Environmental Microbiology (2005) 7(9), 1289–1297 doi:10.1111/j.1462-2920.2005.00808.x

Loss of diversity of ammonia-oxidizing bacteria correlates with increasing salinity in an estuary system

Anne E. Bernhard,[1†] Thomas Donn,[1] Anne E. Giblin[2] and David A. Stahl[1*]

[1]*Department of Civil and Environmental Engineering, University of Washington, 302 More Hall, Box 352700, Seattle, WA 98195-2700, USA.*

[2]*Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02532, USA.*

Summary

Ammonia-oxidizing bacteria (AOB) play an important role in nitrogen cycling in estuaries, but little is known about AOB diversity, distribution and activity in relation to the chemical and physical changes encountered in estuary systems. Although estuarine salinity gradients are well recognized to influence microbial community structure, few studies have examined the influence of varying salinity on the diversity and stability of AOB populations. To investigate these relationships, we collected sediment samples from low-, mid- and high-salinity sites in Plum Island Sound estuary, MA, during spring and late summer over 3 years. Ammonia-oxidizing bacteria distribution and diversity were assessed by terminal restriction fragment length polymorphism (TRFLP) analysis of the ammonia monooxygenase (*amo*A) gene, and fragments were identified by screening *amo*A clone libraries constructed from each site. Most striking was the stability and low diversity of the AOB community at the high-salinity site, showing little variability over 3 years. Ammonia-oxidizing bacteria at the high-salinity site were not closely related to any cultured AOB, but were most similar to *Nitrosospira* spp. Ammonia-oxidizing bacteria at the mid- and low-salinity sites were distributed among *Nitrosospira*-like sequences and sequences related to *Nitrosomonas ureae/oligotropha* and *Nitrosomonas* sp. Nm143. Our study suggests that salinity is a strong environmental control on AOB diversity and distribution in this estuary.

Received 9 August, 2004; accepted 2 February, 2005. *For correspondence. E-mail dastahl@u.washington.edu; Tel. (+1) 206 685 3464; Fax (+1) 206 685 9185. †Present address: Department of Biology, Connecticut College, New London, CT 06320, USA.

Introduction

The productivity of estuaries is, in part, determined by sources and sinks of different nitrogen species (Nixon, 1995). The flux of alternative nitrogen species is determined by a complex interplay of both biological and environmental factors, neither as yet well constrained. Within the biological system, the oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB) is generally thought to play a central role in determining the fate of nitrogen. Ammonia-oxidizing bacteria mediate the first step in nitrification, the oxidation of ammonium to nitrite; this is then oxidized to nitrate by nitrite oxidizing populations. When coupled to denitrification, nitrification promotes a loss of nitrogen from the system. The AOB therefore compete with heterotrophs for available reduced nitrogen. Thus, AOB may be particularly important in estuaries because they are typically nitrogen-limited (Howarth, 1988; Bernhard and Peele, 1997).

Although all chemolithotrophic AOB are united by a very similar metabolic capacity, they are also phylogenetically diverse. Known AOB are affiliated with two major lineages within the *Proteobacteria* (*Betaproteobacteria* and *Gammaproteobacteria*), as inferred from 16S rRNA sequence relationships (Head *et al.*, 1993; Purkhold *et al.*, 2000). This phylogenetic diversity suggests that physiological features determining their habitat range and activity have not been well resolved. Variations in substrate affinity and growth rates are among the few described physiological differences known to influence their distribution and activity (Prosser, 1989). However, other physiological differences that determine the environmental constraints on their distribution and activity are mostly unknown. Because the species composition of AOB undoubtedly exerts significant control of nitrification, impacting estuarine nitrogen dynamics, it is important to refine our understanding of species distribution and activity in relation to possible controlling environmental variables.

Previous studies of AOB diversity in freshwater and marine systems suggest some overlap of AOB species distribution, but these systems appear to be dominated by distinctly different communities (Hiorns *et al.*, 1995; Stephen *et al.*, 1996; Hastings *et al.*, 1998; Speksnijder *et al.*, 1998; Phillips *et al.*, 1999). Ammonia-oxidizing bacteria species distribution in estuaries, however, may be

more complex, primarily because of seasonally and spatially varying mixtures of fresh and marine waters. In estuaries, regular disturbance events such as tidal changes, nutrient pulses and salinity intrusions play a major role in defining the habitat, and undoubtedly play a role in defining AOB species distribution and diversity as well. Only recently have AOB communities in estuaries been targets of molecular studies (de Bie *et al.*, 2001; Caffrey *et al.*, 2003; Cebron *et al.*, 2003; Francis *et al.*, 2003), and the factors that regulate their diversity and distribution in these systems remain unclear.

The mixing of marine and fresh waters in estuaries creates steep physicochemical gradients, and presents a unique environment to refine our understanding of factors controlling AOB species distribution. Salinity has been implicated as a key parameter regulating species composition (de Bie *et al.*, 2001; Bollmann and Laanbroek, 2002) and nitrification activity (Seitzinger, 1988; Rysgaard *et al.*, 1999). Changes in salinity may be particularly important for AOB populations because salinity plays a major role in controlling NH_4^+ adsorption capacity of the sediment (Boatman and Murray, 1982), with increased NH_4^+ efflux as salinity increases (Boynton and Kemp, 1985). Others have shown increased nitrification activity in freshwaters compared with marine waters (Seitzinger, 1988; Rysgaard *et al.*, 1999).

Because AOB are notoriously slow growers and are often difficult to culture, most recent studies of AOB diversity have relied on molecular techniques, targeting the 16S rRNA gene or the ammonia monooxygenase genes (AMO), the enzyme responsible for the first step in the oxidation of ammonia. The phylogeny of *amo*A, which codes for the active subunit of AMO, is generally consistent with the 16S rRNA phylogeny (Purkhold *et al.*, 2000), and provides a highly specific and phylogenetically informative target for AOB community studies (Rotthauwe *et al.*, 1997). Researchers have used *amo*A as a marker to study AOB diversity in a variety of habitats, including marine sediment (Nold *et al.*, 2000), freshwater lakes (Horz *et al.*, 2000) and estuaries (Caffrey *et al.*, 2003; Francis *et al.*, 2003). Additionally, unlike polymerase chain reaction (PCR) primers for *Betaproteobacterial* AOB 16S rRNA genes, the primers developed for *amo*A genes are very specific, thus reducing potential problems of non-specific amplification.

Our study focused on the relationship between AOB community structure and salinity in Plum Island Sound estuary (PIE), a long-term ecological research site in north-eastern Massachusetts. We selected the PIE-LTER system because of its well-defined gradients (Vorosmarty and Loder, 1994; Vallino and Hopkinson, 1998; Hopkinson *et al.*, 1999), allowing us to select sites that encompass a range of physical, chemical and biological attributes. We investigated seasonal changes in AOB community structure by using terminal restriction fragment length polymorphism (TRFLP) analysis of *amo*A genes to track changes in the communities along a salinity gradient over 3 years. Our results suggest a strong link between salinity and AOB population structure.

Results

We sampled three sites in the Plum Island Sound Estuary, a coastal-plain estuary in north-east Massachusetts (Fig. 1), made up of several rivers and surrounded by extensive tidal marshes. Two sampling sites were located in the upper and mid portions of the Parker River Estuary. The salinity of these regions experiences large seasonal changes in response to changes in the discharge of the Parker River (Vallino and Hopkinson, 1998). The third site was located in the lower part of the Rowley River Estuary. This smaller river does not have much effect on the salinity of the lower estuary, which remains high most of the year.

There were significant differences between April and August/September porewater salinity at all three sites (Table 1). Dissolved ammonium concentrations were significantly lower in April than in the late summer at both R8C and P14, but there was no seasonal change at P22. The percentage carbon (%C) and the molar carbon to nitrogen ratio (C/N) of sediment from each site did not show significant changes between sampling periods. When the average annual values at the three stations were compared (data not shown), there was no difference in C/N or dissolved ammonium concentrations among sites. However, there were significant differences among sites for percentage C and salinity.

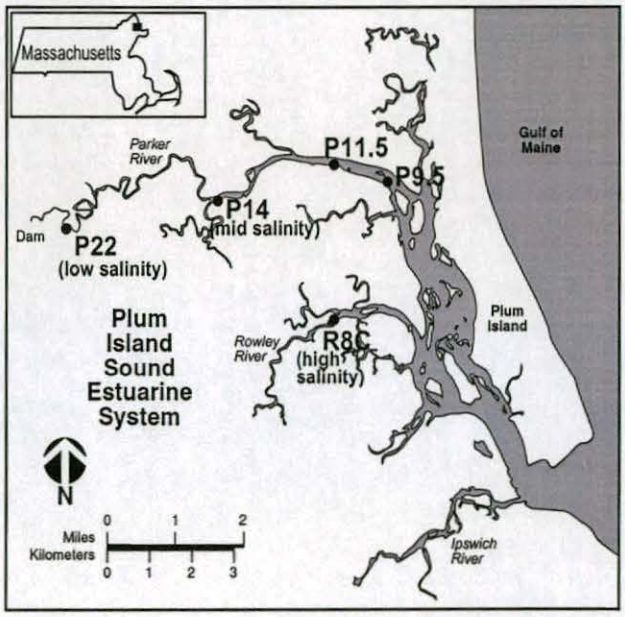

Fig. 1. Location of sampling sites in Plum Island Sound estuary, Massachusetts.

Table 1. Porewater ammonium, salinity, %C and C:N from the top 2 cm of sediment at the three sampling sites in Plum Island Sound, MA.

	Ammonium (μM)		Salinity (psu)		%C		C:N	
Site	April	August/September	April	August/September	April	August/September	April	August/September
R8C	55.0	221.4*	20.5	31.7*	2.84	2.17	12.6	13
P14	86.3	245.3*	6.3	24.7*	1.37	1.85	14.8	14.4
P22	175.5	102.8	0.5	8.7*	3.29	3.48	13.3	14.6

Asterisks indicate August/September values are significantly different from April values.

Distribution patterns of AOB in estuarine sediment were identified by TRFLP analysis of *amo*A genes from *Betaproteobacterial* AOB. Previous attempts to amplify AOB 16S rRNA genes belonging to the *Gammaproteobacteria* were unsuccessful (A.E. Bernhard, A. Schramm, and D.A. Stahl, unpublished), so we did not evaluate *amo*A genes belonging to this group. A total of seven, six and four terminal restriction fragments (TRFs) were detected at P22, P14 and R8C respectively (Fig. 2). Ammonia-oxidizing bacteria diversity at R8C was significantly lower than at P22 and P14 (Table 2), and was dominated by two TRFs (128 and 279). Terminal restriction fragment 128 was also the most abundant TRF at the P14.

We analysed similarities among AOB communities in each sample by cluster analysis, which revealed differences in variability among the three sites (Fig. 3). Ammonia-oxidizing bacteria communities at R8C were relatively stable and showed little variability over time, with all but two samples clustering together. In contrast, AOB populations at P22 and P14 were much more variable among replicates and sampling dates, as evidenced by their distribution throughout all four clusters in Fig. 3.

Ammonia-oxidizing bacteria populations at different depths (0–2 cm in April 2001, 0–4 cm in August 2001) in the sediment showed little variation with depth at the mid- (P14) and high-salinity sites (R8C) (data not shown). At the low-salinity site (P22), AOB populations differed with depth, but showed no consistent trends.

In September 2003, we compared AOB communities from intertidal and subtidal regions at each site. Intertidal samples collected from P22 and P14 were similar to the subtidal samples collected at the same time (Fig. 3). Similarly, the subtidal sample collected at R8C in September 2003 was indistinguishable from the intertidal samples collected at the same time. We also collected two additional samples in September 2003 from sites further down stream in the Parker River (P11.5 and P9.5 in Fig. 1). The AOB community in samples collected near the mouth of the Parker (P9.5) clustered with samples from R8C, while the AOB community from a site slightly higher upstream clustered with samples from P14 (Fig. 3).

The results of our TRFLP analysis indicated site-specific differences for individual TRFs that correlated with salinity. For example, TRF 97 was never detected at R8C, was infrequently (4 out of 28) detected at P14, but was found in over half (19 of 28) of the samples from P22. Terminal restriction fragment 315 was never detected at P14, and only once at R8C, but was detected in almost one third of the samples from P22. Terminal restriction fragment 330 was detected in only half the samples from R8C, but in almost all samples from P14 and P22. Relative abundance of TRF 330 was also correlated with TRF197 ($r = 0.719$). Terminal restriction fragment 197 was found in most samples from P14 (21 of 28) and P22 (15 of 28), but in only 1 of 24 samples from R8C.

Terminal restriction fragments were identified by sequence analysis of the *amo*A gene (Fig. 4). At least one *amo*A sequence was identified for all TRFs. Most of the *amo*A sequences recovered from P22 were related to the *Nitrosomonas ureae/oligotropha* cluster, and were represented by four different TRFs (97, 128, 315 and 330). A few sequences from P22 clustered with another group of *Nitrosomonas*-type sequences most closely related to *Nitrosomonas* sp. Nm143 (Purkhold *et al.*, 2003) and *N. marina/aestuarii.* This cluster also includes sequences from P14 and other mesohaline environments, and is rep-

Table 2. Diversity indices for AOB communities at the three study sites.

Diversity Index	R8C (high salinity)	P14 (mid salinity)	P22 (low salinity)
Richness[1]	3.370[a] (2–5)	4.552[b] (2–6)	4.379[b] (2–6)
Evenness[2]	0.741[a]	0.816[b]	0.803[b]
Shannon's index (H)	0.883[a]	1.213[b]	1.155[b]
Simpson's index	0.532[a]	0.64[b]	0.613[b]

1. Average number of TRFs in each sample.
2. Evenness = H/ln(richness), where $H = \Sigma P_i (\ln P_i)$, where P_i = the proportion of each TRF in a sample.
Average richness for all samples is reported followed by the range in parentheses. Values for each diversity index with the same superscript letters are not significantly different, as determined by ANOVA ($P < 0.05$).

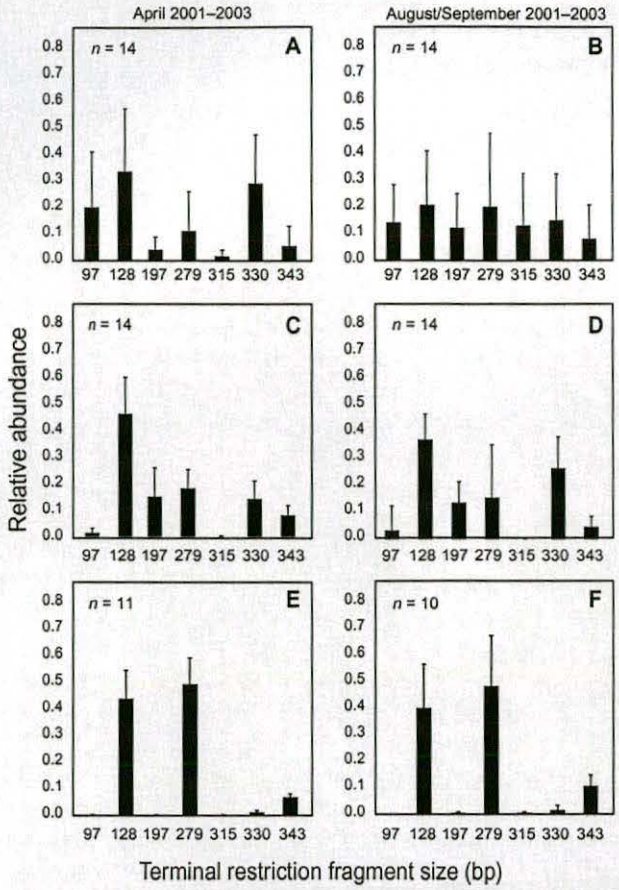

Fig. 2. AOB community profiles based on TRFLP analysis of the *amo*A gene. Bars represent data averaged over all 3 years from each site for April (A, C and E) and August/September (B, D and F). A and B are from P22 (low salinity), C and D are from P14 (mid salinity), and E and F are from R8C (high salinity). Error bars represent one standard deviation.

resented by TRFs 197 and 330. The remaining sequences from P22 did not cluster with any cultured AOB, but were phylogenetically most similar to *Nitrosospira* spp., and corresponded to TRFs 97, 128 and 343. Clones from P14 were equally distributed between *Nitrosomonas*-related sequences and *Nitrosospira*-like sequences. All sequences recovered from R8C clustered in the *Nitrosospira*-like group. Six of the seven sequences representing TRF 330 were most closely related to *N. ureae*, although *N. ureae* has a predicted TRF of 115 bp, which was not detected in any of our TRFLP results. Terminal restriction fragments 279 and 128 (with one exception) and three of the four sequences representing TRF 97 associated with sequences in the clade of *Nitrosospira*-like AOB.

Discussion

Differences in AOB distribution and activity in estuaries are undoubtedly determined by the complex interplay of biological and environmental variables. These variables include differences in substrate affinity (e.g. for ammonia and oxygen), growth rates (see Prosser, 1989 for review) and tolerance to varying salt and pH (Stehr *et al.*, 1995; Bollmann and Laanbroek, 2002). However, these physiological variables are almost certainly very underdetermined. Additionally, hydrodynamic characteristics, such as river discharge and water residence times, may exert control on AOB diversity. The relative importance of these variables, however, is unclear, because many often covary.

The AOB distributed along the estuarine gradient in Plum Island Sound are exposed to changing conditions that include varying chemical (e.g. salinity) and hydrological environments. Changes in AOB population structure in Plum Island Sound were most clearly correlated with salinity, demonstrating decreased diversity and decreased seasonal variability as salinity increased. Ammonia-oxidizing bacteria diversity was not correlated with dissolved ammonium concentrations, or other porewater constitu-

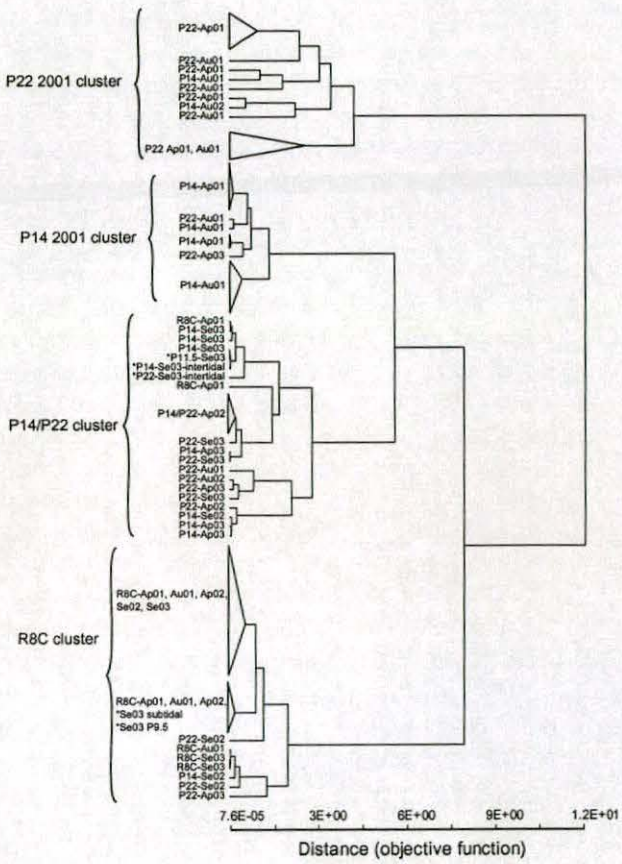

Fig. 3. Cluster analysis of AOB communities based on TRFLP patterns derived from the *amo*A gene. Sample labels correspond to sampling site (P22, P14 or R8C) and sampling date (Ap = April; Au = August; Se = September; 01 = 2001; 02 = 2002; 03 = 2003). Additional sampling sites described in the text are indicated with an asterisk.

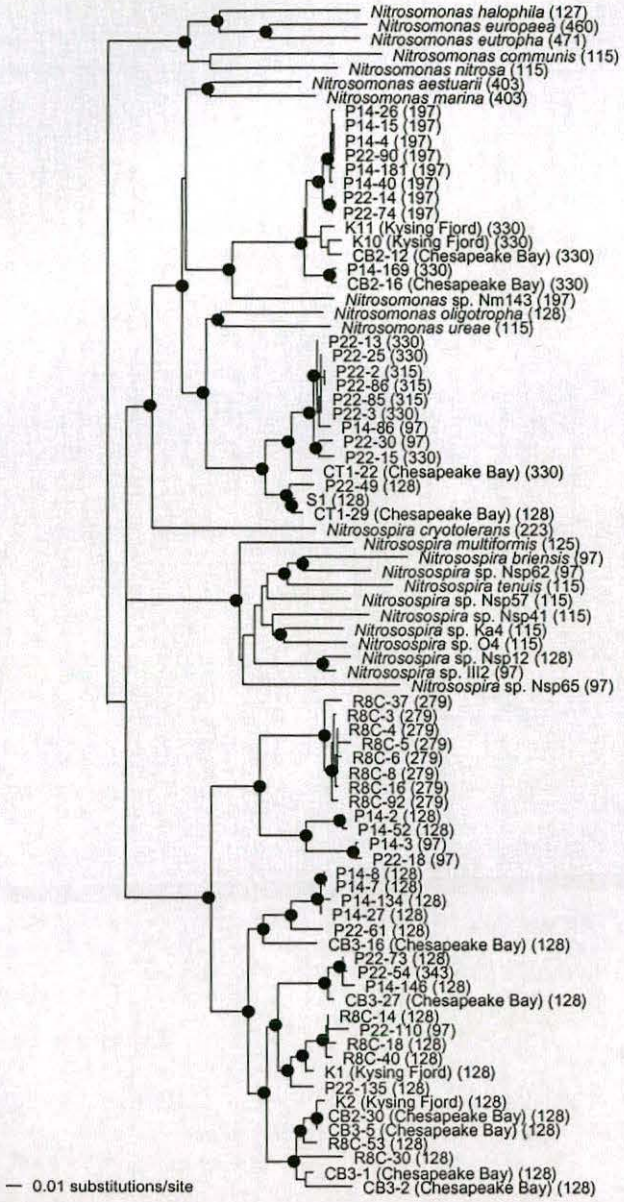

Fig. 4. Phylogenetic relationships of *amo*A sequences recovered from Plum Island Sound estuary, MA. The neighbour-joining tree is based on 376 nucleotides. Sequences from the low-, mid- and high-salinity sites are indicated by the prefixes P22, P14 and R8C respectively. Empirically determined terminal restriction fragment sizes (in base pairs) are shown parenthetically for clones in this study; TRF sizes for other sequences were determined from published sequences. Nodes supported by bootstrap values greater than 50 are indicated by '●'.

ents such as percentage C, or C/N ratios. Although there were seasonal differences in dissolved ammonium, there were no significant differences between sites. Similarly, there were no significant differences in C/N ratios between sites. There were significant differences, however, in percentage C between sites, but these differences did not correlate with differences in diversity, because percentage C at P22 was significantly different from P14, but AOB diversity at these two sites was not significantly different.

Currently there is no consensus on which measure of diversity is most appropriate for molecular microbial studies (Hughes *et al.*, 2001). Undoubtedly, some of these measures may be influenced when using TRFLP data resulting from differences in gene copy number. We cannot predict whether multiple *amo*A copies from one organism would generate the same TRF. However, results from four different diversity indices support our observations, thus moderating any potential bias of a single index.

Because our high-salinity site (R8C) was located in a different river than the mid- and low-salinity sites (P14 and P22), we wanted to confirm that the differences in AOB diversity and distribution were, in fact, related to salinity, and not simply a reflection of hydrological and physicochemical differences between the Parker and Rowley Rivers (Vorosmarty and Loder, 1994; Hopkinson *et al.*, 1999; Tobias *et al.*, 2003). Therefore, in September 2003, we collected several samples further down stream in the Parker where salinity was higher (sites shown in Fig. 1). Ammonia-oxidizing bacteria communities in these samples are consistent with a salinity effect because the mid-river sample (27 psu, water column) was more similar to samples at P14, but the sample collected near the mouth of the Parker (29 psu, water column) was indistinguishable from samples at R8C (Fig. 3).

Differences in AOB communities in Plum Island Sound appear to be correlated with the range of salinities at each site, rather than seasonal shifts in salinity. For example, if AOB populations responded to seasonal changes in salinity, we would expect a shift at the mid-salinity site in August/September to a community more similar to the high-salinity site in April. The absence of apparent seasonal shifts in the population composition of these AOB communities suggests that their population distribution in the Plum Island Sound estuary reflects an adaptation to site-specific characteristics, such as salinity. Our data do not support a cosmopolitan distribution of populations that vary in abundance as conditions change in the estuary.

Sequence analysis of *amo*A clones recovered from all three sites revealed shifts in the dominant sequence types that correlated with salinity. The dominance of *amo*A sequences related to *N. ureae* and *N. oligotropha* at P22 agrees with other studies of freshwater AOB (Speksnijder *et al.*, 1998; de Bie *et al.*, 2001), and pure culture studies, as these species have no salt requirement (Koops and Pommerening-Roser, 2001). Sequences related to *N. ureae* and *N. oligotropha* are also commonly found in other estuaries (de Bie *et al.*, 2001; Bollmann and Laanbroek, 2002; Caffrey *et al.*, 2003; Francis *et al.*, 2003). Another *Nitrosomonas*-type cluster of sequences composed primarily of clones recovered from P14 and other environmental clones from mesohaline sites in Denmark

(Nicolaisen and Ramsing, 2002) and Chesapeake Bay (Francis *et al.*, 2003) suggests a global distribution of this phylotype in brackish environments. Similarly, the *Nitrosospira*-like group is composed exclusively of *amo*A genes recovered from estuaries and includes sequences recovered from estuarine sediment in Chesapeake Bay (Francis *et al.*, 2003), and a Danish estuary (Nicolaisen and Ramsing, 2002). Finding similar sequence types in geographically distinct systems suggests a common control of AOB distribution in estuaries. Unfortunately, no cultured AOB representatives of the majority of *amo*A sequences recovered in this study are available for comparison. Analysis of *amo*A amino acid sequences (not shown) showed only minor differences with the phylogeny based on nucleotide sequences, none of which affected the placement of sequences from Plum Island Sound estuary.

In some cases, *amo*A genes belonging to distantly phylogenetically related lineages had the same TRF (e.g. TRF 97 was found in the *Nitrosomonas* sp. Nm143 cluster and the *Nitrosospira*-like cluster). It is therefore possible for the phylogenetic composition of the community to change without concomitant changes in the TRFLP patterns. Although the lack of phylogenetic resolution among TRFs precludes the use of TRFs as phylogenetic markers, as has been previously reported (Purkhold *et al.*, 2000), it does not diminish its utility as a rapid screening method to monitor changes in the community. Subsequently, TRF patterns must be confirmed by sequence analysis.

Changes in AOB communities in relation to salinity have been reported for other estuarine systems. For example, shifts in the dominant AOB in the Schelde Estuary in Denmark were correlated with increased oxygen and salinity (de Bie *et al.*, 2001). In further studies using continuous enrichment cultures of AOB derived from this estuary, Bollmann and Laanbroek demonstrated that distinct AOB communities were enriched in the presence or absence of salt (Bollmann and Laanbroek, 2002). Analysis of *amo*A clones from sites in Chesapeake Bay also revealed differences in AOB diversity and distribution related to salinity (Francis *et al.*, 2003). These studies provide strong evidence that salinity plays an important role in defining AOB distribution in estuaries.

The reduction in diversity associated with increasing salinity may simply be a consequence of fewer bacterial phylotypes adapted to marine salinity (Stehr *et al.*, 1995). However, diversity patterns more likely reflect a complex spectrum of environmental variables. The high-salinity site may be less heterogeneous, providing for restricted niche differentiation. Alternatively, extreme oscillations of environmental variables may depress diversity (Huston, 1979; Mackey and Currie, 2000). For example, samples from R8C were collected at an intertidal region, while samples from P14 and P22 were collected subtidally. Lower diversity at R8C might reflect the stress of tidal disturbance, contributing to varying oxygen availability, and intermittent drying and UV irradiation. However, a preliminary characterization of intertidal and subtidal samples from all three sites in September 2003 revealed similar community structure, suggesting that the differences were not resulting from tidal exposure. Another explanation for the observed diversity trends could be the presence of populations at the high-salinity site that are sufficiently divergent to not be amplified using the general primer set.

Although salinity appears to be a major factor driving AOB distribution in Plum Island Sound estuary, there was also some evidence for the influence of hydrological forces. Most notably, the estuary experienced near record discharge during spring runoff in March 2001. Population profiles obtained from samples collected that year from sites most directly influenced by the Parker River (P14 and P22) were distinct from those observed at these same sites the following 2 years (Fig. 3). In contrast, only two of 16 samples from the Rowley River collected in 2001 deviated from the other samples collected at this site. Previous studies of the hydrodynamics of Plum Island Sound show that the Parker River experiences significantly larger changes in river discharge, compared with the Rowley (Vallino and Hopkinson, 1998). Thus, the near-record 2001 flooding disturbance may have altered the AOB communities by contributing to a transient change in chemical and nutrient structure (e.g. via nutrient input, import of allocthonous microbes, or intensive disturbance of the sediments).

The patterns of AOB diversity and distribution in Plum Island Sound estuary suggest that salinity is a major controlling factor of their diversity and distribution. Previous studies have also shown that estuarine organisms are often restricted to particular ranges of environmental gradients, resulting in well-developed distribution patterns (Ysebaert *et al.*, 2003). Our study, along with many others of AOB in natural systems, also underscores the need for better efforts in cultivation of AOB that are well represented in clone libraries but not in culture collections. It seems apparent from our studies that the distribution of AOB in Plum Island Sound estuary corresponds with predictions of niche differentiation and resource exploitation, which could be more fully explored by physiological characterizations of the species involved. Additionally, the observation of similar patterns of AOB diversity and distribution in other estuaries suggests that many key controlling variables are common to globally distributed systems. Thus, these data should contribute to a better understanding of environmental factors, in addition to salinity, that determine distribution patterns, and ultimately serve as a framework for relating these patterns to nitrogen cycling in estuaries.

Experimental procedures

Site descriptions

The Plum Island Sound Estuary is a coastal-plain estuary located in north-east Massachusetts (Fig. 1). Tidal range averages 2.6 m (Vorosmarty and Loder, 1994), annual water temperature ranges from –1.0 to 28°C, and mean depth at low tide is less than 2 m (Hopkinson *et al.*, 1999). The upper Parker site (P22) is surrounded by brackish marshes dominated by *Typha*, *Scirpus* and *Carex* while the mid-estuary site (P14) is surrounded by marshes dominated by *Spartina alterniflora* and *S. patens*, vegetation typical for New England salt marshes. The other site (site R8C) was located near the lower end of the Rowley River and surrounded by *Spartina* marshes. During our study, salinity in the overlying water at the time of sampling (all at low tide) ranged from 0 to 12 psu at site P22 (low-salinity site), 1–26 psu at site P14 (mid-salinity site) and 19–34 psu at site R8C (high-salinity site). Both sites in the Parker River were taken on shallow subtidal mudflats. The Rowley site was in the lower intertidal and exposed to air for about 1–2 h per tide. Details of the sediment chemistry, sediment metabolism and sediment nutrient fluxes for P22 and P14 are given in Hopkinson *et al.* (1999), where they are referred to as P2 and P5 respectively. All sites were made up of fine grained muds.

Sample collection

Replicate sediment cores (6.5-cm diameter) were collected from 2001 to 2003 during low tide from sites representing low, mid and high salinity in Plum Island Sound, MA in spring (April), when salinities are at their lowest levels because of high discharge, and late summer (August/September), when salinities are at their highest levels (Fig. 1). Cores were kept dark and transported at *in situ* water temperature to the laboratory (about 2 h). In April 2001, duplicate cores were sectioned into 0.5 cm increments down to 2 cm. Because we did not observe significant changes in community patterns or a significant decrease in potential nitrification rates (A. E. Giblin, unpublished) within the first 2 cm, we sampled to 4 cm in August 2001, and cores were sectioned as follows: 0–0.5, 0.5–1.0, 1.0–2.0 and 2.0–4.0 cm. In 2002 and 2003, the first 2 cm of each core were analysed as a single sample, but replication was increased to triplicate cores. Sections were homogenized, divided into approximately 0.5 g aliquots for community analysis, and stored at –80°C until processing. An additional 5–10 g sample was dried for the measurement of total sediment carbon and nitrogen using a Perkin Elmer 2400 CHN Elemental Analyzer. At each sampling time, an additional 6.5 cm core was taken for porewater analysis. Cores were sectioned into 0–1 or 0–2 cm sections, centrifuged, and the porewater analysed for dissolved ammonium and salinity. Salinity was measured on a refractometer and ammonium was analysed using the (Solorzano, 1969) method modified for small sample size.

In September 2003, one 2.5-cm core was collected intertidally from P14 and P22 and subtidally from R8C. Two additional 2.5 cm cores were collected further down the Parker River from P11.5 and P9.5 (Fig. 1). The top 2 cm were collected from each core and 0.5 g subsamples of each were stored at –80°C for DNA extractions.

DNA extractions from sediment

DNA was extracted from approximately 0.5 g sediment using the Fast DNA kit for Soil (Qbiogene, Carlsbad, CA) following the manufacturer's directions except we used 0.5 g of 0.1 mm zirconium beads instead of the bead matrix supplied with the kit. Extracts were characterized by electrophoresis on 1% agarose gels and DNA concentrations were estimated by comparing the band intensities to a high DNA mass ladder (Invitrogen, Carlsbad, CA) using NIH image analysis software v1.63.

Terminal restriction fragment length polymorphism (TRFLP) analysis

Approximately 20 ng of DNA was amplified using primers specific for the *amo*A gene (Rotthauwe *et al.*, 1997) using the amoA-2R-TC modification (Nicolaisen and Ramsing, 2002). The forward primer (amoA-1F) was fluorescently labelled with FAM (Qiagen, Valencia, CA). Each 20 µl reaction contained 1X buffer with $(NH_4)_2SO_4$ (MBI, Fermentas, Hanover, MD), 320 ng μl^{-1} non-acetylated bovine serum albumin (Sigma, St. Louis, MO), 3.75 mM $MgCl_2$, 0.2 µM each primer, 200 µM dNTP and 0.5 units of *Taq* polymerase (MBI Fermentas). A PTC-100 thermal cycler (MJ Research, Waltham, MA) was used for all reactions with the following cycle sequence: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 45 s, with a final 5 min extension at 72°C. For a few samples that were particularly recalcitrant to PCR amplification of *amo*A, 15 cycles of PCR were performed using unlabelled primers, followed by 25 cycles of PCR using the fluorescently labelled forward primer. This approach was chosen based on the observation that many of these recalcitrant samples amplified better when using non-labelled primer. Polymerase chain reaction product concentrations were estimated following electrophoresis on 1% agarose gels by comparing the band intensities to a low DNA mass ladder (Invitrogen).

Polymerase chain reaction products were digested with 10 units of *Acl*I (New England Biolabs, Beverly, MA) overnight at 37°C. We chose *Acl*I based on *in silico* analysis of 63 published *amo*A sequences using the restriction enzyme cutting program in MacVector v.6.5 (Accelrys, San Diego, CA). Following ethanol precipitation, approximately 15–25 fmols of digested PCR products were resolved on a Long Ranger polyacrylamide gel (FMC, Rockland, ME) on an ABI 377 automated DNA sequencer using GeneScan software (Applied Biosystems, Fremont, CA). The internal size standard, GS500-ROX was loaded in each lane. Terminal restriction fragment sizes and relative abundances were estimated using DAx software (van Mierlo Software Consultancy, the Netherlands).

Clone library construction and analysis

Polymerase chain reaction products from all sediment samples collected in April 2001 were amplified as described above using unlabelled primers. Polymerase chain reaction products were gel purified using the Qiaquick Gel Extraction Kit (Qiagen), pooled for each site, and cloned using the TOPO TA cloning kit (Invitrogen) according to the manufac-

turer's recommendations. Two additional clone libraries were constructed from selected samples in April 2003 from sites P22 and P14. Ninety-six transformants were randomly selected from each clone library and inoculated into 100 µl of Luria–Bertani (LB) broth with 50–100 µg ampicillin μl^{-1} in 96-well microtiter plates and incubated overnight at 37°C. Twenty-four clones from each library from April 2001 and 36 clones from April 2003 libraries were randomly selected for TRFLP analysis as described above. At least two clones from each site representing each TRF size were chosen for sequencing.

Sequence analysis

Fifty clones were amplified as described above, treated with ExoSapIT (Amersham, Piscataway, NJ) according to the manufacturer's recommendations, and sequenced using the vector specific primer, M13r, and dye-terminator chemistry on a MegaBACE 1000 automated sequencer. Sequences were inspected using Sequencher v4.0 (Gene Codes, Ann Arbor, MI) and submitted to BLAST v2.0 to identify closely related sequences. Both nucleotide and amino acid alignments were created using the FastAligner function in ARB (Ludwig *et al.*, 2004) and checked manually. Phylogenetic analyses were performed in PAUP* v4.0 (Swofford, 1991). Phylogenetic trees were constructed using neighbour-joining, maximum parsimony and maximum likelihood methods. The relative confidence in branching order for neighbour-joining and parsimony analyses was evaluated by performing 100 bootstrap replicates. Sequences were checked for possible chimeric structure by comparison of neighbour-joining trees based on different regions of the sequence (Kopczynski *et al.*, 1994).

GenBank accession numbers

These sequence data have been submitted to the GenBank database under the Accession numbers AY702567-AY702615.

Statistical analysis

Regression analyses and ANOVA were performed in StatView, v.5.0.1 (SAS Institute, Cary, NC). Cluster analysis was performed in PC-Ord version 4 (McCune and Mefford, 1999). The relative abundance data from TRFLP analyses were transformed by an arcsine square root function to reduce skew. Cluster analysis was performed using a Euclidean distance measure with Ward's method for group linkage.

Acknowledgements

We thank Jane Tucker and Michele Bahr for sample collection. We also thank Caprice Rosato from the Center for Gene Research and Biotechnology at Oregon State University for TRFLP analyses and Meredith Hullar for help with DAx software. This work was funded by National Science Foundation Grant (DEB-9977897) and a National Science Foundation Postdoctoral Research Fellowship to A.E.B. Salary support to A.E.G. was provided through NSF-OCE-9726921 and NOAA National Sea Grant Program, Department of Commerce, under Grant No. NA16RG2273, Woods Hole Oceanographic Sea Grant Project No. R/M-50. The views expressed here are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies.

References

Bernhard, A.E., and Peele, E.R. (1997) Nitrogen limitation of phytoplankton in a shallow embayment in northern Puget Sound. *Estuaries* **20:** 759–769.

de Bie, M.J.M., Speksnijder, A., Kowalchuk, G.A., Schuurman, T., Zwart, G., Stephen, J.R., *et al.* (2001) Shifts in the dominant populations of ammonia-oxidizing beta-subclass proteobacteria along the eutrophic Schelde Estuary. *Aquat Microb Ecol* **23:** 225–236.

Boatman, C.D., and Murray, J.W. (1982) Modeling exchangeable NH4+ adsorption in marine-sediments – process and controls of adsorption. *Limnol Oceanogr* **27:** 99–110.

Bollmann, A., and Laanbroek, H.J. (2002) Influence of oxygen partial pressure and salinity on the community composition of ammonia-oxidizing bacteria in the Schelde Estuary. *Aquat Microb Ecol* **28:** 239–247.

Boynton, W.R., and Kemp, W.M. (1985) Nutrient regeneration and oxygen-consumption by sediments along an estuarine salinity gradient. *Mar Ecol Prog Ser* **23:** 45–55.

Caffrey, J.M., Harrington, N., Solem, I., and Ward, B.B. (2003) Biogeochemical processes in a small California estuary. 2. Nitrification activity, community structure and role in nitrogen budgets. *Mar Ecol Prog Ser* **248:** 27–40.

Cebron, A., Berthe, T., and Garnier, J. (2003) Nitrification and nitrifying bacteria in the lower Seine River and Estuary (France). *Appl Environ Microbiol* **69:** 7091–7100.

Francis, C.A., O'Mullan, G.D., and Ward, B.B. (2003) Diversity of ammonia monooxygenase (*amoA*) genes across environmental gradients in Chesapeake Bay sediments. *Geobiology* **1:** 129–140.

Hastings, R.C., Saunders, J.R., Hall, G.H., Pickup, R.W., and McCarthy, A.J. (1998) Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. *Appl Environ Microbiol* **64:** 3674–3682.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J., and Saunders, J.R. (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16s ribosomal-RNA gene-sequences. *J Gen Microbiol* **139:** 1147–1153.

Hiorns, W.D., Hastings, R.C., Head, I.M., McCarthy, A.J., Saunders, J.R., Pickup, R.W., and Hall, G.H. (1995) Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. *Microbiology* **141:** 2793–2800.

Hopkinson, C.S.J., Giblin, A.E., Tucker, J., and Garritt, R.H. (1999) Benthic metabolism and nutrient cycling along an estuarine salinity gradient. *Estuaries* **22:** 863–881.

Horz, H.P., Rotthauwe, J.H., Lukow, T., and Liesack, W. (2000) Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of amoA PCR products. *J Microbiol Methods* **39:** 197–204.

Howarth, R.W. (1988) Nutrient limitation of net primary production in marine ecosystems. *Annu Rev Ecol* **19:** 89–110.

Hughes, J.B., Hellmann, J.J., Ricketts, T.H., and Bohannan, B.J. (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl Environ Microbiol* **67:** 4399–4406.

Huston, M. (1979) A general hypothesis of species diversity. *Am Nat* **75:** 406–418.

Koops, H.P., and Pommerening-Roser, A. (2001) Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. *FEMS Microbiol Ecol* **37:** 1–9.

Kopczynski, E.D., Bateson, M.M., and Ward, D.M. (1994) Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Appl Environ Microbiol* **60:** 746–748.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, Yadhukumar, H., Buchner, A., *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363–1371.

McCune, B., and Mefford, M.J. (1999) *PC-ORD, Multivariate Analysis of Ecological Data*. Gleneden Beach, OR, USA: MjM Software.

Mackey, R.L., and Currie, D.J. (2000) A re-examination of the expected effects of disturbance on diversity. *Oikos* **88:** 483–493.

Nicolaisen, M.H., and Ramsing, N.B. (2002) Denaturing gradient gel electrophoresis (DGGE) approached to study the diversity of ammonia-oxidizing bacteria. *J Microbiol Methods* **50:** 189–203.

Nixon, S.W. (1995) Coastal marine eutrophication: a definition, social causes, and future concerns. *Ophelia* **41:** 199–219.

Nold, S.C., Zhou, J.Z., Devol, A.H., and Tiedje, J.M. (2000) Pacific northwest marine sediments contain ammonia-oxidizing bacteria in the beta subdivision of the proteobacteria. *Appl Environ Microbiol* **66:** 4532–4535.

Phillips, C.J., Smith, Z., Embley, T.M., and Prosser, J.I. (1999) Phylogenetic differences between particle-associated and planktonic ammonia-oxidizing bacteria of the beta subdivision of the class proteobacteria in the northwestern Mediterranean Sea. *Appl Environ Microbiol* **65:** 779–786.

Prosser, J.I. (1989) Autotrophic nitrification bacteria. *Adv Microb Physiol* **30:** 125–181.

Purkhold, U., Pommerening-Roser, A., Juretschko, S., Schmid, M.C., Koops, H.P., and Wagner, M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. *Appl Environ Microbiol* **66:** 5368–5382.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Roser, A., and Koops, H.-P. (2003) 16S rRNA and amoA-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. *Int J Syst Evol Microbiol* **53:** 1485–1494.

Rotthauwe, J.H., Witzel, K.P., and 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.

Rysgaard, S., Thastum, P., Dalsgaard, T., Christensen, P.B., and Sloth, N.P. (1999) Effects of salinity on NH_4^+ adsorption capacity, nitrification, and denitrification in Danish estuarine sediments. *Estuaries* **22:** 21–30.

Seitzinger, S.P. (1988) Denitrification in fresh-water and coastal marine ecosystems – ecological and geochemical significance. *Limnol Oceanogr* **33:** 702–724.

Solorzano, L. (1969) Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol Oceanogr* **14:** 799–801.

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

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

Stephen, J.R., McCaig, A.E., Smith, Z., Prosser, J.I., and Embley, T.M. (1996) Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. *Appl Environ Microbiol* **62:** 4147–4154.

Swofford, D.L. (1991) *PAUP: Phylogenetic Analysis Using Parsimony*. Fitchberg, MA, USA: Sinauer Associates.

Tobias, C.R., Cieri, M., Peterson, B.J., Deegan, L.A., Vallino, J., and Hughes, J. (2003) Processing watershed-derived nitrogen in a well-flushed New England estuary. *Limnol Oceanogr* **48:** 1766–1778.

Vallino, J.J., and Hopkinson, C.S. (1998) Estimation of dispersion and characteristic mixing times in Plum Island Sound estuary. *Estuar Coastal Shelf Sci* **46:** 333–350.

Vorosmarty, C.J., and Loder, T.C. (1994) Spring-neap tidal contrasts and nutrient dynamics in a marsh-dominated estuary. *Estuaries* **17:** 537–551.

Ysebaert, T., Herman, P.M.J., Meire, P., Craeymeersch, J., Verbeek, H., and Heip, C.H.R. (2003) Large-scale patterns in estuaries: estuarine macrobenthic communities in the Schelde Estuary, NW Europe. *Estuar Coastal Shelf Sci* **57:** 335–355.