amoA Gene Abundances and Nitrification Potential Rates Suggest that Benthic Ammonia-Oxidizing Bacteria and Not Archaea Dominate N Cycling in the Colne Estuary, United Kingdom

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Nitrification, mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), is important in global nitrogen cycling. In estuaries where gradients of salinity and ammonia concentrations occur, there may be differential selections for ammonia-oxidizer populations. The aim of this study was to examine the activity, abundance, and diversity of AOA and AOB in surface oxic sediments of a highly nutrient-estuary that exhibits gradients of salinity and ammonium. AOB and AOA communities were investigated by measuring ammonia monoxygenase (amoA) gene abundance and nitrification potentials both spatially and temporally. Nitrification potentials differed along the estuary and over time, with the greatest nitrification potentials occurring mid-estuary (8.2 μmol N grams dry weight [gdw]−1 day−1 in June, increasing to 37.4 μmol N gdw−1 day−1 in January). At the estuary head, the nitrification potential was 4.3 μmol N gdw−1 day−1 in June, increasing to 11.7 μmol N gdw−1 day−1 in January. At the estuary head and mouth, nitrification potentials fluctuated throughout the year. AOB amoA gene abundances were significantly greater (by 100-fold) than those of AOA both spatially and temporally. *Nitrosomonas* spp. were detected along the estuary by denaturing gradient gel electrophoresis (DGGE) band sequence analysis. In conclusion, AOB dominated over AOA in the estuarine sediments, with the ratio of AOB/AOA amoA gene abundance increasing from the upper (freshwater) to lower (marine) regions of the Colne estuary. These findings suggest that in this nitrified estuary, AOB (possibly *Nitrosomonas* spp.) were of major significance in nitrification.

Nitrification is central to the global nitrogen cycle, coupling ammonia production from mineralization of organic matter with denitrification. In estuaries, discharge of domestic and industrial waste as well as runoff from mineral fertilizers and nitrogen fixation may contribute to ammonium enrichment. Ammonia oxidation is considered to be the rate-limiting step of nitrification and is catalyzed by ammonia monoxygenase (AMO), which is encoded by the amoA gene. It was previously considered that autotrophic ammonia oxidation is carried out solely by ammonia-oxidizing bacteria (AOB). However, the discovery of a marine archaeon belonging to the thaumarchaea which also oxidizes ammonia showed that this is not the case (1, 2). Ammonia-oxidizing archaea (AOA) belonging to the phylum *Thaumarchaeota* (AOA) are widely distributed in terrestrial and aquatic environments (2, 3) and thus may be more important contributors to nitrification than was previously considered. While previous studies of marine sediments showed different patterns of either AOA or AOB dominance, in estuarine environments where gradients of salinity and ammonia concentrations occur, there may be a differential selection for ammonia-oxidizer populations along these gradients (4–9). It has been previously suggested that AOA are significant in estuarine nitrogen cycling (6) and that AOA were more abundant than AOB along an estuarine salinity gradient (8).

The focus of the current study was the River Colne estuary, a macrotidal, hypernutrified estuary on the east coast of the United Kingdom (Fig. 1) which has very high inorganic nitrogen levels in the upper estuary from inputs from the River Colne and a major sewage treatment works, with decreasing gradients of both ammonium and nitrate downstream (10, 11). Benthic denitrification can remove ~44% of the total oxidized nitrogen load (25% of total inorganic nitrogen) from the estuary before it enters the North Sea; coupled nitrification-denitrification accounts for about 25% of the total denitrification (12). However, little is known about the benthic AOA and AOB communities along the estuary and their potential links with biogeochemical function (10).

It has been suggested that AOA and AOB niches are defined by the concentrations of ammonium present (13), with AOA dominating in low-ammonium environments such as some soils (14) and the open ocean (15) and in some estuaries (6, 16). In the present study, we hypothesized that in the upper estuary of the River Colne at the Hythe, where very high levels of inorganic N occur (12), nitrification is driven by AOB, which predominate over AOA. In contrast, as ammonium concentrations in the water...
column decline downstream, the significance of AOA with respect to nitrification might be expected to increase and AOA may become proportionately more important toward the estuary mouth. In addition, the estuarine salinity gradient might also tend to favor AOA over AOB as salinity increases down the estuary (6). Our study aimed to examine the activity, abundance, diversity, and distribution of these different groups of ammonia oxidizers (AOA and AOB) in the surface oxic sediments where nitrification can occur. The overall goal was to test whether there is spatial and temporal variation in the relative abundances of AOA and AOB amoA genes in relation to sediment nitrification potentials in this hypernutrified estuary that exhibits gradients of salinity and ammonium concentration.

MATERIALS AND METHODS

Field sampling. Samples were taken at approximately three monthly intervals to cover temporal variations between June 2009 and January 2010 at three sites along the Colne estuary, United Kingdom (Fig. 1), the upper estuary at the Hythe (51°52.4'N, 0°55.5'E), the mid-estuary at Alresford Creek (51°50.5' N, 0°58.4'E), and the estuary mouth at Brightlingsea (51°45'N, 1°30'E), as described previously (10, 12). At each site, triplicate surface sediment samples (depth, 0 to 1 cm) were collected using a sterile spatula. (The oxic layers in which nitrification may occur range from a maximum depth of 1.5 mm in winter at the Hythe to approximately 5 mm in winter in the sandier sediment at the estuary mouth at Brightlingsea, and in the summer, oxic layer depths are even shallower [10]). Collected samples were returned to the laboratory on ice within 1 h of sampling. Each replicate was quickly homogenized by mixing, and aliquots (1 g wet weight) of sediment from each replicate were stored at −80°C prior to nucleic acid isolation. Nitrate and ammonium concentrations in the pore water were measured colorimetrically (17) using an autoanalyzer (Skalar Analytical, Netherlands). Sediment water content was determined by oven-drying samples of sediment (5 g wet weight) at 85°C for 48 h to a constant weight.

Nitrification potential measurements. Nitrification potential is the maximum capacity of a soil’s or sediment’s population of nitrifying microorganisms to transform NH₄⁺-N to NO₃⁻-N. Changes in nitrification potentials provide quantitative information on how nitrifying communities respond to changes in environmental conditions and reflect potential changes in the in situ nitrification rates. Nitrification potentials were measured with sediment slurries from each site by mixing each of triplicate 10-g (wet weight) samples of sediment from the depth layer of 0 to 1 cm with 100 ml of sterile EASW medium (18) amended with 300 μM NH₄Cl and 60 μM KH₂PO₄. A further triplicate set of slurries containing allylthiourea (ATU) (172 μM final concentration) were also set up as controls to differentiate autotrophic nitrification from heterotrophic nitrification and examine the relative contributions of AOA and AOB to nitrification activity. All slurries were incubated in the dark at 25°C with gentle shaking (110 rpm) to maintain aeration. Subsamples were removed and analyzed for NH₄⁺ at intervals over 48 h (19). Ammonium concentrations in sediment pore water were analyzed by the indophenol blue spectrophotometric method (20). Rates of ammonium removal were determined by linear regression analysis of the concentrations of ammonium with time.

Real-time quantitative PCR (Q-PCR) of AOB and AOA amoA genes. Total nucleic acids were extracted from 0.5-g (wet weight) sediment samples (21, 22). Unfortunately, the October (autumn) samples for measurements of gene abundances were lost through equipment malfunction, so only three such temporal samples were available; nonetheless, those samples covered the seasonal extremes of temperature. The number of amoA gene copies per g of sediment (dry weight) was measured using primers amoA-1F and amoA-2R (23) to target the amoA gene from AOB and primers CrenamoA23F and CrenamoA616R (24) to target the amoA gene from AOA. DNA standards were created by PCR amplification of sediment DNA extracts. The resulting amplicons were purified using a QiAquick PCR purification kit (Qiagen) prior to quantification using a Nanodrop ND-1000 spectrophotometer. The target abundance for standards was calculated by assuming a molecular mass of 660 Da for double-stranded DNA using the following formula: gene abundance = 6.023 × 10²³ (copies mol⁻¹) × standard concentration (g ml⁻¹)/molecular mass (g mol⁻¹).

Standard curves were created using a dilution series of each DNA standard ranging from 10⁷ to 10⁰ target genes ml⁻¹ for AOB amoA and from 10⁶ to 10¹ target genes ml⁻¹ for AOA amoA. Standards, samples, and no-template controls (NTC) were amplified in triplicate with each primer set. Reactions were performed on a CFX96 real-time system (Bio-Rad) with initial denaturation for 5 min at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each 20-μl reaction mixture contained 20 ng of DNA template, 10 μl of × SensiFAST SYBR No-ROX master mix (Bio-line), and a 100 nM concentration of each primer. A dissociation curve analysis was performed at the end of each reaction to verify amplification of a single PCR product. The samples were quantified against the corresponding standard curve using CFX Manager version 2.0 software (Bio-Rad).

PCR-DGGE analysis of the AOB 16S rRNA and amoA genes. PCR amplification of the amoA gene was undertaken (to further define the AOB present) using primers amoA-1F-GC and amoA-2R and PCR cycling conditions as described previously (23). PCR amplification of the AOB 16S rRNA gene used primers CTO189f-GC and CTO654r and PCR cycling conditions as described previously (23). PCR-DGGE analysis of the AOB 16S rRNA and amoA genes was performed as previously described (27) except that the gels were silver stained (22). DGGE bands containing amplicons with a molecular mass of 660 Da for double-stranded DNA using the following formula: gene abundance = 6.023 × 10²³ (copies mol⁻¹) × standard concentration (g ml⁻¹)/molecular mass (g mol⁻¹)
were excised and sequenced using a reverse primer (either amoA-2R or CTO654r) (23, 24) by Geneservice Ltd. (Cambridge, United Kingdom). Phylogenetic analysis of amoA and 16S rRNA gene sequences was performed using PHYLIP 3.4 (28) with the Jukes-Cantor DNA distance and neighbor-joining methods (29, 30). Bootstrap analysis was based on 1,000 replicates. Trees were constructed using Treeview (WIN32 version 1.5.2) (31).

454 pyrosequencing. In order to determine whether the total microbial community followed spatiotemporal patterns similar to those seen with the AOA and AOB taxa, we used a broad community-screening approach targeting 16S rRNA phylogenetic marker genes. Samples were PCR amplified and pyrosequenced by the Research and Testing Laboratory (Lubbock, Texas, USA) using a Roche 454 FLX instrument with Titanium reagents for tag-encoded FLX amplicon pyrosequencing on the basis of their standard PCR methods and protocols. For bacterial 16S rRNA gene libraries, primers Gray28F and Gray519R were applied, producing a fragment of 491 bp (32). For archaeal 16S rRNA gene libraries, primers ARCH349F and ARCH806R were applied, producing a fragment of 457 bp (33).

High-throughput community pyrosequencing results were analyzed using the QIIME pipeline and its associated modules (34). All sequences were checked for the presence of correct pyrosequencing adaptors, 10-bp barcodes, and taxon-specific primers, and those containing errors in these regions were removed. Sequences less than 450 bp in length were removed, and sequences over 550 bp in length were removed. Sequences with low (>10) quality scores and sequences containing homopolymer inserts were also removed. All pyrosequence reads were clustered into operational taxonomic units (OTUs) at the 95% similarity level using the UCLUST algorithm (35), and any chimeras present were removed using ChimeraSlayer. Representative sequences from each OTU were identified using RDP Classifier (36). Finally, all singletons were removed before further analysis was performed (37).

**Statistical analysis.** Bacterial and archaeal amplicon libraries were analyzed separately, treating these taxa as two distinct assemblages. Data were analyzed via nonmetric multidimensional scaling (NMDS) based on Jaccard’s index as a measure of community dissimilarity. NMDS was supported by permutational multivariate analysis of variance (PERMANOVA), which was also based on Jaccard’s index. Species richness was calculated using rarefaction and normalized to the sample with the fewest amplicon reads. All community analyses were conducted using R statistical language version 2.7.2 and the R standard libraries and the community-analysis-specific package “vegan” (38).

A paired-samples t test was used to compare AOB and AOA amoA gene abundances, and two-way ANOVAs and Tukey’s honestly significant difference (HSD) post hoc analysis at a 95% confidence interval were used to determine the effect of site and season on AOB and AOA amoA gene abundance (39). Pearson’s correlation analyses were performed to determine whether there was a correlation between AOB and AOA amoA gene abundance and nuclear paramagnetic resonance (NPR) (39).

**Nucleotide sequence accession numbers.** Sequences were submitted to GenBank under the following accession numbers: JX567314 to JX567343.

**RESULTS**

**Potential nitrification rates.** There were significant differences, both spatial and temporal, between nitrification potentials along the estuary ($P < 0.001$, Tukey HSD test) (Fig. 2). In the absence of ATU, nitrification potentials tended to be greatest in the mid-estuary and lowest in the upper estuary at the Hythe, where oxic layer depths were shallower than at the other sites. Nitrification potentials were significantly ($P < 0.009$, Tukey HSD test) higher in January than in the warmer summer and autumn months, when the surface oxic layers were shallow (Fig. 2A).
In the presence of ATU, nitrification potentials decreased significantly compared to control results ($P < 0.001$, Tukey HSD test) and were detectable only in the summer months at all sites and also from Brightlingsea in October (Fig. 2B). The nitrification potentials were completely inhibited by ATU in October and January and decreased to about 80% in June and August.

ATU-insensitive nitrification was of greatest significance (at up to 33% of the measured nitrification potential) in the upper estuary and mid-estuary sediments during high-temperature periods (e.g., June to August) (Fig. 2C). Within the oxic layer of all the sediments, nitrification was almost entirely ATU sensitive in October and January (Fig. 2C).

AOA and AOB amoA gene abundances. AOB amoA gene abundances were significantly higher (by approximately 2 orders of magnitude) than AOA amoA gene abundances across all sites and seasons (Fig. 3) [paired-samples $t$ test; $t_{(25)} = 3.92$, $P = 0.001$]. Moreover, AOB amoA gene abundances differed significantly across sites [$F_{(2,25)} = 42.28$, $P < 0.001$] and seasons [$F_{(2,25)} = 14.86$, $P < 0.001$], and there were significant spatial and temporal interaction effects on AOB amoA gene abundance [$F_{(4,25)} = 16.68$, $P < 0.001$]. Post hoc analysis revealed significantly higher AOB amoA gene abundance at the estuary mouth at Brightlingsea than at the Hythe ($P < 0.001$) and Alresford ($P = 0.001$). In addition, AOB amoA abundances were significantly higher across sites in June than in August ($P < 0.001$). In contrast to AOB results, there were no significant spatial or temporal differences in AOA amoA gene abundances. In general, the ratio of AOB/AOA amoA gene copy numbers in surface sediments increased strongly from the upper to lower regions of the estuary throughout the seasons (Table 1). In the present study, there was no significant correlation between potential nitrification rates and AOB amoA gene abundance across the different sites in the Colne estuary ($r = 0.044$, $P = 0.831$).

AOA and AOB community structures. Since AOB were both temporally and spatially dominant over AOA in the Colne estuary, AOB communities were further analyzed by DGGE band sequencing of the amoA and 16S rRNA genes from extracted DNA and reverse-transcribed RNA from ammonia-oxidizing bacteria. In total, 30 bands with distinct positions in the DGGE fingerprints (amoA gene, 14 bands; 16S rRNA gene, 16 bands) from across sites and sample time points were obtained (see Fig. S1 and S2 in the supplemental material). Based on both DNA and RNA profiles from amoA and 16S rRNA genes, AOB communities from the Hythe were more distinct than those from Alresford and Brightlingsea (see Fig. S1 and S2 in the supplemental material). In general, the DNA profiles of the amoA gene showed that there were a greater number of bands at the Hythe and that the number of bands decreased downstream and with time (see Fig. S1). In the RNA profiles for both the amoA and 16S rRNA genes, there were a number of unique fragments which were absent in the DNA profiles that had high sequence identity to Nitrosomonas spp. (bands 5 to 7 [see Fig. S1A]; bands 4 to 5 [Fig. S1B]; band 4 [Fig. S1C]; bands 5 to 6 [Fig. S2B]; and band 6 [Fig. S2C]), suggesting that there was a less complete picture of the AOB community in the DNA fingerprints.

Phylogenetic analysis of amoA gene sequences revealed that several DGGE bands retrieved from the Hythe and Brightlingsea in June and August grouped with 100% bootstrap confidence to a Nitrosomonas clade (see Fig. S3A in the supplemental material). In addition, amoA gene sequences recovered from the Hythe (in June and October) and Brightlingsea (in August and October) formed two discrete clades which grouped with Nitrosomonas cryotolerans (see Fig. S3A). Phylogenetic analysis of the 16S rRNA gene sequences from DGGE bands corroborated the amoA gene sequence data showing a clustering with Nitrosomonas spp. (with the exception of one DGGE band recovered from Brightlingsea in August which clustered within a Nitrospira clade) (see Fig. S3B).

Observed differences between AOB and AOA communities may also be accounted for by their respective proportions of the total bacterial and archaeal communities. While DGGE analysis provided putative identification of potential key AOB species, it did not provide a robust analysis of total bacterial and archaeal communities. In order to examine whether AOB and AOA populations follow general trends of bacterial and archaeal communi-

**TABLE 1** Ratio of AOB/AOA amoA gene abundance and physicochemical characteristics of the estuarine sites

<table>
<thead>
<tr>
<th>Site (mo)</th>
<th>AOB/AOA ratio</th>
<th>Salinity</th>
<th>Water content (%)</th>
<th>NH$_4^+$-N$^*$ (µM)</th>
<th>NO$_3^-$-N + NO$_2^-$-N$^*$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hythe (June)</td>
<td>10.0</td>
<td>3.0</td>
<td>64.3</td>
<td>14.13</td>
<td>20.11</td>
</tr>
<tr>
<td>Alresford (June)</td>
<td>16.5</td>
<td>20.0</td>
<td>51.2</td>
<td>4.46</td>
<td>3.52</td>
</tr>
<tr>
<td>Brightlingsea (June)</td>
<td>121.0</td>
<td>33.5</td>
<td>70.0</td>
<td>2.10</td>
<td>0.94</td>
</tr>
<tr>
<td>Hythe (August)</td>
<td>15.0</td>
<td>3.0</td>
<td>66.6</td>
<td>6.69</td>
<td>6.56</td>
</tr>
<tr>
<td>Alresford (August)</td>
<td>6.9</td>
<td>20.0</td>
<td>53.2</td>
<td>6.31</td>
<td>2.99</td>
</tr>
<tr>
<td>Brightlingsea (August)</td>
<td>18.2</td>
<td>33.5</td>
<td>61.4</td>
<td>3.21</td>
<td>1.30</td>
</tr>
<tr>
<td>Hythe (January)</td>
<td>5.6</td>
<td>3.0</td>
<td>70.3</td>
<td>4.48</td>
<td>4.98</td>
</tr>
<tr>
<td>Alresford (January)</td>
<td>15.8</td>
<td>20.0</td>
<td>57.5</td>
<td>5.01</td>
<td>2.53</td>
</tr>
<tr>
<td>Brightlingsea (January)</td>
<td>1340.6</td>
<td>33.5</td>
<td>34.9</td>
<td>3.68</td>
<td>1.39</td>
</tr>
</tbody>
</table>

* Sediment pore water was used to measure nutrient concentrations.
ties, we examined total bacterial and archaeal communities using 454 pyrosequencing of 16S rRNA genes. NMDS analysis of total bacterial and archaeal pyrosequencing libraries (6,904 bacterial operational taxonomic units [OTUs] and 234 archaeal OTUs from 118,800 and 3,381 amplicon reads, respectively, following quality control checks) showed that both total bacterial and archaeal communities changed in composition along the estuary (see Fig. S4A and C in the supplemental material; PERMANOVA) for bacteria, F(2,9) = 1.31, P = 0.054; for archaea, F(2,8) = 1.19, P = 0.084] but not across seasons (see Fig. S4A and C; PERMANOVA) (P > 0.1 in all cases). In general, the total bacterial communities were twice as rich as those of the archaea (see Fig. S4B and D), even when normalized (rarefied) for differences in sequencing intensity across kingdoms.

OTUs that were assigned to known AOB species represented only a very small fraction of the total 16S rRNA bacterial libraries. *Nitrosospira*-like sequences assigned to a single OTU were detected across all samples and comprised <0.07% of the bacterial libraries. A further OTU assigned to the family *Nitrosonomonadaceae* was found in most libraries but was more abundantly detected in August in Arlesford (0.11%) and the Hythe (0.05%). Nitrite-oxidizing bacteria were generally more abundant than AOB, with seven OTUs assigned to the genus *Nitrospina* and six to the genus *Nitrosopina* which together represented up to a maximum of 0.66% and 0.85% of 16S rRNA sequences in the January Hythe and Brightlingsea samples, respectively. AOA sequences were undetected in the archaeal 16S rRNA libraries, suggesting that they represent only a very small proportion of the total archaeal communities at each site, and this supports the idea of a lower measured abundance of AOA than AOB amoA genes.

**DISCUSSION**

Nitrification potentials measured in the Colne estuary sediments in the absence of ATU increased in winter in a manner commensurate with increases in sediment oxic zone depth stimulating nitrification. Indeed, previous work (10) has reported sediment-water export of nitrate from sandy sediments at the mouth of the estuary during winter, when, despite the low temperature, the oxic layer depth is maximal. Addition of 172 μM ATU resulted in drastic reductions of nitrification potentials at all sites and times: virtually complete inhibition in October and January and 60% to 80% inhibition in warmer months. AOB are reportedly more sensitive to ATU than AOA, with seven OTUs assigned to the genus *Nitrospina* and six to the genus *Nitrosopina* which together represented up to a maximum of 0.66% and 0.85% of 16S rRNA sequences in the January Hythe and Brightlingsea samples, respectively. AOA sequences were undetected in the archaeal 16S rRNA libraries, suggesting that they represent only a very small proportion of the total archaeal communities at each site, and this supports the idea of a lower measured abundance of AOA than AOB amoA genes.

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marsh sediments (48) and between AOA amoA gene abundance and potential nitrification rates in the open ocean and some estuaries (6, 9) have been found. In the Colne estuary, AOB amoA gene abundances generally increased in the lower reaches of the estuary (Table 1). In contrast to the results of the present study, AOA amoA gene abundance has been shown to be greater than AOB gene abundance along a different estuarine salinity gradient (8). Although the potential rates did not correlate significantly with salinity, AOB amoA gene abundance was generally significantly higher at the marine end of the estuary (at Brightlingsea), where salinities are typically between 28 and 32 compared to the levels seen in brackish water at the top of the estuary (the Hythe), with salinities typically between 2 and 17 (12). Salinity variations also play a major role in ammonium adsorption/desorption in sediments (47, 49), and ammonium efflux to the oxic layer from deeper, high-ammonium estuarine sediments is enhanced by tidal changes in salinity (47, 49), with salinity variations being greater at the estuary head than at the estuary mouth. This again tends to favor AOB over AOA. However, other environmental variables such as trace metal and pH levels could also be significant in shaping AOB and AOA communities and potential nitrification rates.

In conclusion, differences in nitrification potential rates occurred both spatially and temporally in the Colne estuary, with the greatest potential autotrophic nitrification rates occurring mid-estuary in January. Although several factors (such as levels of trace metals, pH, and salinity) might selectively promote autotrophic activity by AOB or AOA in an estuarine environment, the sensitivity of AOA to high ammonium concentrations (13, 44) might explain the dominance of nitrification by AOB in this highly fertilized estuary. Furthermore, the greater temporal and spatial abundance of AOB amoA genes suggests that AOB (possibly Nitrosomonas spp.), rather than AOA, were of major significance in nitrogen cycling in the Colne estuary.

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REFERENCES


