Vol. 23: 225–236, 2001 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published February 28

Shifts in the dominant populations of ammonia-oxidizing β-subclass Proteobacteria along the eutrophic Schelde estuary

Monique J. M. de Bie[1], Arjen G. C. L. Speksnijder[2], George A. Kowalchuk[3,*], Tim Schuurman[1], Gabriël Zwart[2], John R. Stephen[4], Onno E. Diekmann[5], Hendrikus J. Laanbroek[2]

[1]Netherlands Institute of Ecology, Center for Estuarine and Coastal Ecology, Korringaweg 7, 4401 NT Yerseke, The Netherlands

[2]Netherlands Institute of Ecology, Center for Limnology, Rijksstraatweg 6, 3631 AC Nieuwersluis, The Netherlands

[3]Netherlands Institute of Ecology, Center for Terrestrial Ecology, Boterhoeksestraat 48, PO Box 40, 6666 ZG Heteren, The Netherlands

[4]Center for Environmental Biotechnology, 10515 Research Dr., Unit 300, University of Tennessee, Tennessee 37932, USA

[5]University of Groningen, Department of Marine Biology, 9750 AA Groningen, The Netherlands

ABSTRACT: The community structure of ammonia-oxidizing bacteria of the β-subclass Proteobacteria was investigated with respect to environmental gradients along the Schelde, a eutrophic estuary system. A dominance of *Nitrosomonas*-like sequences was detected using molecular techniques targeting the 16S rRNA gene on 3 separate sampling dates, and different *Nitrosomonas*-like populations were most dominant at different locations along the estuary. The most frequently detected ammonia oxidizer-like sequences in the freshwater part of the estuary were associated with a sequence cluster previously designated as *Nitrosomonas* Cluster 6a. This group, which is closely affiliated with the cultured species *N. ureae*, has previously been detected as the dominant ammonia-oxidizer group in various freshwater systems, and was also the dominant recovered sequence cluster from a contributory, untreated sewage effluent sample. The 16S rDNA recovered from brackish locations further downstream was dominated by a group of novel *Nitrosomonas*-like sequences. *Nitrosospira*-like sequences represented only a small minority of those detected for all samples. The shift in dominant ammonia-oxidizer populations occurred in the estuarine region with the sharpest observed gradients in salinity, oxygen, and ammonia. These results provide evidence in support of the differential selection of physiologically distinct *Nitrosomonas*-like groups according to the environmental gradients encountered along the estuary

KEY WORDS: Ammonia-oxidizing bacteria · Estuaries · DGGE · *Nitrosomonas*

INTRODUCTION

The discharge of large amounts of nitrogen into the environment has led to the eutrophication of many estuarine systems. In the Schelde estuary, domestic and industrial waste products (both treated and untreated) as well as runoff from fertilized agricultural lands have contributed to nitrogen enrichment. Nitrogen enters the estuary predominantly in its reduced form, ammonia (NH_3, or ammonium [NH_4^+] in its protonated form), which can be oxidized to nitrite (NO_2^-) by chemolithotrophic ammonia-oxidizing bacteria. Ammonia oxidation is the first, and often rate-limiting, step in the removal of nitrogen from environmental systems (Prosser 1989), and nitrification is quantitatively important in the estuary, in terms of both oxygen and ammonia consumption (Soetaert & Herman 1995a). Estuarine environments contain gradi-

*Corresponding author. E-mail: gkowal@cto.nioo.knaw.nl

ents of salinity, ammonia concentration, and dissolved oxygen levels. Thus, as bacteria travel with the residual seaward current, they encounter changing environmental conditions. The residence time of water in the total estuary is about 60 d (Soetaert & Herman 1995b), although this may be extended by attachment to particles or by (temporary) sedimentation (Owens 1986). The mean residence time of particles in one compartment of the estuary (see Fig. 1) is comparable with the generation time of many cultured ammonia-oxidizing bacteria (Helder & de Vries 1983). Thus, competition and selection may occur between distinct ammonia-oxidizer populations as they travel through the Schelde estuary. Alternatively, ammonia-oxidizing bacteria may possess the ability to adapt to the environmental gradients encountered. Clues as to which of these processes most affect ammonia-oxidizer populations might therefore be gained by examining their community structure along the estuarine region where these key environmental gradients are observed.

Ecological studies of ammonia-oxidizing bacteria have been hampered by the difficulties and biases associated with the isolation and manipulation of these organisms in pure culture (Koops & Harms 1985, Prosser 1989). The monophyletic nature of the β-subclass ammonia-oxidizing bacteria has however facilitated the development of nucleic acid-base techniques for their detection and characterization (McCaig et al. 1994, Voytek & Ward 1995, Mobarry et al. 1996, Wagner et al. 1996, Kowalchuk et al. 1997, Rotthauwe et al. 1997, Schramm et al. 1998, Stephen et al. 1998). Phylogenetic analysis of 16S rDNA sequence data defines 2 genera within this clade, *Nitrosospira* and *Nitrosomonas*, each of which can be further subdivided into at least 4 distinct sequence clusters (see present Fig. 3 and Stephen et al. 1996 and Maidak et al. 1999). Members of the species *Nitrosococcus oceani*, of the γ-subclass Proteobacteria, also possess the property of autotrophic ammonia oxidation. Although these bacteria have been described in marine habitats (Ward 1982), very few cultured strains have been described using molecular tools, impeding the development of similar nucleic acid-based analyses for their detection and phylogenetic characterization.

The separation of mixed polymerase chain reaction (PCR) products, generated by specific amplification of 16S rRNA genes, by denaturing gradient gel electrophoresis (DGGE) has become a powerful technique for the rapid comparison of multiple bacterial communities over space and time (Muyzer et al. 1993, Muyzer & Smalla 1998). The interpretation of DGGE banding patterns has been facilitated by hybridization using specific oligonucleotide probes for band identification (Teske et al. 1996). Alternatively, excision of DGGE bands, followed by DNA extraction, re-amplification, and sequence analysis, has also aided in the phylogenetic placement of DGGE bands (Ferris et al. 1996). These techniques have recently been applied to the analysis of β-subclass ammonia oxidizer-like 16S rDNA sequences recovered by PCR specifically targeting the *Nitrosospira/Nitrosomonas* clade (Kowalchuk et al. 1997, Stephen et al. 1998). These studies have correlated the dominance of certain phylogenetic clusters in connection with specific environmental factors, suggesting that physiological differences between clusters affect their distribution across environmental gradients (Stephen et al. 1996, 1998, Kowalchuk et al. 1998, Speksnijder et al. 1998, McCaig et al. 1999, Whitby et al. 1999). This study continues the process of relating the structure of environmental β-subclass Proteobacteria ammonia-oxidizer communities to ecological parameters.

The specific aim of this study was to relate the community structure of ammonia-oxidizing bacteria to the dynamic environmental conditions encountered along the Schelde estuary. Eight study locations were chosen to sample across the region of the estuary with the sharpest gradients with respect to salinity, ammonia availability, and dissolved oxygen content (Fig. 1). An untreated sewage sample, typical of that discharged into the estuary, was also included in the investigation. Estuarine samples were taken on 3 separate occasions to investigate seasonal and year-to-year differences, and key environmental factors were monitored for all samples. PCR, specifically targeting β-subclass ammonia oxidizer-like 16S rDNA, and DGGE were used to

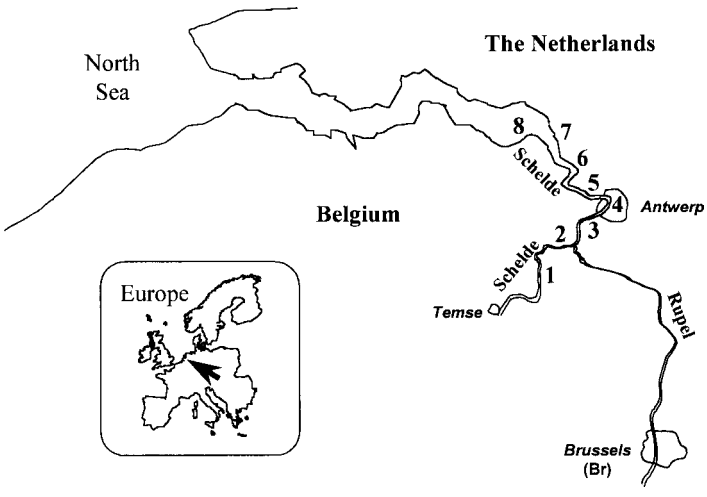

Fig. 1. Map of River Schelde and Schelde estuary. Numbers indicate sample locations and correspond to estuary compartments used previously in a modeling study of nitrogen dynamics (Soetaert & Herman 1995b). Distance from the city of Antwerp to the mouth of the estuary is approximately 100 km

analyze changes in ammonia-oxidizer community structure. Specific hybridization and sequence analysis of DGGE bands were also employed to determine the phylogenetic cluster composition of the samples examined. Ammonia-oxidizer community composition, as judged by PCR-assisted sequence retrieval, is discussed with reference to the observed environmental gradients along the dynamic estuarine system.

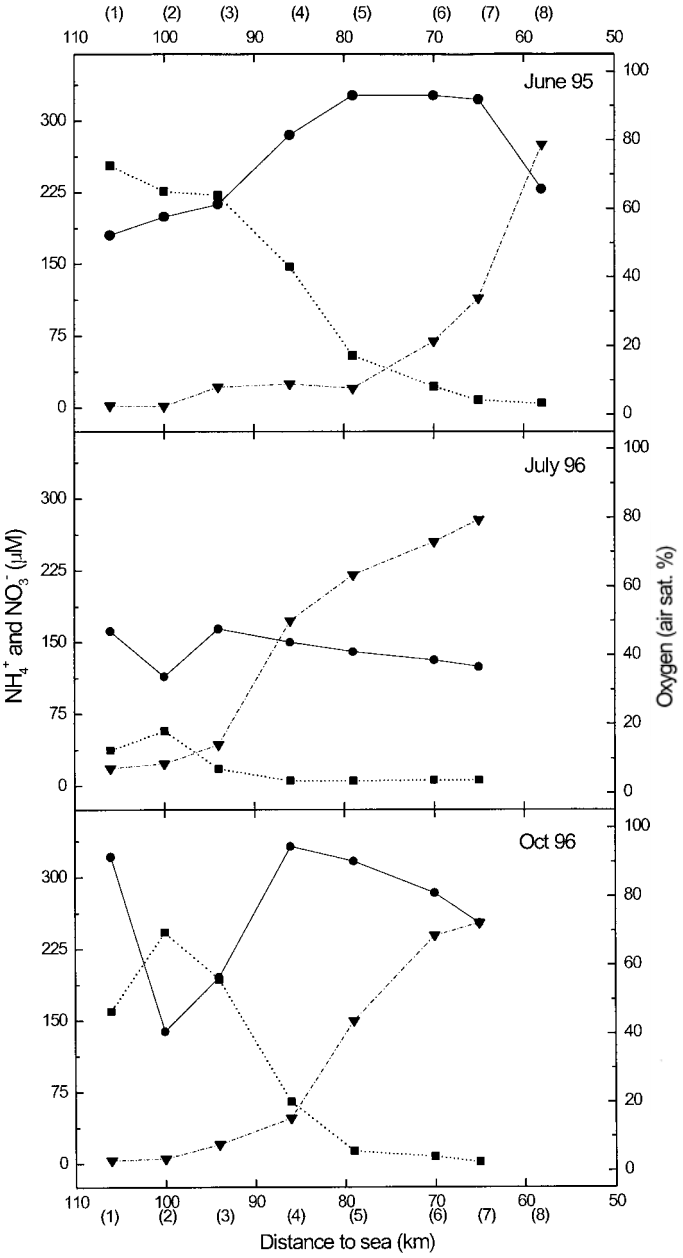

Fig. 2. Oxygen (▼), ammonia (■) and nitrate (●) concentrations as a function of distance from mouth of the estuary in June 1995 and July and October 1996. Stations numbered as in Fig. 1 (in parentheses, above top abscissa and below bottom abscissa)

MATERIALS AND METHODS

Description of research area. The Schelde estuary (also known as the 'Western' Scheldt) drains an estimated 21000 km^2 of Northern France, Belgium and The Netherlands, an area with approximately 10 million inhabitants and a nitrogen load of 56000 t N yr^{-1} (Soetaert & Herman 1995b). The estuary proper is defined to extend from Temse, the uppermost point of salt-water intrusion, to the mouth into the North Sea near Vlissingen (Fig. 1). Sampling stations were selected in the upper part of the estuary, where gradients of oxygen, ammonia and nitrate are steepest (Fig. 2, Table 1). The pH of all samples ranged between 7.5 and 7.9 (results not shown). An additional sample was taken from untreated wastewater of the city of Brussels, at its point of entry into the Rupel, a tributary of the Schelde (N. Brion pers. comm.; see also present Fig. 1). The Brussels wastewater is one of the main contributors to pollution in the upper Schelde estuary.

Sample collection. Surface water samples were taken aboard the RV 'Luctor' at the sample sites (indicated in Fig. 1) in June 1995, July 1996 and October 1996. The amount of vertical stratification in this system is minimal, and surface water samples were therefore regarded as providing a good representation of the entire water column (Wollast 1988). Samples in different years and seasons were taken to provide a gross estimate of the stability of the β-proteobacterial ammonia-oxidizing community along the estuary. Sampling was performed within the navigational channel by use of a sterile 1 l screw-cap bottle. It should be noted that the sample furthermost downstream used for the molecular analysis of the June 1995 analysis was taken at Stn 8 (Fig. 1) instead of Stn 7. Samples were filtered through 0.2 µm pore-size nitrocellulose filters (approximately 250 ml water per filter; Schleicher and Schuell BA83, diam. = 25 mm). Filters were wrapped in aluminum foil and frozen immediately (–20°C). Filters were transferred to –80°C upon arrival at the laboratory. Salinity, temperature and oxygen concentrations were measured with a CTD (conductivity, temperature, depth) system equipped with a polarographic oxygen sensor (THISHYDRO H2O, Lokeren, Belgium). Nutrient analyses were performed with a segmented flow auto-analyzer system (SKALAR, Breda, The Netherlands). The amount of suspended particulate matter (SPM) in the water samples was estimated by filtering a known volume of sample through preweighed glass-fiber (GF/F) filters. Filters with SPM were dried for 24 h at 40°C, cooled in a desiccator, and weighed again. SPM was calculated by the increase in weight of the filters.

DNA isolation. DNA isolation used a mechanical disruption protocol (Stephen et al. 1996). Half of each

Table 1. Salinity (Sal), nitrite (NO_2) and suspended particulate matter (SPM) values in water samples along the Schelde estuary. Stations numbered as in Fig. 1

Stn	Jun 1995			Jul 1996			Oct 1996		
	Sal (ppt)	NO_2 (µM)	SPM (mg l^{-1})	Sal (ppt)	NO_2 (µM)	SPM (mg l^{-1})	Sal (ppt)	NO_2 (µM)	SPM (mg l^{-1})
1	0.5	35	12	1.1	12	131	0.8	18	65
2	0.7	29	18	1.7	11	122	1	24	77
3	1.2	39	30	4.9	10	42	1.9	23	43
4	2.9	34	65	10.3	4	20	6	26	27
5	4.1	35	14	12.7	3	21	10.3	12	19
6	5.8	29	77	14.6	3	22	14	4	29
7	7.5	24	11	16	3	19	14.3	3	15
8	14.4	18	43						

nitrocellulose sample filter was added to a 2 ml screw-cap tube containing 0.5 ml TE (Tris 10 mM/EDTA 1 mM pH 7.6) buffer, 0.5 ml TE-saturated phenol, pH 8.0 (Gibco Laboratories, Detroit, MI, USA) and 0.5 g 0.1 mm-diam. acid-washed zirconium beads (Biospec Products, Bartlesville, OK, USA). The tubes were shaken at 5000 rpm for 3 times 30 s in a Mini-Beadbeater (Biospec Products) and kept on ice between shaking intervals. After centrifugation for 5 min at 5000 × *g*, 0.5 ml of the aqueous layer was removed and extracted twice with 0.5 ml phenol/chloroform/isoamylalcohol 25:24:1 (pH 8.0; Gibco). The remaining aqueous layer (0.4 ml) was recovered, and DNA was precipitated with 0.1 vol 3 M sodium acetate (pH 5.2), 2 vol. 96% ethanol, and 2 µl glycogen (Boehringer, Mannheim, Germany). Precipitation was for 16 h at –20°C. DNA was pelleted at 13 000 × *g* for 30 min and washed once with ice-cold 70% ethanol. After drying (2 min Savant Speedvac DNA 110), the pellet was resuspended in 100 µl TE buffer (pH 8.0). Further purification was performed using the Wizard DNA clean up kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA was eluted from the columns with 100 µl 80°C TE buffer, and stored at –80°C.

PCR, DGGE and hybridization analyses. PCR was conducted using the expand high-fidelity polymerase system mix (Boehringer, Mannheim, Germany) according to the manufacturer's specifications using the conditions described below. Each 50 µl reaction mixture contained 5 µl template DNA (approximately 30 ng), 5 µl 10 × reaction buffer, 200 µM of each deoxynucleotide, 5 pM each of the CTO189f-GC and CTO654r primers (Kowalchuk et al. 1997), 2 µl 10 mg ml^{-1} non-acetylated bovine serum albumin (New England Biolabs, Beverley, MA, USA), and 2.5 units expand DNA polymerase. Reaction mixtures were overlaid with an equal volume of molecular biology grade mineral oil (Sigma, St. Louis, MO, USA) and PCR was performed on a Hybaid Omnigene thermocycler (Teddington, UK) in simulated tube mode according to the following thermocycling regime: 1 × (60 s 94°C); 35 × (30 s 92°C, 60 s 57°C, and 45 s + 1 s $cycle^{-1}$ 68°C); and 1 × (5 min 68°C). PCR products were examined by 2% agarose gel electrophoresis (2% agarose; 0.5 × TBE; 1 × TBE = 90 mM Tris-borate, 2 mM EDTA, pH 8.3), with standard ethidium bromide staining to confirm product size and estimate DNA concentration.

Approximately 200 ng PCR product was loaded per sample for DGGE analysis according to the protocol described by Muyzer et al. (1993) as modified by Kowalchuk et al. (1997). DGGE gels used a gradient of 35 to 50% denaturant (100% denaturant = 7 M urea and 40% formamide) and were run at 60°C on a Protean II electrophoresis system (BioRad Laboratories; Hercules, CA, USA) for 16 h at 75 V. DNA fragments of known ammonia-oxidizer sequence cluster affinity (present Fig. 3 and Stephen et al. 1996) were run alongside environmental samples to act as controls for subsequent hybridization analysis. DNA was stained using ethidium bromide and rinsed twice for 15 min in 0.5 × TAE buffer (48.22 g Tris base, 2.05 g anhydrous sodium acetate, 1.86 g $Na_2EDTA \cdot 2\ H_2O$, pH 8 in 1 l deionized water) prior to UV transillumination. Gel images were captured digitally using The Imager System (Appligene, Illkirch, France). DNA from DGGE gels was transferred to Hybond-N^+ nucleic acid transfer membranes (Amersham International, Bucks, UK), using a BioRad semi-dry transblotter SD according to Muyzer et al. (1993). Transferred DNA was subsequently denatured (DNA-side down) and simultaneously cross-linked to the membrane by soaking in 0.4 M NaOH, 0.6 M NaCl on Whatman 3 mm (Whatman, Kent, UK) filter paper. Membranes were similarly neutralized with 1 M NaCl, 0.5 M Tris/HCl (pH 8.0). Hybridization analyses and quantification of hybridization signals were conducted according to Stephen et al. (1998), using a hierarchical set of oligonucleotide probes designed for the identification of the previously recognized sequence clusters within the β-subclass ammonia-oxidizer clade (Fig. 3). Recent evidence now allows the specific detection of sequence subgroups within *Nitrosomonas* Cluster 6, as proposed by Stephen et al. (1996). This sequence cluster has previously been detected in a variety of environments (McCaig et al. 1994, Stephen et al. 1996), and a subgroup of this cluster, currently termed Cluster 6a, has recently been postulated (Speksnijder et al. 1998). This monophyletic group of 16S rDNA sequences has been recovered from soil and freshwater sediment environments, and the oligonucleotide probe NmoCL6a_205 has been

used in conjunction with specific PCR-DGGE for their detection (Stephen et al. 1996, 1998, Speksnijder et al. 1998). The phylogenetic relationship of strains and sequences grouped into *Nitrosomonas* Cluster 6b is as yet uncertain, and this group currently represents all *Nitrosomonas* Cluster 6 sequences that do not fall within the *Nitrosomonas* Cluster 6a clade (Fig. 3). The DGGE pattern obtained for the Brussels wastewater sample was not subjected to hybridization analysis. The relative intensities of DGGE bands for this sample were estimated by quantification of ethidium bromide staining using the ImageMaster Elite software package (Version 3.01, Amersham Pharmacia Biotech, Uppsala, Sweden).

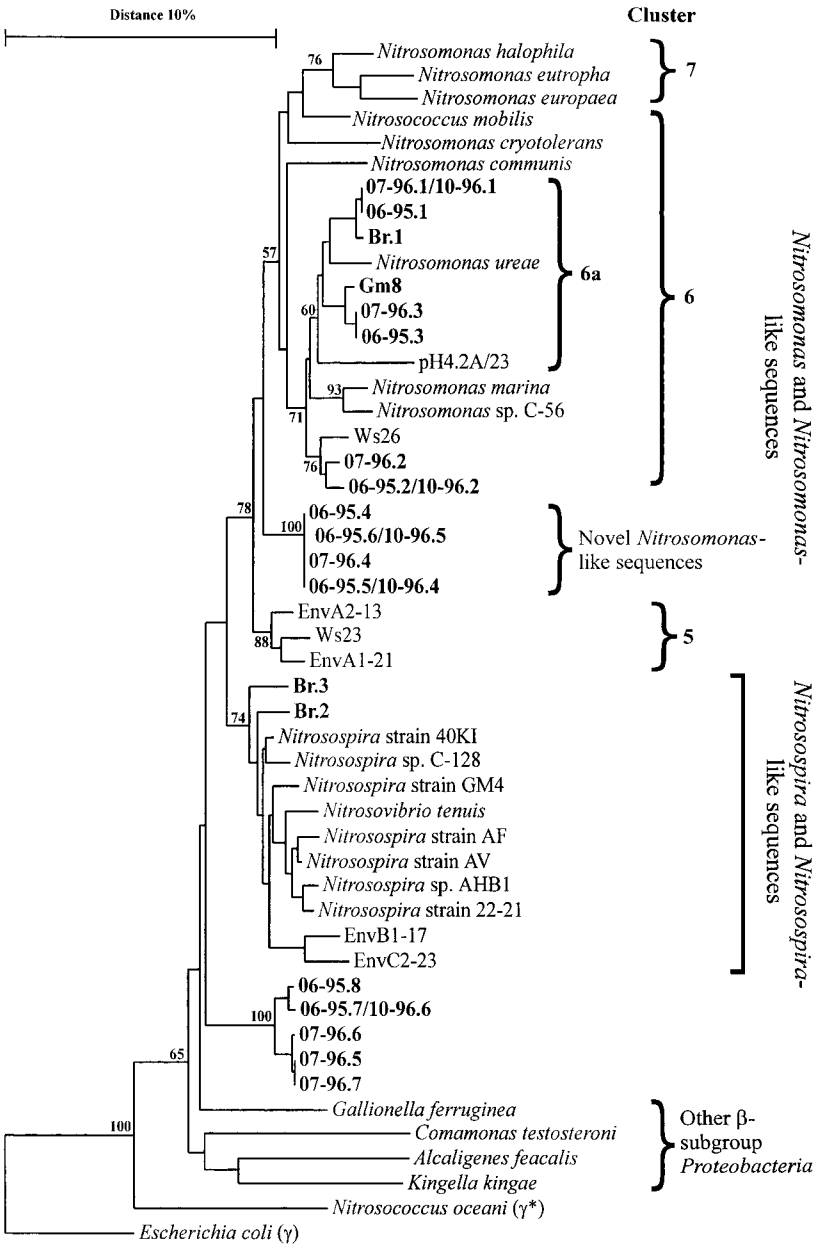

Fig. 3. Neighbor-joining tree of selected β-subclass Proteobacteria 16S rDNA sequences. Tree highlights the ammonia-oxidizer-like sequences found, and is constructed using partial 16S rDNA sequences as described in 'Materials and methods'. Sequences with number designations correspond to the denaturing gradient gel electrophoresis (DGGE) bands in Fig. 4. Band and sequence designations are: month-year.band no., e.g. 07-96.4 = fourth DGGE band position from the July 1996 samples. Br designations correspond to excised bands from the Brussels wastewater DGGE pattern in Fig. 4C. Comparative environmental clone sequences were recovered from marine sediments (prefixed with Env: Stephen et al. 1996), soil with low pH (pH4.2A/23: Stephen et al. 1996), freshwater lake sediment (Gm8: Speksnijder et al. 1998), and estuarine water (Ws26 and Ws23: Speksnijder et al. 1998)

DGGE band excision and sequence analysis. Only the central section of selected DGGE bands was excised for subsequent DNA re-amplification. Each gel fragment (approximately 2 mm^3 acrylamide) was placed in a 2.0 ml screw-cap tube, containing 300 µl TE buffer and 0.3 g 1 mm-diam. zirconium beads. Tubes were shaken for 30 s (5000 rpm in a mini-beadbeater and incubated for 3 h at 4°C. After gel fragments had been collected at the bottom of the tube by 15 s centrifugation, 5 µl of the supernatant was recovered to act as template DNA for subsequent PCR. Each 25 µl PCR mixture contained 1.25 units Taq DNA polymerase (Boehringer), 1.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 0.01% w/v gelatin, 200 µM of each deoxynucleotide, and 5 pM each of the CTO 189f-GC and CTO654r primers (Kowalchuk et al. 1997). Amplification was performed using a thermocycling regime of 28 × (60 s 95°C, 60 s 57°C, 45 s + 1 s $cycle^{-1}$ 72°C). From each PCR, 5 µl was subjected to DGGE as described above to confirm recovery of the desired band. Sequencing reactions used 5 µl of each reaction template without further purification. Sequencing was performed with the Thermosequenase kit (Amersham) according to the manufacturer's recommendations using the bacterial-specific 16S rDNA primers, 357f and 518r (Edwards et al. 1989) labeled with Texas Red. Sequencing reactions were run and analyzed on a Vistra DNA sequencer 725 (Amersham). Sequence data were edited and assembled in the Sequencer 3.0 software package (Gene

Codes Corporation, Ann Arbor, MI, USA), and format conversions were carried out in Seqapp 1.9a169 (D. G. Gilbert 1993: available by ftp.bio.indiana.edu).

Phylogenetic analysis of recovered 16S rDNA sequences was performed as described previously (Speksnijder et al. 1998). Sequence alignments included sequences from the ribosomal database project (RDP: Maidak et al. 1999) and spanned 430 nucleotide positions. Optimization of alignments was performed in the dedicated comparative sequence editor program (de Rijk & de Wachter 1993, van de Peer et al. 1997) using recognized 16S rRNA secondary structures (van de Peer et al. 1997). Sequence comparisons used 432 informative positions of 16S rDNA sequence that could be unambiguously aligned for all recovered sequences and selected databank reference sequences. Tree construction was performed with the Treecon program (van de Peer & de Wachter 1994) using neighbor-joining analysis and matrix calculation according to the method of Jukes & Cantor (1969). Gaps were not taken into account, and bootstrap analysis was based upon 100 replicates. Novel partial 16S rDNA sequences determined in this study have been deposited into the EMBL sequence databank under Accession Numbers AJ132047–AJ132062.

RESULTS

Characterization of environmental conditions along the estuary

The upper reaches of the estuary typically contained low dissolved oxygen levels, low salinity, and a high ammonia to nitrate ratio (Fig. 2, Table 1). The situation was reversed in the lower, more saline parts of the estuary. Variation between sampling dates with respect to salinity and other environmental gradients was mostly influenced by tides, as previously found in the Schelde (Soetaert & Herman 1995a,b). The brackish zone (near the city of Antwerp) contained sharp gradients in all these variables (Fig. 2, Table 1). It is in this portion of the estuary that maximum nitrification has been observed (Somville 1984, de Wilde & de Bie 2000). The locations of the steepest gradients in nutrients and oxygen concentration were between Stns 4–8, 3–5, and 3–6 for the June 1995, July 1996, and October 1996 sampling dates, respectively.

Recovery of 16S rDNA and DGGE analysis

All DNA extractions from filtered samples were performed at the same time. Thus, DNA extractions were performed using samples that had been stored at –80°C for different periods of time. Although it was observed that older filters yielded slightly less DNA than fresher filters, it is not known whether the 1995 samples contained less DNA, or if prolonged storage affected the efficiency of DNA recovery (results not shown). Specific PCR amplification of 16S rDNA using the CTO189f-GC and CTO654r primers yielded positive results in all cases.

DGGE analysis of recovered PCR products revealed 3 to 10 bands per sample (Fig. 4). For upstream sample sites, the most dominant bands occurred relatively high in the gel, showing migration within the range of controls for *Nitrosomonas* Clusters 6a, 6b, and 7 (*N. europaea* and *N. eutropha* belonging to the last). At more seaward sites, bands lower in the gel, comparable in migration to *Nitrosomonas* Cluster 5 and several *Nitrosospira* sequence cluster controls, became most dominant. Although all sampling dates showed this trend, the exact position and extent of the shift varied in a fashion similar to the nutrient profiles described above. The Brussels wastewater sample produced a very strong band high in the gel, suggestive of *Nitrosomonas* Clusters 6a, 6b or 7, as well as 3 additional bands lower in the gel, migrating within the range of several *Nitrosospira* controls (Fig. 4C).

Hybridization and sequence analysis of DGGE banding patterns

Previous DGGE studies have shown that migration behavior alone is not a good predictor of phylogenetic affinity with respect to ammonia-oxidizer 16S rDNA fragments (Kowalchuk et al. 1997, 1998). We therefore characterized DGGE bands by hybridizing DGGE banding patterns with a battery of 16S rDNA-targeted oligonucleotide probes, specific at different taxonomic levels within the β-subclass ammonia-oxidizing bacteria (Stephen et al. 1998). However, an all β-subclass ammonia oxidizer-specific probe, β-AO233 (Stephen et al. 1998), failed to hybridize with some DGGE bands, suggesting that these might not contain sequences falling within the *Nitrosospira/Nitrosomonas* radiation. Excision, re-amplification, sequence determination, and phylogenetic analysis of such bands (06-95.7–9', 07-96.5–7, and 10-96.6: Figs. 3 & 4) placed their sequences outside the *Nitrosospira/Nitrosomonas* clade. These bands were excluded from further analyses. The recovery of some non-ammonia-oxidizer sequences with these primers has been noted previously (Kowalchuk et al. 1998, 2000), and their presence did not interfere with the further analysis of sequences within the β-subclass ammonia-oxidizer radiation.

The diversity within 16S rDNA sequences belonging to the genus *Nitrosomonas* is greater than that found

for the *Nitrosospira* genus (Pommerening-Röser et al. 1996). Given the current available sequence information, it is not yet possible to assess the specificity and accuracy of probes designed for the detection of phylogenetic lineages within the genus *Nitrosomonas* (Stephen et al. 1998, Utaaker & Nes 1998). We therefore excised all the bands from environmental DGGE patterns for sequence analysis to confirm hybridization results (see below) and allow for the phylogenetic placement of recovered *Nitrosomonas*-like sequences. Bands with the same DGGE mobility across sample locations produced identical sequences, and were included only once in the phylogenetic analysis (Fig. 3). Quantification of hybridization signals and sequence analysis of excised bands revealed a shift in the predominant β-subclass ammonia-oxidizer-like sequences detected along the estuary transect (Fig. 5). Upstream regions of the estuary displayed a dominance of 16S rDNA sequences classified as *Nitrosomonas* Cluster 6a (Fig. 5). In contrast, a novel *Nitrosomonas*-like sequence group was observed in lower reaches of the estuary. This shift was observed for all 3 sampling dates, although not always to the same degree or at the exact same position along the estuary. Moving seaward, the point in the estuary where the novel *Nitrosomonas*-like sequences first became abundantly detected was between Stns 6 and 8 for the June 1995 sample, at Stn 4 for the July 1996 sample, and at Stn 5 for the October 1996 sample. The position of the sharpest ammonia-oxidizer community shift for all 3 dates corresponded to the point in the estuary where salinity values were approximately 10 ppt, oxygen saturation levels were around 40%, and ammonia concentration dropped below 15 µM (Fig. 2, Table 1).

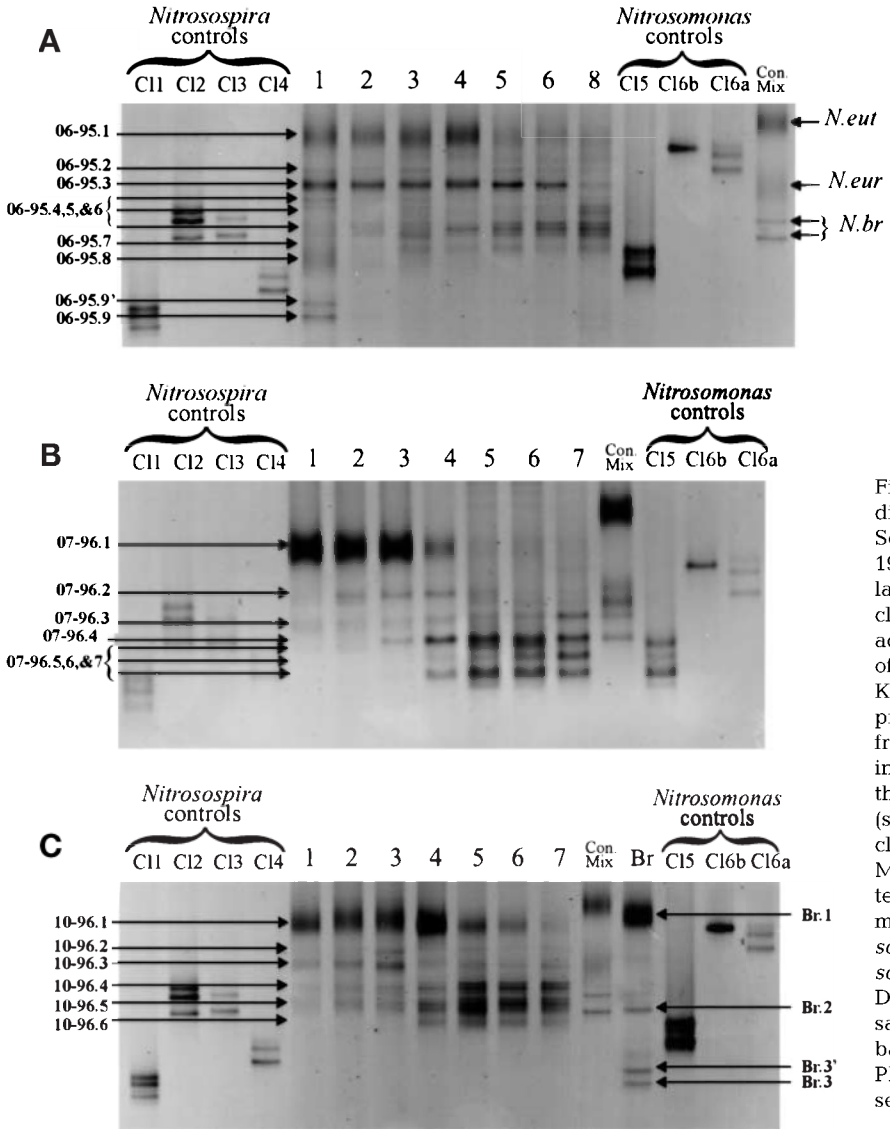

Fig. 4. PCR-DGGE analysis of ammonia-oxidizer-like 16S rDNA fragments along the Schelde estuary for (A) June 1995, (B) July 1996 and (C) October 1996; Numbers above lanes (1–8): stations numbered in Fig. 1; C: cloned sequence cluster-controls, numbered according to sequence cluster designations of Stephen et al. (1996), as described in Kowalchuk et al. (1997); band labels with a prime: bands contain a 1 base pair difference from their namesake sequences that was introduced due to an ambiguous position in the CTO654r primer (Kowalchuk et al. 1997) (sequences from such bands were not included in the phylogenetic analysis); Con. Mix: lanes from control PCRs, for which the template consisted of a mixture of chromosomal DNA from 3 pure culture strains (*Nitrosomonas eutropha*, *N. europaea* and *Nitrosospira briensis*); Br: lane in (C) contains DNA recovered from Brussels wastewater sample; arrows indicate position of the bands excised for sequence determination. Phylogenetic placement of DGGE band sequences and description of band labeling is given in Fig. 3

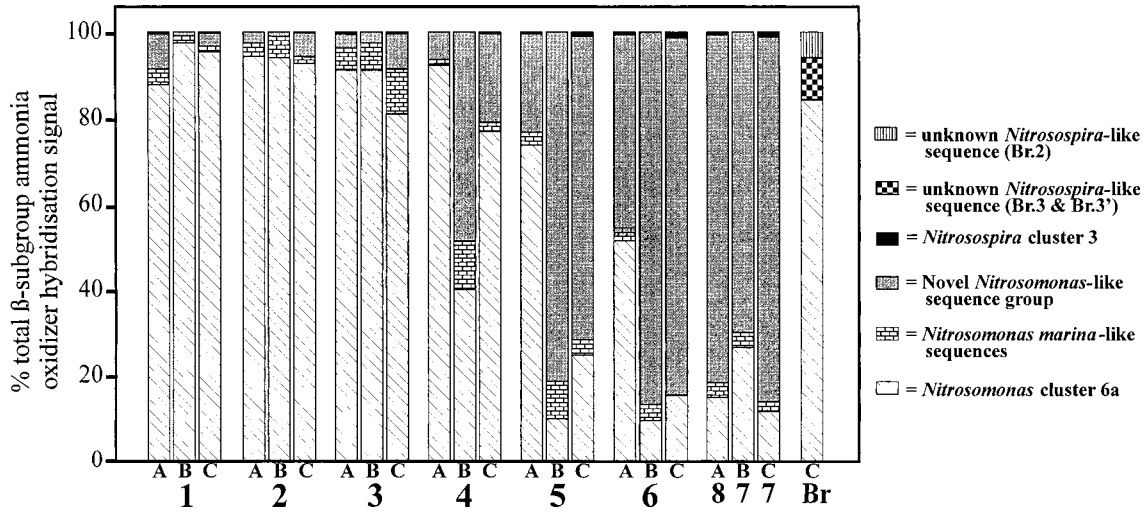

Fig. 5. Distribution of detected β-subclass ammonia-oxidizer-like sequences across Schelde estuary. DGGE gels shown in Fig. 4 were hybridized with an hierarchical set of oligonucleotide probes for identification and quantification of DGGE bands (Stephen et al. 1998). 1–8: sampling stations; A, B, C: sampling dates June 1995, July 1996, and October 1996, respectively. Relative amount of each specific sequence cluster, as detected by specific hybridization, is given with respect to the hybridization signal with probe β-AO233, which targets all ammonia-oxidizers (Stephen et al. 1998). The cumulative signal from sequence cluster-specific probes could explain 95 to 103% of the all β-subclass ammonia-oxidizer hybridization signal. Results were standardized to 100% for graphical presentation. Values for the Brussels wastewater sample were determined by the quantification of ethidium bromide fluorescence

Nitrosomonas-like sequences, which were most closely related to the culture strain *N. marina* Nm22 (06-95.2, 07-96.2, and 10-96.2), were also detected along the entire estuary transect sampled. Hybridization signals for these bands represented 0 to 10% of the total β-subclass ammonia-oxidizer-specific signal, and no trends with respect to location or sampling date were apparent. No clear *Nitrosospira*-like DGGE bands were related in the estuarine samples by ethidium bromide staining, due to the weak nature of the signal and the overlap with other, more dominant, DGGE bands. *Nitrosospira*-specific hybridization signals were characterized as *Nitrosospira* Cluster 3. These signals constituted less than 2% of the total recovered PCR product in all estuarine samples. Sequence results from excised bands agreed with the phylogenetic predictions based upon hybridization analysis in all cases examined.

DGGE bands from the Brussels sample were also sequenced. The sequence of the uppermost band (Br.1) placed it within *Nitrosomonas* Cluster 6a (Fig. 3). The remaining 3 bands (Br.2, Br.3, and Br.3') produced *Nitrosospira*-like sequences. The Br.2 sequence contained the probe site characteristic of members of *Nitrosospira* Cluster 4 (Stephen et al. 1998). However, the existence of several ambiguities in the nucleotide sequence did not allow accurate phylogenetic analysis to the sequence cluster level. The lowermost bands (Br.3 and Br.3') also contained several ambiguous nucleotide positions, which corresponded to highly variable regions of the 16S rRNA gene; here again, sequence cluster level characterization was not possible. These bands may have consisted of multiple *Nitrosospira*-like sequences.

DISCUSSION

A shift was observed in the β-subclass ammonia-oxidizer populations detected along the estuary within the region where gradients with respect to salinity, dissolved O_2, and ammonia were sharpest, and where ammonia oxidation was highest (de Wilde & de Bie 2000). The physiological significance of detecting different phylogenetic groupings of ammonia-oxidizer-like 16S rDNA sequences is not yet known. However, the fact that different ammonia-oxidizer groups were detected under the different environmental conditions along the estuary, and that these changes were stable with regard to the steepest environmental gradients over sampling dates, implies that these changing conditions may affect ammonia-oxidizer growth, activity and/or survival in the estuary. Bacteria showing affinity with *Nitrosomonas* Cluster 6a were the most dominantly detected β-subclass ammonia oxidizers in the upper reaches of the estuary, where freshwater, low oxygen and high ammonia conditions prevail. Sequences showing affinity with this sequence cluster

have previously been detected in soil, freshwater, and freshwater sediment (Stephen et al. 1996, 1998, Speksnijder et al. 1998). These authors suggested that the distribution of this specific *Nitrosomonas* lineage may be restricted to non-marine environments, and the decrease in *Nitrosomonas* Cluster 6a detection in the lower estuary samples would support this hypothesis. Speksnijder et al. also detected this group of sequences in nearly anoxic sediment layers, suggesting a level of tolerance to low oxygen conditions. Although it has been demonstrated that ammonia-oxidizing bacteria can adapt to low-oxygen environments, this ability may not be equally present in all phylogenetic groups (Smorczewski & Schmidt 1991, Bodelier et al. 1996). Given the methods used, the activity of the detected bacteria could not be assessed, and some may be present in inactive forms, potentially more resistant to adverse environmental conditions. The dominance of *Nitrosomonas* Cluster 6a in upstream estuary samples may also be affected by input of ammonia-oxidizing bacteria from untreated wastewater into the estuary, although it should be stressed that the Brussels wastewater represents but one of many potential sources of nitrifying bacteria introduced into the estuary. Such wastewater samples have previously been shown to contain high ammonia-oxidizer biomass (Brion 1997), and a representative wastewater sample was also dominated by *Nitrosomonas* Cluster 6a. It may be that such organisms survive well after being released into the upper estuary, despite the long stretch of the estuary before entering the region studied. Their decline lower in the estuary might be explained by either a lack of tolerance to changing environmental conditions or a decreased ability to compete with other organisms for substrate. Previous studies of activated sludge samples have also detected high numbers of *Nitrosomonas*-like bacteria, although these populations belonged to the *Nitrosomonas* Cluster 7 lineage (Mobarry et al. 1996, Wagner et al. 1996).

Nitrosomonas Cluster 6a is displaced by a novel *Nitrosomonas*-like group further down the estuary system. This shift coincides with sharp increases in salinity and O_2 concentration, as well as a sharp decrease in ammonia concentration in the estuary. The exact curve of the nutrient and salinity values is the result of several biological and physical processes (Soetaert & Herman 1995b). While nitrogen salts in the upper estuary are mainly controlled by microbiological activity, the location of maximum activity, both with regard to station position and salinity, is influenced by the tidal regime, freshwater discharge, and wind. As shown in Table 1, suspended matter values vary along the estuary, and the distribution of the novel *Nitrosomonas*-like group coincides with lower SPM values in the downstream reaches of the estuary. Thus, the distribution of different ammonia-oxidizer populations throughout the estuary may be influenced by differential strategies of either free-living or particle-bound lifestyles. Separate analyses of particulate and pelagic samples would address this issue (Phillips et al. 1999). Strains affiliated with the novel *Nitrosomonas* lineage may be well adapted to the environmental conditions present in the lower regions of the estuary. However, the relative shift between phylogenetic groupings is not strong evidence of growth of specific nitrifier types, as the decrease in the overall number of nitrifiers at the most downstream locations may lead to the dilution of certain populations (Billen 1975). The increase in the relative proportion of these *Nitrosomonas*-like sequences lower in the estuary could also be due to ammonia-oxidizer inoculation or nutrient addition via land run-off or other point or non-point sources along its way to the sea. Although one cannot exclude this possibility, 2 lines of evidence speak against this explanation. Firstly, previous studies on nutrient concentration along the region of the estuary under study have never revealed nutrient point sources that measurably increased local nutrient concentrations (Soetaert & Herman 1995a,b). Also, the city of Antwerp, situated at the point where ammonia-oxidizer population shifts were observed, utilizes advanced waste-treatment systems, which release only negligible bacterial biomass and nutrients into the estuary. In contrast, waste from the urban area of Brussels is untreated, providing a far greater potential source of both nitrifying organisms and nutrients (Brion & Billen 1997). Secondly, terrestrial environments, similar to the agricultural soil systems adjacent to the Schelde estuary, have previously been shown to contain ammonia-oxidizer communities dominated by *Nitrosospira*-like bacteria (Stephen et al. 1996, 1998, Hastings et al. 1998).

The physiological differences between the 2 main *Nitrosomonas* lineages detected across the estuarine transect studied are not yet known, and no pure cultures are available yet to examine their differential responses to the environmental gradients encountered in the Schelde estuary. However, knowledge of the distribution of these populations should help in the development of the necessary enrichment and isolation strategies.

Minority populations of *Nitrosospira* Cluster 3 and a *Nitrosomonas marina*-like bacterium were also identified across the estuarine system. The former group has previously been detected in terrestrial and freshwater environments (Hiorns et al. 1995, Stephen et al. 1996, 1998, Hastings et al. 1998), but only represents a small fraction of the total recovered β-subclass ammonia-oxidizer sequences in this estuary environment. As the name suggests, *N. marina* Nm22 and closely related strains have previously been detected in marine envi-

ronments and are obligatorily halotrophic (McCaig et al. 1994, 1999, Pommerening-Röser et al. 1996). The detection of the sequences 06-95.2, 07-96.2, and 10-96.2 in the freshwater reaches of the estuary suggests that strains closely related to *N. marina* might not be strictly limited to saline environments.

Despite the importance of nitrification in estuarine habitats (Owens 1986, Soetaert & Herman 1995b), few studies to date have addressed ammonia-oxidizer community structure in this habitat (Murray et al. 1996). A previous study, based on the cloning of ammonia-oxidizer-like 16S rDNA sequences after semi-specific PCR, also detected *Nitrosomonas*-like sequences at Sampling Stn 4 within the Schelde estuary (Speksnijder et al. 1998). Among the limited number of ammonia-oxidizer-like clones examined, only sequences showing affinity with the genus *Nitrosomonas* were recovered, including sequences from the *Nitrosomonas* Cluster 6a and *N. marina* lineages detected in the present study (Fig. 3). Nitrifying bacteria have also been studied in the Elbe estuary, where several *Nitrosomonas*-like lineages have been isolated (Stehr et al. 1995) or detected by fluorescent *in situ* hybridization (Wagner et al. 1996). Although *Nitrosospira*-like bacteria were observed, most detected strains were related to either *Nitrosomonas ureae* or *N. europaea*.

As is the case with most molecular studies of ammonia oxidizers, the present study has focussed upon bacteria of the β-subclass Proteobacteria. However, ammonia-oxidizing bacteria of the genus *Nitrosococcus*, within the γ-subclass Proteobacteria, may also be present in the estuarine system examined. This possibility is especially relevant to the lower, more saline reaches of the estuary, given the currently recognized distribution of this genus (Ward & Carlucci 1985). The development of similar methods to those used here for the direct detection of γ-subclass ammonia-oxidizing bacteria may prove essential in improving our understanding of their role in the environment. It should also be stressed that the present study targeted 16S rDNA, and therefore could not discriminate between active and dormant cells. Studies designed to detect active cells, for instance by targeting 16S rRNA (Felske et al. 1996) or ammonia-oxidizer-specific mRNAs by reverse transcriptase PCR, should be helpful in this respect.

The efficiency of DNA extraction may affect the accuracy of community fingerprinting techniques such as PCR-DGGE. Ammonia-oxidizing bacteria tend to form tight clusters or attach to particulate matter, which can prevent their lysis during DNA isolation procedures (Schramm et al. 1998). For this reason, we used a highly rigorous method for DNA isolation, the addition to which of freeze-thaw steps or additional bead-beating did not lead to the liberation of more DNA or additional DGGE bands (results not shown). In addition to environmental gradients, the distribution of other microbial community members may influence ammonia-oxidizer populations. Although a previous study of ammonia-oxidizing bacteria in freshwater sediment environments demonstrated that ammonia-oxidizer and eubacterial communities can vary independently (Speksnijder et al. 1998), this possible influence certainly cannot be discounted.

Several environmental parameters vary across the estuarine transect studied. Given the bacterial residence time in the estuary, both adaptation and selection are possible within the ammonia-oxidizer communities present. Although some ammonia-oxidizing bacteria are known to be able to adapt to low oxygen environments (Bodelier et al. 1996, Kowalchuk et al. 1998, Speksnijder et al. 1998), exposure to low oxygen conditions can also affect ammonia-oxidizer diversity and community structure (Smorczewski & Schmidt 1991). Similarly, adaptation to increasing salt concentrations is possible for some ammonia oxidizers (Helder & de Vries 1983, Somville 1984). However, certain strains appear to be particularly well suited to high salt conditions (Pommerening-Röser et al. 1996), and different ammonia-oxidizer populations have been detected in the comparison of freshwater and saltwater aquaria (Hovanec & DeLong 1996). Ammonia-oxidizing bacteria also undergo physiological adaptations in response to low ammonia availability (Laanbroek & Woldendorp 1994). Despite these adaptive capabilities, phylogenetic differences within the genus *Nitrosomonas* are known to be reflected in their ammonia sensitivities and half-saturation constant (K_s) values of ammonia oxidation (Suwa et al. 1997). In addition to the environmental gradients discussed above, other less readily apparent environmental factors may also influence the distribution of ammonia-oxidizer populations in this estuarine system. Thus, although the current study suggests that the environmental gradients encountered in the estuarine environment affect ammonia-oxidizer community structure, it is not yet possible to determine the influence of individual environmental factors. However, knowledge of the distribution and diversity of β-subclass ammonia-oxidizer populations along the Schelde estuary now allows for the design of experiments to test which environmental factors most influence their distribution, and provides clues into strategies for their enrichment and isolation.

Acknowledgements. The authors thank Nico Goosen for early ideas on the project and Natacha Brion (Free University of Brussels) for taking the Brussels wastewater sample. The crew of the RV 'Luctor' is acknowledged for helping with the sampling and Miranda van Agterveld for help with the sequence analysis. G.A.K. was funded by an NWO grant to the Netherlands Graduate School of Fundamental Ecology. This represents publication no. 2529 of the Netherlands Institute of Ecology.

LITERATURE CITED

Billen G (1975) Nitrification in the Scheldt estuary (Belgium and the Netherlands). Estuar Coast Mar Sci 3:79–89

Bodelier PLE, Libochant JA, Blom CWPM, Laanbroek HJ (1996) Dynamics of nitrification and denitrification in root-oxygenated sediments and adaptation of ammonia-oxidizing bacteria to low-oxygen or anoxic habitats. Appl Environ Microbiol 62:4100–4107

Brion N, Billen G (1997) Wastewater as a source of nitrifying bacteria in river systems: the case of the River Seine downstream from Paris. In: Brion N (ed) Etude de processus de nitrification á l'échelle de grands réseaux hydrographiques antrhopisés. PhD thesis, Université Libre de Bruxelles, p 26–37

de Rijk P, de Wachter R (1993) DCSE v2.54, an interactive tool for sequence alignment and secondary structure research. Comput Appl Biosci 9:735–740

de Wilde HPJ, de Bie MJM (2000) Nitrous oxide in the Schelde estuary: production by nitrification and emission to the atmosphere. Mar Chem 69:203–216

Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of gene coding for 16S ribosomal RNA. Nucleic Acids Res 17:7843–7853

Felske A, Engelen B, Nübel U, Backhaus H (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. Appl Environ Microbiol 62:4162–4167

Ferris MJG, Muyzer G, Ward DM (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62:340–346

Hastings RC, Saunders JR, Hall GH, Pickup RW, McCarthy AJ (1998) Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl Environ Microbiol 64:3674–3682

Helder W, de Vries RTP (1983) Estuarine nitrite maxima amd nitrifying bacteria (Ems-Dollard estuary). Neth J Sea Res 17(1):1–18

Hiorns WD, Hastings RC, Head IM, McCarthy AJ, Saunders JR, Pickup RW, Hall GH (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology 141:2793–2800

Hovanec TA, DeLong EF (1996) Comparitive analysis of nitrifying bacteria associated with freshwater and marine aquaria. Appl Environ Microbiol 62:2888–2896

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic Press, New York, p 21–132

Koops HP, Harms H (1985) Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidising bacteria. Arch Microbiol 141:214–218

Kowalchuk GA, Stephen JR, de Boer W, Prosser JI, Embley TM, Woldendorp JW (1997) Analysis of ammonia-oxidizing bacteria of the β-subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbiol 63: 1489–1497

Kowalchuk GA, Bodelier PLE, Heilig GHJ, Stephen JR , Laanbroek HJ (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen-availability in soils and root-oxygenated sediments, usin PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol Ecol 27: 339–350

Kowalchuk GA, Stienstra A, Stephen JR, Speksnijder AGCL, Heilig GHJ, Woldendorp JW (2000) Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcereous grasslands. Environ Microbiol 2:99–110

Laanbroek HJ, Woldendorp JW (1994) Activity of chemolithotrophic nitrifying bacteria under stress in natural soils. In: Gwynfryn Jones J (ed) Advances in microbial ecology. Plenum Press, New York, p 275–304

Maidak BL, Cole JR, Parker CTJ, Garrity GM, Larsen N, Lilburn TG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM , Woese CR (1999) A new version of the RDP (ribosomal database project). Nucleic Acids Res 27:171–173

McCaig AE, Embley TM , Prosser JI (1994) Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol Lett 120:363–368

McCaig AE, Phillips CJ, Stephen JR, Kowalchul GA, Harvey M, Herbert M, Embley TM, Prosser JI (1999) Nitrogen cycling and community structure of β-subgroup ammonia-oxidising bacteria within polluted, marine fish-farm sediments. Appl Environ Microbiol 65:213–220

Mobarry BK, Wagner M, Urbain V, Rittmann BE , Stahl DA (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl Environ Microbiol 62:2156–2162

Murray AE, Hollibaugh JT, Orrego C (1996) Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturating gel electrophoresis of 16S rDNA fragments. Appl Environ Microbiol 62:2676–2680

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

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

Owens NJP (1986) Estuarine nitrification: a naturally occurring fluidized bed reaction? Estuar Coast Shelf Sci 22:31–44

Phillips CJ, Smith Z, Embley TM, Prosser JI (1999) Phylogenetic differences between particle-associated and planktonic ammonia-oxidizing bacteria of the β-subdivision of the class Proteobacteria in the Northwestern Mediterranean Sea. Appl Environ Microbiol 65:779–786

Pommerening-Röser A, Rath G, Koops HP (1996) Phylogenetic diversity within the genus *Nitrosomonas*. Syst Appl Microbiol 19:344–351

Prosser JI (1989) Autotrophic nitrification in bacteria. Adv Microbiol Physiol 30:125–181

Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine scale analysis of natural ammonia oxidizing populations. Appl Environ Microbiol 63:4704–4712

Schramm A, de Beer D, Wagner M, Amann R (1998) Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl Environ Microbiol 64:3480–3485

Smorczewski WT, Schmidt EL (1991) Numbers, activities, and diversity of autotrophic ammonia-oxidizing bacteria in a freshwater, euthrophic lake sediment. Can J Microbiol 37: 828–833

Soetaert K, Herman PMJ (1995a) Estimating estuarine residence times in the Westerschelde (The Netherlands) using a box model with fixed dispersion coefficients. Hydrobiologia 311:215–224

Soetaert K, Herman PMJ (1995b) Nitrogen dynamics in the

Westerschelde estuary (SW Netherlands) estimated by means of the ecosystem model MOSES. Hydrobiologia 311:225–246

Somville M (1984) Use of nitrifying activity measurements for describing the effect of salinity on nitrification in the Scheldt estuary. Appl Environ Microbiol 47:424–426

Speksnijder AGCL, Kowalchuk GA, Roest K, Laanbroek HJ (1998) Recovery of a *Nitrosomonas*-like 16S rDNA sequence group from freshwater habitats. Syst Appl Microbiol 21:321–330

Stehr G, Böttcher B, Dittberner P, Rath G, Koops HP (1995) The ammonia-oxidizing nitrifying population of the River Elbe estuary. FEMS Microbiol Ecol 17:177–186

Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley TM (1996) Molecular diversity of soil and marine 16s rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. Appl Environ Microbiol 62:4147–4154

Stephen JR, Kowalchuk GA, Bruns MA, McCaig AE, Phillips CJ, Embley TM, Prosser JI (1998) Analysis of β-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. Appl Environ Microbiol 64: 2958–2965

Suwa Y, Sumino T, Noto K (1997) Phylogenetic relationships of activated sludge isolates of ammonia oxidizers with different sensitivities to ammonium sulfate. J Gen Appl Microbiol 43:373–379

Teske A, Wawer C, Muyzer G, Ramsing NB (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariagerfjord, Denmark) as evaluated by most-probable number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl Environ Microbiol 62:1405–1415

Utaaker JB, Nes IF (1998) A qualitative evaluation of the published oligonucleotides specific for the 16S rRNA gene sequences of the ammonia-oxidizing bacteria. Syst Appl Microbiol 21:72–88

van de Peer Y, de Wachter R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 10:569–570

van de Peer Y, Jansen J, de Rijk P, de Wachter R (1997) Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res 24:111–116

Voytek MA, Ward BB (1995) Detection of ammonium oxidising bacteria in the beta-subclass of the class Proteobacteria in aquatic samples with the PCR. Appl Environ Microbiol 61:1444–1450

Wagner M, Rath G, Koops HP, Flood J, Amann R (1996) *In situ* analysis of nitrifying bacteria in sewage treatment plants. Water Sci Technol 34:237–244

Ward BB (1982) Oceanic distribution of ammonia oxidizing bacteria determined by immunofluorescence. J Mar Res 40:1155–1172

Ward BB, Carlucci AF (1985) Marine ammonia- and nitrite-oxidizon bacteria: sereological diversity determined by immunofluorescence in culture and in the environment. Appl Environ Microbiol 50:194–201

Whitby CB, Saunders, JR, Rodriguez J, Pickup RW, McCarthy, A (1999) Phylogenetic differentiation of two closely related *Nitrosomonas* spp. that inhabit different sediment environments in an oligotrophic freshwater lake. Appl Environ Microbiol 65:4855–4862

Wollast R (1988) The Scheldt estuary. In: Salomons W, Bayne BL, Duursma EK, Förstner U (eds) Pollution of the North Sea, an assessment. Springer-Verlag, Berlin, p 183–193

Editorial responsibility: Bess Ward, Princeton, New Jersey, USA

Submitted: July 8, 2000; Accepted: December 4, 2000
Proofs received from author(s): February 7, 2001