNM-1007, designed for the detection of non-marine *Nitrosomonas* sequences, shows multiple mismatches in its target site, and the *Nitrosomonas*-specific NSM1B (HOVANEC et al., 1996) probe contains a single mismatch. The monophyletic group formed by the water and sediment clones within *Nitrosomonas* cluster 6 can be targeted by a probe or specific primer designed to recognise the signature sequence 5'ATCGAAAGACCTTATGC3' (202-219). This probe, designated S-*-Nsm6a-0205-a-S-17, also matches *Nitrosomonas ureae* and the terrestrial *Nitrosomonas*-like clones (Table 4) of STEPHEN et al. (1996), which are inclusive to this group (Figure 1). This target sequence might also be of use for *in situ* analyses as it contains at least three mismatches with all other known 16S rDNA sequences, except for *Nitrosomonas communis*, which shows a single mismatch and *Nitrosococcus mobilis*, *Zooglea ramigera* which each contain two mismatches (Table 4). Within the target group, clones Ijs13 and Gm8 also show a single mismatch and clone Vm6 has two mismatches. Comparison of clones whose inserts were fully sequenced revealed that there were no appropriate probe sites downstream of the CTO reverse primer binding site. Given this and the stability of phylogenetic trees determined from 465 bp (see above), full length sequencing was not deemed necessary for the Lake IJsselmeer (Medemblik), the river IJssel, and Lake Gooimeer winter clones.

Discussion

DGGE profiling of the total bacterial communities (Figure 3) showed large differences between the different sample sites but not between sediment layers at a single site. This latter result was somewhat surprising as the resident microbial communities are likely subjected to a steep O_2 gradient. O_2 concentrations decline from 10–12 mg/l at the sediment surface to under 0.1 mg/l at a depth of 5mm for comparable freshwater sediments (DE BEER et al., 1991). In the anaerobic part other bacteria were expected as in aerobic top layer. But the very similar bacterial DGGE profiles may be due to the constant disruption and resedimentation of top layers of the sediment. The negative result of hybridisation analyses for the detection of β-subgroup ammonia oxidiser-like sequences suggests that these bacteria are in low numbers in comparison to other Eubacterial populations, although one cannot rule out the possibility of biases against the former group due to differences in DNA isolation and PCR efficiency (PICARD et al., 1992).

The differences in DGGE profiles between sample sites indicate differences in the total bacterial communities present. However, this appears to have no influence on the ammonia-oxidising bacteria populations present, as *Nitrosomonas* cluster 6 was the only sequence cluster of β-subgroup ammonia-oxidising bacteria to be recovered for all water and sediment sites. The tight group formed by these sequences also contains clones previously recovered from soil and is most similar to the cultured strain *Nitrosomonas ureae*. The phylogenetic distance separating the freshwater *Nitrosomonas* cluster 6 sequences is in some cases greater than those observed between the *Nitrosospira* clusters, which are known to differ in physiology and distribution (Figure 1; STEPHEN and MCCAIG, personal communication; KOWALCHUK et al., 1997). Thus, although there is no evidence supporting the further subdivision of cluster 6, it cannot be ruled out that important physiological differences might exist between the detected *Nitrosomonas* cluster 6 bacteria. The same PCR conditions were able to detect the presence of other β-subgroup ammonia oxidiser sequence clusters in rhizosphere and brackish water samples, suggesting that the detection of only *Nitrosomonas* cluster 6 sequences was not simply due to a bias in the PCR for this group. Biases in PCR and DNA isolation are difficult to dismiss, but previous experiments have shown no preference of such *Nitrosomonas* sequences over other β-subgroup ammonia oxidiser sequences during the PCR (KOWALCHUK et al., 1997), and the mechanical lysis method used is thought to be one of the most rigorous available for the disruption of even highly recalcitrant organisms (JOHNSON, 1991).

For each sample site, only a single insert for each DGGE "type" was sequenced from the clones which were positive for the CTO primer PCR screening. However this does not prove that all clones of a particular DGGE "type" are in fact identical. KOWALCHUK et al. (1997) showed that 16S rDNA inserts with different sequences can produce indistinguishable DGGE patterns. Thus, it may be that the CTO-positive clones which were not sequenced contain additional variation not represented in Figure 1. Any such variation would probably consist of only very small differences, and there is no reason to believe that it might effect the overall tree topology.

As expected, PCR amplification with the βAMO primers recovered sequences which fell outside the β-subgroup ammonia oxidiser clade, and the proportion of non-ammonia oxidiser clones varied greatly between samples. Several factors may influence the relative recovery of ammonia oxidiser-like clones with the βAMO primers, including the total number ammonia-oxidising present and the ratio of ammonia-oxidising bacteria to total bacteria, especially of closely related β-subgroup Proteobacteria. The relative influence of these factors is not known at present. The screening of clones with the CTO primer set (KOWALCHUK et al., 1997) was highly predictive in terms of identifying ammonia oxidiser-like sequences. This screening was not perfect however. Although no false positive clones were detected, the CTO screening did give a negative result with the Ws23 clone, whose sequence placed it within *Nitrosomonas* cluster 5.

This is the first report of dominant detection of a *Nitrosomonas*-like group of bacteria among the ammonia-oxidising bacterial community in freshwater habitats. SMORCZEWSKI and SCHMIDT (1991) did report the presence of *Nitrosomonas* bacteria in enrichment cultures derived from the highest positive MPN dilution's from freshwater, eutrophic lake sediment. They identified both *Nitrosospira* and *Nitrosomonas* bacteria in oxygen-rich systems, yet only the latter was observed under oxygen-poor conditions. However, it is not known how well these results mirror the *in situ* situation, as the laboratory culture conditions used are thought to select for *Nitrosomonas europaea* strains (PROSSER, 1989).

Examination of the target sites for primers and probes previously used for the detection of *Nitrosomonas*-like sequences reveals some discrepancies with the freshwater clones examined in this study (Table 3). Interestingly, HIORNS et al. (1995) reported a dominance of *Nitrosospira*-like sequences in freshwater lake samples determined both by specific probing of eubacterial PCR products with genus-specific probes and nested PCR with genus-specific primers. However, the presence of *Nitrosomonas*-like sequences similar to those detected in our freshwater and sediments samples would not have been detected by PCR with the Nm-75 / RNM-1007r primer pair due to multiple mismatches in the reverse primer (Table 3). It could not be determined if the Nm-75 primer / probe site matches that of this *Nitrosomonas* group because it falls outside the region amplified by the β-AMO primers. Since we failed to recover *Nitrosospira*-like sequences from our freshwater and sediment samples, we cannot exclude the possibility that *Nitrosospira* are also present in relatively low numbers in these samples. A small proportion of *Nitrosospira*-like sequences might go undetected in a background dominated by *Nitrosomonas*. Thus, the contrasting results between the freshwater environments analysed by HIORNS et al. (1995) and those in this investigation may either be due

to differences in the resident ammonia oxidiser communities or to the specific probes and primers used in the analyses.

Similarly, PCR detection relying on the specificity of the NitA and NitF primers (WARD et al., 1997) would not register the contribution of this potentially dominant ammonia oxidiser clade to the total ammonia oxidiser community. The fact that *in situ* hybridisation probes may miss certain target ammonia oxidisers due to mismatches in the binding site was recognised by MOBARRY et al. (1996) and HOVANEC et al. (1996). The former authors also demonstrated that the Nso190 probe, which contains two mismatches with the freshwater *Nitrosomonas* cluster 6 clones, is less inclusive to the β-subgroup ammonia-oxidising bacteria than the Nso1225 probe, which has a perfect match. In situ hybridisation analysis would provide more direct evidence as to the role of *Nitrosomonas*-like organisms in fresh water environments.

These results illustrate how the continued recovery of sequence information from environmental samples obtained using primers with broad specificity can aid in primer and probe refinement. Furthermore, the tight clustering of the freshwater pelagic and sediment sequences in this study allows for the design of oligonucleotide probes highly specific for this group, thus adding to our ability to define ammonia oxidiser populations in the environment.

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