Changes in the community structure and activity of betaproteobacterial ammonia-oxidizing sediment bacteria along a freshwater–marine gradient

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Summary

To determine whether the distribution of estuarine ammonia-oxidizing bacteria (AOB) was influenced by salinity, the community structure of betaproteobacterial ammonia oxidizers (AOB) was characterized along a salinity gradient in sediments of the Ythan estuary, on the east coast of Scotland, UK, by denaturant gradient gel electrophoresis (DGGE), cloning and sequencing of 16S rRNA gene fragments. Ammonia-oxidizing bacteria communities at sampling sites with strongest marine influence were dominated by *Nitrosospira* cluster 1-like sequences and those with strongest freshwater influence were dominated by *Nitrosomonas oligotroph* like sequences. *Nitrosomonas* sp. Nm143 was the prevailing sequence type in communities at intermediate brackish sites. Diversity indices of AOB communities were similar at marine- and freshwater-influenced sites and did not indicate lower species diversity at intermediate brackish sites. The presence of sequences highly similar to the halophilic *Nitrosomonas marina* and the freshwater strain *Nitrosomonas oligotroph* at identical sampling sites indicates that AOB communities in the estuary are adapted to a range of salinities, while individual strains may be active at different salinities. Ammonia-oxidizing bacteria communities that were dominated by *Nitrosospira* cluster 1 sequence types, for which no cultured representative exists, were subjected to stable isotope probing (SIP) with $^{13}$C-HCO$_3^-$, to label the nucleic acids of active autotrophic nitrifiers. Analysis of $^{13}$C-associated 16S rRNA gene fragments, following CsCl density centrifugation, by cloning and DGGE indicated sequences highly similar to the AOB *Nitrosomonas* sp. Nm143 and *Nitrososomas cryotolerans* and to the nitrite oxidizer *Nitrosospira marina*. No sequence with similarity to the *Nitrosospira* cluster 1 clade was recovered during SIP analysis. The potential role of *Nitrosospira* cluster 1 in autotrophic ammonia oxidation therefore remains uncertain.

Introduction

The intertidal areas in coastal zones include some of the most productive marine ecosystems and are thus vital as breeding and feeding grounds for many species of birds, fish and crustaceans. These areas are under threat from anthropogenic activities, through land reclamation, fisheries and in particular through eutrophication caused by nutrient input through terrestrial runoff of nitrogen. Estuarine intertidal systems are especially vulnerable because the river catchments in areas of intensive farming can result in the transport of highly enriched nutrients (Raffaelli, 1999). The low species diversity of brackish-water estuarine ecosystems (Remane and Schlieper, 1971; Attriill, 2002) may additionally enhance effects of elevated nutrient levels and promote growth of antagonistic phytoplankton or macroalgal species, resulting in low-diversity, high-density ecosystems. Nitrification, the conversion of ammonia to nitrate via nitrite, is central to the global cycling of nitrogen and, when linked to anoxic environments, where NO$_2^-$ can serve as alternative electron acceptor for denitrification, is a major regulating factor alleviating eutrophication processes (Kemp et al., 1990; Rysgaard et al., 1994). However, nitrification processes depend on the availability of free dissolved oxygen and may be totally eliminated if hypoxia occurs through eutrophication (Kemp et al., 1990).

The first, usually rate-limiting oxidation step in nitrification, is carried out by ammonia-oxidizing bacteria (AOB), which can also reduce nitrite to nitric oxide (NO) and nitrous oxide (N$_2$O) at low oxygen tension. Ammonia-oxidizing bacteria consist of three evolutionary distant groups, two of which belong to the class *Proteobacteria* (Head et al., 1993; Koops et al., 2003). One group forms a deep branch within the Gammaproteobacteria and comprises only three recognized, closely related marine *Nitrosococcus* species. The second group, which includes...
the majority of cultured strains, forms a monophyletic group within the Betaproteobacteria and consists of two genera, *Nitrosomonas* and *Nitrosospira*. The third group, belonging to the order Planctomycetales, is associated with the Anammox process and its importance in marine systems has been suggested, particularly for oxic–anoxic interfaces (Jetten et al., 2003). Molecular studies of AOB communities in freshwater and estuarine systems suggest the dominance of betaproteobacterial AOB (Caffrey et al., 2003). Laboratory isolates of freshwater and marine betaproteobacterial AOB laboratory isolates (Koops and Pommerening-Röser, 2001), and AOB 16S rRNA gene betaproteobacterial AOB laboratory isolates (Koops and 2003). Laboratory isolates of freshwater and marine betaproteobacterial AOB laboratory isolates (Koops and Pommerening-Röser, 2001), and AOB 16S rRNA gene sequences from freshwater and estuarine environments (Speksnijder et al., 1998; de Bie et al., 2001; Caffrey et al., 2003) fall predominantly within *Nitrosomonas* lineages. In marine systems, prevalent sequence types are associated with the *Nitrosomonas eutropha* lineage (Phillips et al., 1999) or belong to the *Nitrosospira* cluster 5 or *Nitrososphaera* cluster 1 clone groups, for which no cultured representative has yet been isolated (McCaig et al., 1999; Hollibaugh et al., 2002; Freitag and Prosser, 2003; 2004).

Salt requirement is a major ecophysiological parameter in determining the niche specialization of many bacteria. Characterization of both laboratory isolates (Koops and Pommerening-Röser, 2001; Koops et al., 2003) and clone libraries (Stephen et al., 1996) suggests that the influence of salt concentration is a distinguishing feature within both *Nitrosomonas* and *Nitrosospira* lineages. This suggests that patterns of diversity, community composition and activity of different sequence types of AOB will differ in estuaries in response to salinity gradients. The aim of this study was to test this hypothesis in a well-characterized estuarine environment, the Ythan estuary, for which data on seasonal and spatial distributions of salinity, hydrography, nutrients and flushing times are available (Balls et al., 1995; Gillibrand and Balls, 1998; Raffaelli et al., 1999). Ammonia-oxidizing bacteria communities were analysed by amplification of 16S rRNA gene fragments from environmental DNA and subsequent analysis by denaturing gradient gel electrophoresis (DGGE), cloning, sequencing and phylogenetic analysis. These techniques were combined with stable isotope probing (SIP; Radajewski et al., 2000; Lueders et al., 2004) to characterize the active AOB community. Application of SIP to determine AOB activity involves incubation of environmental samples with 13C-labelled CO₂ and fractionation of extracted 13C- and 12C-labelled nucleic acids. Molecular analysis of labelled and unlabelled nucleic acids characterizes active and inactive community members, respectively, and thereby provides a more reliable measure of links between community structure and ecosystem function.

**Results**

**16S rRNA gene analysis of environmental AOB communities**

Denaturing gradient gel electrophoresis analysis of the CTO189f-CTO654r 16S rRNA gene polymerase chain reaction (PCR) products nested with 357f-GC-518r, spanning the hypervariable V3 region only, produced almost identical patterns for replicate samples from each sampling site, demonstrating more than 30 clearly distinguishable sequence types (Fig. 1A). Denaturing gradient gel electrophoresis migration patterns varied significantly

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**Fig. 1.** Analysis of betaproteobacterial ammonia oxidizer communities in Ythan estuary sediments at sampling locations indicated in Fig. 5, with salinities ranging from close to seawater (A) to freshwater (E). Betaproteobacterial AOB-like 16S rRNA gene sequences were amplified from extracted DNA using CTO189f and CTO654r PCR primers, nested with 357f-GC and 518r. Closest BLAST matches of clone sequences shown in lanes C₁ and C₂ (marked by arrows) are shown in Table 2.

A. Denaturant gradient gel electrophoresis analysis of amplification products.
B. UPGMA dendrogram assessing the similarity of DGGE profiles illustrated in (A).

Salinities (%) according to Gillibrand and Balls (1998), calculated from conductivity measurements.

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between the sampling sites. Dominant bands of different migration behaviour were present at each site and indicated a general shift from 16S rRNA gene fragments with relatively low migration at freshwater sites to higher migration at marine sites. These differences are reflected in the unweighted pair group method with arithmetic mean (UPGMA) dendrogram, based on a similarity matrix calculated from the presence/absence of DGGE bands. Profiles from sites A and E were least similar, while those for sites B, C and D clustered together with higher similarity (Fig. 1B). Ammonia-oxidizing bacteria richness, in terms of the number of different DGGE bands, was similar (8–9) for all sampling sites. Evenness and Shannon diversity index values were similar (0.929–0.97 and 2.84–2.94 respectively; Table 1) for sampling sites B–E, but evenness at marine station A (0.864) was outside the standard deviation range for all other samples except station B.

To determine the identity of DGGE bands, clone libraries were obtained using CTO189f-CTO654r primers with secondary amplification using 357f-GC-518r primers. CTO clones showing identical migration characteristics to dominant environmental DGGE bands were sequenced and closest relatives determined by comparison with GenBank database sequences, using BLAST (Table 2). Putative identifications of individual bands are indicated in Fig. 1A and show a shift from Nitrosospira cluster 1-associated sequences (clones 8 and 12–15; Fig. 1A), in samples with highest marine influence, to Nitrosomonas marina- and Nitrosomonas oligotropha-associated sequences (clones 1–5 and 9; Fig. 1), with increasing freshwater influence. The AOB sediment community of marine station A was dominated by four sequence types associated with Nitrosospira cluster 1 with several faint bands matching sequences from N. marina/oligotropha lineages also detectable. The AOB community in station B was also strongly influenced by Nitrosospira cluster 1 sequence types but Nitrosomonas cluster 5-, Nitrosomonas sp. Nm143- and N. marina/oligotropha-associated sequences types were present at similar relative abundances. Intermediate brackish station C profiles were dominated by a Nitrosomonas sp. Nm143-associated sequence type and three of the Nitrosospira cluster 1-associated bands were no longer detectable. The same sequence types were present at

<table>
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<tr>
<th>Sampling site</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tr>
<td>Shannon</td>
<td>2.67 (± 0.15)</td>
<td>2.85 (± 0.36)</td>
<td>2.95 (± 0.10)</td>
<td>2.84 (± 0.08)</td>
<td>2.91 (± 0.007)</td>
</tr>
<tr>
<td>Evenness</td>
<td>0.86 (± 0.14)</td>
<td>0.95 (± 0.040)</td>
<td>0.929 (± 0.03)</td>
<td>0.95 (± 0.03)</td>
<td>0.97 (± 0.002)</td>
</tr>
</tbody>
</table>

Table 2. CTO189f-CTO654r 16S rRNA gene sequence fragments (0.45 kb) amplified from Ythan estuary sediments identified by BLAST closest matches.

<table>
<thead>
<tr>
<th>Representative clone/library</th>
<th>Closest relative Name/putative associated lineage</th>
<th>Reference</th>
<th>BLAST closest match accession number</th>
<th>% identity</th>
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<td>CL-1/E</td>
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<td>98</td>
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<td>CL-2/E</td>
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<td>CL-3/C</td>
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<td>CL-5/E</td>
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<td>5, 7, 1</td>
<td>AJ003756</td>
<td>98</td>
</tr>
<tr>
<td>CL-6/C, D</td>
<td>Clone LD1-B6/Nitrosomonas sp. 143</td>
<td>2</td>
<td>AY114347</td>
<td>98</td>
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<tr>
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<td>Clone LD1-B6/Nitrosomonas sp. 143</td>
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<td>98</td>
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<td>CL-8/E</td>
<td>Clone LD1-B28/Nitrosospira cluster 1</td>
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<td>AY114345</td>
<td>98</td>
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<tr>
<td>CL-9/B</td>
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<tr>
<td>CL-11/B</td>
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<td>CL-15/A</td>
<td>Clone LD1-A10/Nitrosospira cluster 1</td>
<td>2</td>
<td>AY114348</td>
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</tbody>
</table>

Clone sequences were assembled from libraries generated from each sampling station (A–E) as DGGE marker lanes (CL1 and CL2; Fig. 1) on the basis of co-migration with DGGE bands in environmental samples.

References: 1, Purkhold and colleagues (2003); 2, Freitag and Prosser (2003); 3, McCaig and colleagues (1999); 4, Kowalchuk and colleagues (1997); 5, Speksnijder and colleagues (1998); 6, Bollmann and Laanbroek (2001); 7, Burrell and colleagues (2001). © 2005 The Authors

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comparable relative abundances at station D but with the additional presence of a band associated with the *N. marina* lineage. The AOB communities in freshwater station E were characterized by five dominant sequence types associated with the *N. marina*/*oligotropha* lineages. The AOB communities in freshwater station E were characterized by five dominant sequence types associated with the *N. marina*/*oligotropha* lineages. The Nitrosospira cluster 1-associated sequence type present as one distinct band in all samples (clones 8 and 13; Fig. 1A) was associated with two different Nitrosospira cluster 1 sequences (LD1-A1 and LD1-B28; Table 2) that were not resolved by DGGE analysis and were observed predominantly in marine (A and B) or freshwater clone libraries (E) respectively. The two additional bands common to marine stations A and B were also associated with Nitrosospira cluster 1, while bands common to DGGE migration patterns from sites B, C and D were associated with the *N. marina* and *Nitrosomonas* sp. Nm143 lineages. The additional band common in freshwater-influenced sites D and E was also associated with the *N. marina* lineage.

**Stable isotope probing analysis of AOB sediment microcosm communities**

Calibration studies were performed with density gradient centrifugation of mixtures of DNA from fully 13C-labelled *Nitrosomonas europaea* cells and 12C-Escherichia coli cells. Use of ethidium bromide at a concentration lower than those previously reported (Radajewski et al., 2000) increased sensitivity of UV visibility and sharpness of 12C and 13C bands, and buoyant densities of 200 µl fractions were established by measurement of refractive index. In most cases linear gradients were established (Fig. 2) but frequently the first and last two to three fractions did not show linearity, and were discarded. In addition, in some cases, the gradient of refractive index was not linear, indicating disruption through disturbance, and these were also discarded. 12C- and 13C-DNA were separated well with highest concentrations in buoyancies of 1.68 and 1.73 respectively (Fig. 2). However, both were also detectable throughout several fractions, exhibiting Gaussian-like distributions.

Following extraction, centrifugation and fractionation of SIP microcosms, distinct bands of 13C- and 12C-DNA could not be visualized in density centrifugation tubes by standard ethidium bromide staining and fluorimetry. Stable isotope probing analysis was performed on samples from sites A and B amplifying 16S rRNA genes using bacterial primers to enable inclusion of sequence types not detected by AOB-specific primers. Denaturant gradient gel electrophoresis profiles (Fig. 3A and B) therefore contained considerably more bands than those in Fig. 1A, particularly for low buoyant density fractions, containing

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**Fig. 2.** Fractionation of unlabelled and 100% 13C-labelled nucleic acids within a buoyant density gradient in 5.1 ml centrifugation tubes with an initial CsCl buoyant density of 1.7019 g ml⁻¹ subjected to centrifugation at 227 640 g for 24 h. A. Nucleic acids of individual buoyant density fractions resolved and quantified by standard agarose gel ethidium bromide fluorescence. B. Distribution of unlabelled and 13C-labelled nucleic acids (●) and buoyant density gradient (○). Inset: CsCl density gradient tube containing approximately 2 µg of unlabelled *E. coli* nucleic acids (top) and 100% 13C-labelled nucleic acids extracted from *N. europaea* respectively (bottom).
Clone libraries (CLA for microcosm sediment station A and CLB for station B) were assembled from nested 27f- and 160 bp eubacterial 16S rRNA gene sequences amplified from sediment microcosms amended with 30 μg ml⁻¹ NH₄Cl, N and Na₂¹³CO₃ and incubated for 20 days. Nucleic extracts were subjected to isopycnic CsCl density centrifugation and fractionated according to their buoyant density. Lanes represent amplicons generated with the general bacterial 27f-1492r PCR primers nested with 357f-GC and 518r primers from individual buoyant density fractions. Clone libraries (CLₐ for microcosm sediment station A and CLₐ for microcosm sediment station B) were assembled from nested 27f-1492r and 357f-pf1053r PCR products of the three fractions showing the highest buoyant densities. Individual clones were reamplified with 357f-GC plus 518r for DGGE analysis and clone library assembly. Closest BLAST matches of clone sequences shown in lanes CLₐ and CLₐ (marked by arrows) are given in Table 3. ¹³C enrichment of clone-associated bands is indicated by ¹³C/¹²C proportionate ranking positions (R; see text); bold typeface indicates ¹³C/¹²C ratios > 1. No clone was isolated with association with the bands ranked at position 1, for station A, and position 4, for station B.

Fig. 3. Stable isotope probing-denaturant gradient gel electrophoresis (SIP-DGGE) analysis of ammonia oxidizer communities in sediments from Ythan estuary station A (A) and station B (B). Analysis was performed on 160 bp eubacterial 16S rRNA gene sequences amplified from sediment microcosms amended with 30 μg ml⁻¹ NH₄Cl, N and Na₂¹³CO₃ and incubated for 20 days. Nucleic extracts were subjected to isopycnic CsCl density centrifugation and fractionated according to their buoyant density. Lanes represent amplicons generated with the general bacterial 27f-1492r PCR primers nested with 357f-GC and 518r primers from individual buoyant density fractions. Clone libraries (CLₐ for microcosm sediment station A and CLₐ for microcosm sediment station B) were assembled from nested 27f-1492r and 357f-pf1053r PCR products of the three fractions showing the highest buoyant densities. Individual clones were reamplified with 357f-GC plus 518r for DGGE analysis and clone library assembly. Closest BLAST matches of clone sequences shown in lanes CLₐ and CLₐ (marked by arrows) are given in Table 3. ¹³C enrichment of clone-associated bands is indicated by ¹³C/¹²C proportionate ranking positions (R; see text); bold typeface indicates ¹³C/¹²C ratios > 1. No clone was isolated with association with the bands ranked at position 1, for station A, and position 4, for station B.

1²C-DNA, where complex banding patterns were observed. These profiles are typical of those in traditional DGGE analysis generated by bacterial 16S rRNA gene primers and, as expected, were similar throughout low buoyant density fractions. Principal components analysis of SIP-DGGE banding intensities (59 and 53 bands for sites A and B respectively) (Fig. 4) clearly discriminated buoyant density fractions that were associated with the ¹²C and ¹³C distribution (Fig. 2), resulting in separation of the tightly clustered ¹³C fractions from the ¹²C fractions. ¹³C fractions were also grouped together but to a lesser degree than the ¹²C fractions.

In analysing changes in DGGE banding patterns along the buoyant density gradient, emphasis was placed on bands that appeared with high relative intensity in high buoyant density fractions. This was achieved by ranking the ratios of the relative intensities of each band averaged over the buoyant density ranges established by calibration trials for ¹³C and ¹²C incorporation (Fig. 2). Bands that appeared in only one fraction were ignored. Profiles from fractions obtained from both sites show the presence of several bands in high buoyant density ¹³C fractions alone, the appearance of some bands in all fractions and the presence of others in ¹²C fractions only. The clone library generated from high buoyant density fractions from the most marine station A demonstrated 10 clones with different DGGE migration behaviour and corresponding bands in ¹³C fractions (Table 3, Fig. 3A). These clones were associated with bands that showed ¹³C/¹²C banding intensity ratios ranked between 2 and 47, with six clones ranked between 2 and 8 and three further clones ranked 12, 13 and 15. With the exception of clone 10 (ranked 47) that corresponded with a band that was present in all fractions, all clones showed ¹³C/¹²C banding intensity ratios > 1. The band with the highest ¹³C/¹²C banding intensity ratio (i.e. ranked 1) was not retrieved in the clone library (Fig. 3A). BLAST searches of 0.7 kb clone

Fig. 4. Principal component scores of correlation matrices produced from DGGE band intensities of bacterial 16S rRNA gene fragments from ¹³C-amended marine microcosms (station A, squares, and station B, circles) after isopycnic CsCl density centrifugation and fractionation. Open symbols represent ¹²C buoyant density fractions (≤ 1.7; see Fig. 3B); solid symbols represent ¹³C buoyant density fractions (≥ 1.7; see Fig. 3B). PC1 represents 59.3% and 60% of the total variance for ¹³C microcosms A and B, respectively, and PC2 represents 17.6% and 17.2% of the total variance respectively. Ellipses (A, dotted lines; B, closed lines) demonstrate the 70% confidence region of the principle component scores of the ¹³C and ¹²C buoyant density fractions.
sequences produced closest match associations between 92% and 99% similarity with database sequences, most of which were associated with clones or pure cultures from marine environments. Two clones were closely associated with pure culture sequences of the marine ammonia oxidizer, *Nitrosomonas cryotolerans* (clone 4, ranked 15), and the marine nitrile oxidizer, *Nitrospira marina* (clone 6, ranked 3) (Table 3, Fig. 3A). Two further clones (clones 7 and 2, ranked 2 and 8) were associated with clone sequences isolated in studies on nitrifying biofilms.

For station B, eight clones were sequenced corresponding to DGGE migration behaviour of bands from 13C buoyancy fractions. These were associated with bands with 13C/12C banding intensity ratios ranked between 1 and 31, with seven ranked between 1 and 10 and 13C/12C banding intensity ratios > 1 (Table 3, Fig. 3B). Again, BLAST searches showed two clones with sequences closely associated with pure cultures of marine nitrifying strains, the betaproteobacterial ammonia oxidizer *Nitrosomonas* sp. Nm143 (clone 17, ranked 5) and the nitrile oxidizer *Nitrospira marina* (clone 15, ranked 1) (Table 3, Fig. 3B). All other clones showed closest match associations with clones and isolates from marine environments. As for station A, no sequence was generated with similarity to the *Nitrosospira* cluster 1 clade that dominated environmental DGGE migration patterns from these stations.

**Table 3.** BLAST closest matches of Ythan estuary sediment microcosm 0.7 kb 357f-pf1053r 16S rRNA clone sequences generated from high buoyancy nucleic acid fractions.

<table>
<thead>
<tr>
<th>Representative clone/library</th>
<th>Closest relative Name/putative lineage</th>
<th>Reference</th>
<th>BLAST closest match accession number</th>
<th>% identity</th>
<th>Rank</th>
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<tr>
<td>CLA-1 Arctic sea ice clone ARK9971/Pseudomonas</td>
<td>4</td>
<td>AF468404</td>
<td>98</td>
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<td>CLA-2 Cold seep sediment clone/Bacteroidetes</td>
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<td>AY592607</td>
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</tr>
</tbody>
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BLAST searches showed two clones with sequences closest to the *Nitrosospira* cluster 1 clade that dominated environmental DGGE migration patterns from these stations.

**Discussion**

The aim of this study was to test the hypothesis that the distribution patterns and activities of AOB communities in estuarine environments could be explained in terms of salinity gradients. At the study site, the Ythan estuary, the high tide salinity gradient (Gillibrand and Balls, 1998) ranges from marine values at the river mouth (site A) to a predominant freshwater influence at site E. At low tides, salinities in the entire estuary up to site A decrease considerably, especially when the tidal range is small. Additional fluctuations result from seasonal rainfall, the influence of river flow and high water levels at neap and spring tides, when the marine water front can reach site E (Gillibrand and Balls, 1998). Changes in community structure along the estuary may therefore result from selection of organisms adapted to particular salinities or to changes in salinity, even at sites with an otherwise overriding marine or freshwater influence. Related studies on the diversity of bacterial communities along salinity gradients often focus on the distribution of planktonic assemblages (Cerbon et al., 2004; Crump et al., 2004) and the distinction of true planktonic riverine, estuarine and marine communities can be obscured by mixing of water bodies and temporal changes. However, these influences will be less for sediment communities, where organisms are attached to particles or occupy interstitial...
pore space, where pore water salinities may be buffered against the peak changes over a tidal cycle.

Changes in community structure along the estuarine salinity gradient

Communities of betaproteobacterial AOBs, comprising communities of active and inactive AOB, were characterized by DGGE profiling and sequencing of 16S rRNA genes amplified with the CTO primer set. This primer set is specific for betaproteobacterial AOBs, but with potential bias through mismatches in the CTO189f sequence against some Nitrosomonas lineages, and there is also the possibility of amplification from naked DNA (Demaneche et al., 2001). Stable isotope probing employing eubacterial primers was used to characterize active organisms. The first of these approaches demonstrated significant differences within AOB communities at sampling sites along the estuary, with Nitrosospira cluster 1-like sequences dominating AOB communities close to the river mouth and 

*Nitrosomonas* species. Along intermediate sites, sequences at freshwater sites. Denaturant gradient gel electrophoresis profiles from intermediate sites were dominated by *Nitrosomonas* sp. Nm143 sequences and, to a lesser extent, by *Nitrosomonas* cluster 5-like sequences. These trends in community structure are consistent with previous studies of marine and freshwater environments and with physiological characteristics of laboratory culture representatives (where available), and suggest selection for organisms with different responses to salinity. With the exception of the freshwater/soil strain *N. oligotropha*, all BLAST closest match sequences from cultured members of the *N. oligotropha/marina* group are obligate halophiles (with salt optima of 300–400 mM NaCl) or, for *Nitrosomonas* 143, have been isolated only from coastal environments (Jones et al., 1988; Koops et al., 2003). The presence of *N. oligotropha*-like sequences in sediments strongly influenced by marine water influx (station B), and of halophilic *Nitrosomonas aestuarii* and *N. marina* sequences from sediments at freshwater sites (E), may therefore reflect adaptation to a range of salinities, while individual strains may be active at different salinities. The uncultured *Nitrosospira* cluster 1- and *Nitrosomonas* cluster 5-like sequences dominate AOB sequences amplified from estuarine and marine sediments (McCaig et al., 1999; Bano and Hollibaugh, 2000; Freitag and Prosser, 2003; 2004). Pure culture representatives of these groups have not been obtained, and their role in ammonia oxidation is based on clustering of their 16S rRNA genes within a monophyletic group for which all cultivated representatives are autotrophic AOB, and on reports of non-persisting enrichment cultures of *Nitrospora* cluster 5 (Stephen et al., 1996). Unequivocal evidence of their assumed role as AOB requires rigorous characterization of laboratory enrichment or pure cultures. De Bie and colleagues (2001) also detected *N. oligotropha-* and *N. marina*-like sequences in the Schelde estuary and Bollmann and Lanbroek (2002) concluded from culture experiments that salinity was responsible for differences in AOB communities. However, the maximum salinity of their study sites was only 19‰, and no *Nitrosospira* cluster 1- or *Nitrosomonas* cluster 5- or *Nitrosomonas* 143-like sequences were retrieved. Caffrey and colleagues (2003) reported similar sequences in the Elkhorn Slough estuary in California, but with no evidence of a relationship with salinity or sampling. Recently Bernard et al. (2001) analysed the distribution of the functional gene responsible for the first step in the oxidation of ammonia, the ammonia monoxygenase gene (*amoA*) along a salinity gradient in an estuarine system. Their results confirm the dominance of *Nitrosospira*-associated sequences at marine sites and the prevalence of *N. oligotropha-* and *Nitrosomonas* sp. Nm143-associated sequences at freshwater and intermediate sites. Evidence from distributions of benthic invertebrates along a salinity gradient in the Baltic (Remane and Schlieper, 1971) has led to the hypothesis that diversity will be lower in brackish-water ecosystems than in marine or freshwater systems, but this was not supported here. The number and relative intensity of individual 16S rRNA gene sequences resolved on DGGE suggest similar richness of AOB communities at all sampling sites but significantly reduced evenness and, to some extent, reduced Shannon diversity indices at the marine site. Similarities in richness along the gradient may result from temporal changes in salinity and diversity indices at sites A and B are strongly influenced by high relative abundance of *Nitrosospira* cluster 1 and *Nitrosomonas* cluster 5 sequences, whose roles in ammonia oxidation are not yet confirmed.

Application of SIP to estuarine AOB community analysis

Stable isotope probing analysis was carried out to determine which AOB were active at sites A and B, which had contrasting communities, and to assess whether *Nitrosospira* cluster 1 sequences in marine sites contributed to activity. 13C-DNA-derived sequences will identify organisms that have incorporated 13C-CO2 or secondary utilizers, incorporating organic compounds released by autotrophs or biomass following death. Molecular analysis was carried out using Bacteria-specific primers to ensure that all active autotrophs were detected, in addition to secondary utilizers. Principle components analysis clearly discriminated bacterial DGGE profiles derived from 13C- and 14C-associated buoyant densities, indicating successful density centrifugation and fractionation, distinct microbial communities and minimal cross-contamination. The tight clustering of 13C fractions reflects microbial com-

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munities unaffected by $^{13}$C incorporation spanning the buoyant density gradient according to the Gaussian-like distribution observed during calibration spins. The clustering of $^{13}$C buoyant density fractions was less pronounced, reflecting more heterogeneity in DGGE profiles, due to the presence of naturally abundant $^{12}$C-carbonate in sediments and turnover of $^{13}$C substrates. This will dilute $^{13}$C incorporation, leading to accumulation of nucleic acids not fully labelled in fractions with a relative lower buoyant density. In addition, low copy numbers in $^{13}$C buoyant density fractions, due to low label incorporation, may lead to stochastic amplification of individual templates, generating more random DGGE profiles (Taberlet et al., 1996).

Stable isotope probing calibration trials demonstrated shallow gradient curves close to the minimum possible slope, resulting in maximum interband distance and sharply defined $^{12}$C and $^{13}$C bands. However, the distribution of nucleic acids in density fractions implies contamination of $^{13}$C-associated fractions by $^{12}$C nucleic acids, with potential for generation of false-positive results from $^{13}$C-associated fractions when the $^{13}$C/$^{12}$C nucleic acid ratio is low and highly sensitive PCR strategies are used. In this case, the concentration of nucleic acids loaded onto individual density centrifugation tubes is of considerable importance, as higher loads will increase the likelihood of contamination. However, confidence in interpretation of SIP data was increased by ranking DGGE bands according to the ratio of the averaged relative intensities in $^{13}$C and $^{12}$C buoyant density fractions and facilitated identification of sequence types indicative of $^{13}$C incorporation, which could otherwise only be achieved by extensive comparison of clone libraries. Ranking of $^{13}$C/$^{12}$C relative intensity ratios may bias against active organisms present at high relative abundance. Nevertheless, the study provides confidence in the use of SIP for analysis of active AOB in natural environments and omission of DGGE bands present in one fraction only enables further discrimination of sequence types representing label incorporation from those resulting from stochastic PCR effects.

**Stable isotope probing analysis of AOB**

Screening of a relatively low number of clones generated with bacterial primers from nucleic acids in SIP buoyant density fractions indicating $^{13}$C incorporation yielded some sequences with closest BLAST matches to ammonia and nitrite oxidizers. This demonstrates the sensitivity of the approach, as nitrifiers will constitute a small proportion of the total bacterial community. A 16S rRNA gene fragment highly similar (98%) to the nitrite oxidizer *Nitrospira marina* was isolated from the station B sediment microcosm that co-migrated with the highest ranking DGGE band and the clone sequence of the fifth highest ranking band had high sequence similarity (98%) to the *Nitrosomonas* sp. Nm143 lineage. Detection of *Nitrospira marina* sequence type indicates high rates of autotrophic nitrite oxidation after 20 days of microcosm incubation. The concurrent high ranking of sequence types generally associated with heterotrophs suggests significant turnover of biomass generated from nitrifiers, the existence of previously uncharacterized heterotrophs, or may be indicative of heterotrophic CO$_2$ assimilation (Hesselsoe et al., 2005). However, Hesselsoe and colleagues (2005) estimated that heterotrophic CO$_2$ assimilation accounted for 3% of the total cell carbon in *Pseudomonas putida*, whereas Manefield and colleagues (2002) suggested that 20% nucleic acid isotope incorporation is the minimum for successful fractionation during SIP-based approaches. Furthermore, the appearance of members of Burkholderiales-like sequence types (*Comamonas* and *Oxalobacteriaceae*) at higher ranking positions than the AOB *Nitrosomonas* sp. Nm143 suggests their degradative rather than syntrophic role within autotrophic communities.

Clone sequences related to *Nitrosomonas cryotolerans* and *Nitrospira marina* were observed in microcosm A. The obligate halophilic *N. cryotolerans* grows at temperatures as low as $-5^\circ$C (Jones et al., 1988) and survives for prolonged periods at low ammonia concentrations (Jones and Morita, 1985; Johnstone and Jones, 1988). Few 16S rRNA gene clones with high similarity to *N. cryotolerans* have been recovered from marine systems (Tal et al., 2003) and nothing is known of its importance in marine nitrification. Other clone sequences were associated with an *Acidobacter*-like sequence from a lithotrophic biofilm, with high abundance of *Nitrospira*-related clone sequences (Holmes et al., 2001) and with sequences similar to *Pseudomonas*, *Actinobacter*, the uncharacterized candidate division OP11 and a *Sphingomonas* sequence from a nitrifying biofilm with high relative abundance of Anammox and *Nitrospira*-like sequence types (Egli et al., 2003). The physiological characteristics of members of these groups are largely unknown and a potential role in autotrophic metabolism cannot be excluded. The respective low and high rankings of AOB and *Nitrospira marina* sequence types suggest that nitrite oxidation was the predominant autotrophic process after 20 days of incubation and that AOB biomass had been degraded. The closest database relative of the AOB sequence type was *Nitrosomonas* sp. Nm143, which was also dominant in the environmental DGGE and clone survey. Only four cultured strains of *Nitrosomonas* sp. Nm143 have been isolated from marine environments (Purkholt et al., 2003) but 16S rRNA gene sequences associated with this lineage have been isolated from various marine environments, including anoxic sediments (Freitag and Prosser, 2003), suggesting its abundance in marine and estuarine ecosystems. Nothing is known about its importance within the
marine nitrogen cycle and this is the first study confirming the activity of *Nitrosomonas* sp. Nm143 within an estuarine sediment ecosystem. The BLAST closest match AOB sequence type identified by SIP in the microcosm from the most marine station A was *N. cryotolerans*, which was not identified in the environmental DGGE and clone survey. However, this may have been due to the high relative abundance of *Nitrospira* cluster 1 sequence type. No clone with similarity to the *Nitrospira* cluster 1 or *Nitrosomonas* cluster 5 sequence types was recovered from SIP microcosms, which may be due to the relatively high ammonia concentrations and temperature during incubation. However, their role in ammonia oxidation therefore remains uncertain. The high proportion of non-nitrifier sequences in $^{13}$C fractions suggests that shorter incubation periods are required for better discrimination of active nitrifiers.

The study therefore provides evidence for the selection of different ammonia oxidizer ecotypes along an estuarine salinity gradient which correlates with the physiological characteristics of representative cultured strains, where these are available. The study also demonstrates the ability of stable isotope probing to distinguish active and inactive ammonia and nitrite oxidizers at different sites and the activity of other organisms, presumed to be secondary utilizers.

**Experimental procedures**

*Site description and sampling*

Sediment samples were collected from the Ythan estuary on the east coast of Scotland, UK (Fig. 5), approximately 8 km long and with average and maximum widths of approximately 300 m and 620 m, respectively, containing a total intertidal area of approximately 1.85 km$^2$ (Leach, 1971). Flushing times of the estuary range from one tidal cycle (see above) to 5–12 days (Balls, 1994) and approximately 95% of the catchment area (approximately 680 km$^2$) is agricultural (Lyons et al., 1993). Depending on seasonal rainfall and river flow, salinity over a 6 km stretch ranges from close to marine values (25–30‰; averaged over the tidal cycle) at the river mouth (Fig. 5, station A) to 0–5‰ before the Logie Buchan bridge, and approaches freshwater values shortly beyond the bridge (Fig. 5; station E; Gillibrand and Balls, 1998). Ammonia concentrations show a negative correlation with salinity and median values range between 8 µM at freshwater sites to approximately 1 µM at marine salinity (Balls et al., 1995). Average temperatures in Ythan river water and coastal surface water at the sampling time were 5°C and 6°C respectively (Scottish Environmental Protection Agency, Water Data, Harmonized Monitoring Scheme, http://www.sepa.org.uk/data/hm/hm.asp; Coastal Long-term Monitoring Programme, FRS Marine Laboratory, Aberdeen, http://www.frs-scotland.gov.uk/Delivery/standalone.aspx?contentid=543). Sediment samples were taken at low tide in March 2004 at five locations covering a 6 km section along the estuary. Two replicate surface samples were collected within a 5 m radius at each sampling station close to the water line. Sampling stations were (Fig. 5): A: 57°18′32″N, 1°59′30″W; B: 57°18′42″N, 1°59′41″W; C: 57°19′20″N, 1°59′43″W; D: 57°20′04″N, 2°00′10″W; and E: 57°21′37″N, 2°01′00″W. Sediment samples were freed from larger particles and processed on the same day. Subsamples for molecular analysis were frozen at −80°C until required.

*Stable isotope probing microcosm studies*

Microcosms consisted of 25 g of sediment, bulked from two samples from stations with the strongest marine influence (A and B), in 250 ml Erlenmeyer flasks and amended with 5 ml of artificial sea water (g l$^{-1}$ distilled water: NaCl, 25; MgCl$_2$·7H$_2$O, 5; CaCl$_2$·6H$_2$O, 1; KCl, 1) supplemented with ammonium sulfate at 30 µg ml$^{-1}$ NH$_4^+$-N, giving a final concentration of approximately 10 µg ml$^{-1}$ NH$_4^+$-N. Flasks were sealed with butyl rubber stoppers and the headspace was purged with CO$_2$-free air (Whitby et al., 2001). A $^{13}$C-labelled carbon source was provided by dropwise addition of 1 ml of 5% (w/v) Na$_2^{13}$CO$_3$ (99% atom enriched; CK-Gas, Cambridge,
UK) to the sediment slurry, while simultaneously titrating with an equimolar solution of HCl to give a final pH of 7.5. Flasks were incubated at 20°C for 3 weeks with weekly flushing of the headspace and replenishment of Na213CO3. Flasks were sampled destructively for extraction of nucleic acids.

**DNA extraction**

Nucleic acids were extracted from sediments and SIP microcosms according to Griffiths and colleagues (2000) with previously described modifications (Freitag and Prosser, 2003). Nucleic acid extracts from microcosms were visualized by ethidium bromide staining in standard agarose gel electrophoresis with Hyperladder DNA fragment size markers (Bioline, London, UK) and quantified by densitometry analysis (TN-Image, freeware by Thomas J. Nelson, http://entropy.brneurosci.org/timage.html).

**Isopycnic density centrifugation and gradient fractionation**

13C-labelled and unlabelled nucleic acids extracted from SIP microcosms were fractionated by density centrifugation in CsCl gradients (Schildkraut et al., 1962; Radajewski et al., 2000) in 5.1 ml Quick-Seal polyallomer tubes in a VTi65.2 vertical rotor (Beckman Coulter, Palo Alto, CA, USA). CsCl gradients and ultracentrifugation conditions were initially calibrated with 4 µg of DNA per tube, extracted from pure cultures of *N. europaea* grown in Skinner and Walker medium (Skinner and Walker, 1961) at 500 µg ml−1 NH413CO3-N (Powell and Prosser, 1985) supplemented with Na213CO3 as described above, and *E. coli*, cultivated in standard Luria–Bertani nutrient broth (g l−1, thoroughly mixed, and then suspended in a total volume of 200 µl of sterile H2O. For verification of the buoyant density range of maximum concentrations of 13C-labelled and unlabelled DNA, precipitated nucleic acids were re-eluted in 30 µl of sterile H2O. For verification of the buoyant density range of maximum concentrations of 13C-labelled and unlabelled DNA, precipitated nucleic acids from calibration tubes containing labelled *N. europaea* and unlabelled *E. coli* DNA were visualized and concentrations estimated by ethidium bromide fluorescence in standard agarose gel electrophoresis with Hyperladder DNA fragment size markers (Bioline).

**Analysis of sediment AOB communities**

Extracted DNA was amplified by nested PCR using 16S rRNA gene primer sets specific for the majority of betaproteobacterial AOB (CTO189f and CTO654r) (Kowalchuk et al., 1997). Polymerase chain reaction products were reamplified for DGGE analysis using the general bacterial 357f-GC and 518r primers (Muyzer et al., 1993). Before secondary amplification, PCR products were diluted 1:20 to prevent amplification of non-target sequences without affecting DGGE profiles. Cloning inserts of 0.45 kb, specific for betaproteobacterial AOB, were created by PCR amplification of DNA extracts with the β-AMO161f and β-AMO1301r primer set (McCag et al., 1994), followed by PCR amplification with the CTO189f and CTO654r primer set. Primer pairs and PCR conditions for all amplifications were used as described previously (Freitag and Prosser, 2003). Denaturant gradient gel electrophoresis was carried out as described previously (Kowalchuk et al., 1997) using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hemel Hempstead, UK) and 1.5 mm, 8% polyacrylamide gels containing denaturant gradients of 35–62%, for analysis of 357f-GC and 518r PCR products. Polycrylamide gels were silver-stained (McCag et al., 2001) for 15 min and destained for 10 min in dH2O before digital image analysis (Phoretix 1-D gel analysis software; Phoretix International, Newcastle-Upon-Tyne, UK). To compensate for variations in DNA loading between DGGE lanes, the total band intensity for each lane was normalized to that of the lane with the lowest DNA loading (McCag et al., 2001; Nicol et al., 2003). Similarity matrices, based on band presence, were produced using the Dice coefficient, from which similarity dendrograms were constructed by UPGMA. Additionally, the Shannon diversity index, which describes both species richness and evenness, was calculated by counting the numbers of different band positions and their respective relative intensity per sample (McCag et al., 1999).

**Community analysis of 13C microcosms**

Nucleic extracts from individual fractions obtained from CsCl using a Gilson MiniPlus3 peristaltic pump (Anachem, Luton, UK) at a flow rate of 0.5 ml min−1. Density gradients were aliquoted into 200 µl fractions with a LKB 8Romma 2112Rediar fraction collector (Pharmacia LKB, Cambridge, UK). Buoyant density was determined by measuring the refractive index in a 10 µl aliquot of each fraction and centrifugation tubes showing a disturbed linear density gradient were discarded. Nucleic acids were separated from CsCl by overnight precipitation in 2 vols of polyethylene glycol at 4°C (Griffiths et al., 2000) followed by centrifugation at 20 000 g for 30 min. Residual salts were removed by washing with 70% ethanol, pellets were air-dried and precipitated nucleic acids were re-eluted in 30 µl of sterile H2O. For verification of the buoyant density range of maximum concentrations of 13C-labelled and unlabelled DNA, precipitated nucleic acids from calibration tubes containing labelled *N. europaea* and unlabelled *E. coli* DNA were visualized and concentrations estimated by ethidium bromide fluorescence in standard agarose gel electrophoresis with Hyperladder DNA fragment size markers (Bioline).

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density fractionation were analysed by nested PCR amplification using general bacterial primers 27f and 1492r (Lane, 1991) for primary amplification, followed by secondary amplification with primers 357f-GC and 518r (Muyzer et al., 1993) for DGGE analysis. Polymerase chain reaction amplicons (0.7 kb) of sufficient quality for use as cloning inserts were created by nested PCR amplification of the 27f-1492r PCR product with the 357f primer in conjunction with the general bacterial primer pf1053r (Edwards et al., 1989). Denaturant gradient gel electrophoresis analysis of general bacterial 16S rRNA gene fragments was carried out as described above, but with polyacrylamide gels containing denaturant gradients of 35–70%. Denaturant gradient gel electrophoresis banding profiles derived from DNA with buoyant densities corresponding to 13C and 12C fractions were analysed in three ways to assess active AOB: (i) qualitative assessment of increases in relative intensity, or appearance of bands, in 13C fractions in comparison with 12C fractions, (ii) calculation, for each band, of the ratio of relative intensity averaged across 13C and 12C fractions, with 13C:12C ratios > 1 indicating sequences derived from organisms incorporating 13C-CO2 and (iii) principal components analysis of normalized relative intensities of bands from 13C and 12C fractions (Systat 8.0; Systat Software UK Limited, Hounslow, UK).

Cloning and sequence analysis

For analysis of betaproteobacterial AOB, one clone library per sampling point was created using PCR amplicons generated with the CTO primer set, as described above. Two PCR amplicons from each sediment sample were pooled to compensate for PCR drift and to ensure that dominant sequences were represented in the clone library. Polymerase chain reaction fragments were purified by standard agarose gel electrophoresis and excised DNA bands were cleaned as described by Boyle and Lew (1995), ligated into the pGEM T-vector system (Promega, Southampton, UK) and transformed into XL1-Blue MRF Kan supercompetent E. coli cells (Stratagene, Cambridge, UK) according to the manufacturers’ instructions. Transformed colonies were screened for inserts of the correct size by PCR amplification with the CTO PCR assay, as described above, and 15 clones per library with the correct fragment size insert were subjected to nested PCR with the 357f-GC-518r (Muyzer et al., 1993) primers. Polymerase chain reaction products were screened by DGGE for sequence differences within the hypervariable V3 region. Clones selected for sequencing represented at least two replicate sequence types with DGGE migration patterns identical to individual DGGE bands in amplicons of environmental samples.

Clone libraries were also generated from three gradient fractions from DNA extracted from each Na13CO3-amended microcosm, within the buoyant density range of 13C-labelled N. europaea DNA. Bacterial 16S rRNA gene fragments were generated, as described above, from at least three pooled amplicons of each density fraction, to ensure recovery of dominant sequence types. Ligation and transformations were performed as described above and transformed colonies screened for correct size inserts by PCR amplification with the M13f-M13r vector primers (Promega). Thirty clones with the correct fragment size insert of each library were subjected to nested PCR with the 16S rRNA gene primers 357f-GC-518r (Muyzer et al., 1993) and screened by DGGE. The 0.7 kb 357f-pf1053r PCR amplification products of clones showing DGGE migration patterns identical to bands in amplicons generated from density fractions were selected for sequencing. Representatives of environmental and microcosm clone libraries were amplified with vector primers M13f-M13r and M13 PCR products were purified by standard preparative agarose gel electrophoresis as described above. To ensure sequence read without ambiguities, M13 vector products were sequenced with sufficient overlap of sequencing reads using the SP6 and T7 vector primers (Promega). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Warrington, UK), and the cycle sequencing products were analysed with a Model ABI377 automated sequencer (PE Biosystems). 16S rRNA gene clone sequences were compared with closely related sequences retrieved from the GenBank database using the BLAST algorithm (Altschul et al., 1990). Sequences have been deposited at GenBank under Accession Nos DQ068702–DQ068740.

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Ammonia oxidizers in a freshwater–marine estuarine gradient


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