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Functionally distinct communities of ammonia-oxidizing bacteria along an estuarine salinity gradient

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Summary

The relationship between ammonia-oxidizing bacteria (AOB) and potential nitrification rates was examined along a salinity gradient in a New England estuary in spring and late summer over 3 years. Ammonia-oxidizing bacteria abundance was estimated by measuring gene copies of the ammonia monooxygenase catalytic subunit (*amo*A) using real-time polymerase chain reaction. Ammonia-oxidizing bacteria abundance ranged from below detection to 6.0×10^7 *amo*A copies (gdw sediment)$^{-1}$. Mean potential nitrification rates ranged from 0.5 to 186.5 nmol N (gdw sediment)$^{-1}$ day^{-1}. Both AOB abundance and potential rates were significantly higher in spring than late summer. Correlations between rates and abundance varied significantly among sites, but showed site-specific ammonia oxidation kinetics related to AOB community structure. The effect of salinity on potential nitrification rates was evaluated by incubating sediment from each site under four salinity conditions (0, 5, 10 and 30 psu). At all sites, rates were generally highest in the intermediate salinity treatments, but rates at the upstream site were inhibited at high salinity, while rates at the two downstream sites were inhibited at the lowest salinity. Although salinity appears to be an important factor in determining AOB distribution, it may not be the primary factor as AOB exhibited a broad range of salinity tolerance in our experiments. Our results indicate that there are significant differences in abundance and community composition of AOB along the salinity gradient, and the differences are reflected in community function.

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Introduction

Nitrification, the sequential oxidation of ammonium to nitrite and then nitrate, is a key part of nitrogen transformations in the environment and is fundamental to ecosystem processes. For example, the rates of nitrite and nitrate production often determine the rate of denitrification, leading to the removal of N from the system. Thus, environmental variables influencing ammonia oxidation in productive systems such as estuaries have received considerable attention. Temperature (Berounsky and Nixon, 1993), light (Horrigan and Springer, 1990), oxygen (Usui *et al.*, 2001), ammonium (Stehr *et al.*, 1995; Magalhaes *et al.*, 2005), sulfide (Joye and Hollibaugh, 1995) and residence time (Brion *et al.*, 2000) are all reported to affect nitrification rates in estuaries. Other investigators have examined how the environment shapes community structure, demonstrating that some of the same factors influence the community composition of ammonia-oxidizing bacteria (AOB) (de Bie *et al.*, 2001; Bollmann and Laanbroek, 2002; Francis *et al.*, 2003; Bernhard *et al.*, 2005; Freitag *et al.*, 2006). However, as yet few studies have reported on relationships among physical/chemical variables, population structure and activity in estuaries (Bollmann *et al.*, 2002; Caffrey *et al.*, 2003; O'Mullan and Ward, 2005).

Nitrification was long thought mediated by two functionally distinct groups of bacteria – AOB and nitrite-oxidizing bacteria (NOB). Although known AOB associate with both the Gammaproteobacteria and Betaproteobacteria, most described species are affiliated with the latter, and environmental studies have focused primarily on the Betaproteobacteria because of their monophyletic nature and relative abundance in many environments. More recent microbiological and environmental studies have revealed that our current understanding of ammonia-oxidizing microorganisms is incomplete in both breadth and depth of their diversity. Comparative sequencing of genes encoding the 16S rRNA and the catalytic subunit of the ammonia monooxygenase (AmoA) recovered from environmental samples has shown that proteobacterial AOB in culture represent only a fraction of the natural diversity within this division (Kowalchuk and Stephen, 2001). A far greater breadth of evolutionary diversity was revealed by the discovery of anaerobic ammonia oxidation (anammox) (Strous *et al.*, 1999) and ammonia-oxidizing

Crenarchaea (Könneke *et al.*, 2005). These discoveries further underscore the importance of linking diversity, activity, and controlling environmental variables.

Previously, we demonstrated that the AOB communities in Plum Island Sound estuary in north-eastern Massachusetts differed significantly along a salinity gradient, with communities at the high-salinity site significantly less diverse than at the mid- or low-salinity sites (Bernhard *et al.*, 2005). Many studies have shown a significant effect of salinity on nitrification rates and AOB community composition in estuarine sediments, but the cause remains unclear. It is known that salinity plays a major role in ammonium adsorption in sediment (Boatman and Murray, 1982; Seitzinger *et al.*, 1991; Rysgaard *et al.*, 1999), but many other environmental factors co-vary with salinity, making it difficult to associate individual variables with nitrification activity. The salinity effect may also reflect physiological changes in AOB or shifts in community composition brought about by changes in salinity (Bollmann and Laanbroek, 2002). In this study, we investigated: (i) the direct effect of salinity on potential nitrification rates; (ii) the link between potential nitrification rates and abundance of the ammonia monooxygenase gene (*amo*A); and (iii) the relationship between potential rates and AOB community composition. These studies underscored the complexity of the process, but demonstrated strong correlations among AOB community structure, salinity and potential nitrification rates in this natural system.

Results

We measured potential nitrification rates and AOB abundance in sediment samples collected in spring and late summer from sites representing low-, mid- and high-salinity conditions. Ammonium and phosphate were added to each sample and NO_3^- production was monitored over 3 days. Rates over the 3-year sampling period ranged from 0 to 350.2 nmol N gdw^{-1} day^{-1}. Mean rates in April were generally about an order of magnitude higher (21.5–186.5 nmol N gdw^{-1} day^{-1}) than in August or September (0.5–14.3 nmol N gdw^{-1} day^{-1}). We observed similar seasonal patterns of AOB abundance, with AOB abundance [estimated by real-time polymerase chain reaction (PCR) quantification of the *amo*A gene] ranging from below detection to 6.0×10^7 gene copies gdw^{-1} (Fig. 1). Abundance of *amo*A was generally much lower in August and September compared with April for all sites, and *amo*A abundance was always lowest at the low-salinity site (P22) for all sampling dates. In 2001, *amo*A abundance was highest at the high-salinity site (R8C) in April and August. In April 2003, although *amo*A abundance between the low- and mid-salinity sites differed by two orders of magnitude, the differences were not significant (Student's *t*-test, $P = 0.07$, d.f. = 4).

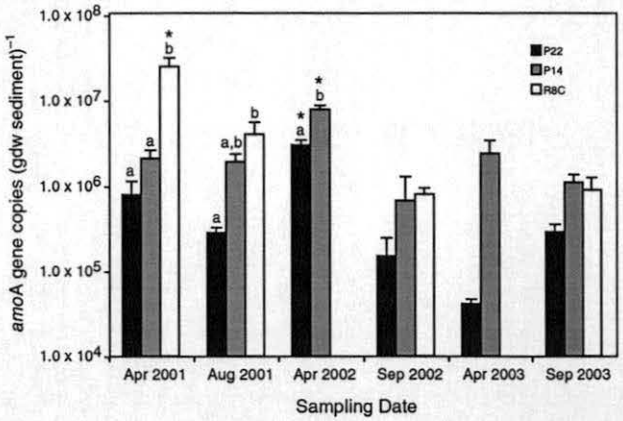

Fig. 1. Mean *amo*A gene copies per gram of dry weight of sediment in the top 0–2 cm at low- (P22), mid- (P14) and high-salinity (R8C) sites in Plum Island Sound, MA. Means for April 2001 represent duplicate samples from 0–0.5, 0.5–1.0, 1.0–1.5 and 1.5–2.0 cm horizons averaged together. Means for August 2001 represent duplicate samples from 0–0.5, 0.5–1.0 and 1.0–2.0 cm horizons averaged together. All other data represent triplicate samples from 0–2 cm. No data were available for R8C in April 2002 or April 2003. Error bars represent the standard error of the mean. Within each sampling date, bars with different letters are significantly different. Asterisks indicate significance ($\alpha = 0.05$) among samples from the same site.

To further assess the salinity effect, potential nitrification rates were measured under four salinity conditions: 0, 5, 10 and 30 psu. During April, the highest potential nitrification rates were usually detected at 10 psu for all sites (Fig. 2), although on one sampling date, the highest rates at P22 were recorded in the 0 psu treatment. Additionally, potential rates at P22 were significantly inhibited at 30 psu compared with the maximum response. In contrast, potential nitrification at P14 and R8C was inhibited at

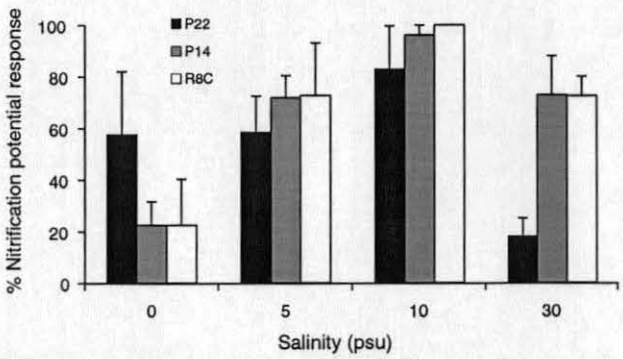

Fig. 2. Nitrification potential, as mean per cent response versus salinity at low- (P22), mid- (P14) and high-salinity (R8C) sites in Plum Island Sound, MA. Rates for each site were scaled to the maximum rate at any salinity for each sampling period and then averaged for the 3 years. Rates were measured under four salinity conditions (0, 5, 10 and 30 psu) in April 2001, 2002 and 2003 for all sites except R8C where data were taken in 2001 and 2003 only. Error bars represent one standard deviation. Average *in situ* salinity (psu) ranged from 0.5 to 8.7 (P22), 6.3 to 24.7 (P14) and 20.5 to 31.7 (R8C).

Table 1. Mean potential nitrification rates [nmol N (gdw sediment)$^{-1}$ day^{-1}] (± SD) at different sediment depths from samples collected in 2001.

Depth (cm)	April 2001			August 2001		
	P22	P14	R8C	P22	P14	R8C
0–0.5	21.4 (9.7)	60.9 (19.7)	76.0 (82.8)	0.4 (0.6)	2.1 (2.9)	1.6 (0.7)
0.5–1.0	2.6 (3.6)	40.0 (22.3)	96.5 (30.1)	1.5 (2.1)	29.0 (29.6)	3.2 (0.7)
1.0–1.5	5.5 (2.9)	29.6 (13.8)	210.0 (27.2)	–	–	–
1.5–2.0	1.8 (2.6)	24.6 (11.1)	135.5 (56.6)	–	–	–
1.0–2.0	–	–	–	1.1 (1.6)	24.7 (22.9)	2.5 (3.5)
2.0–4.0	–	–	–	1.7 (2.5)	21.3 (23.6)	4.7 (0.1)

Rates were determined in salinity treatments closest to *in situ* salinity.

0 psu. In August and September in all 3 years, rates were low and highly variable and no meaningful trends in response to salinity were detected (data not shown). In 2001, we also measured potential nitrification rates at different sediment depths (Table 1). Rates were measured under near *in situ* salinity conditions. In April 2001, rates generally decreased with depth at P22 and P14. At R8C, however, we observed the opposite trend, with rates increasing with depth.

To further investigate site-specific differences and the link between ammonia oxidation and specific populations, we used *amo*A abundance and potential nitrification rates to calculate cellular oxidation rates for each site. Assuming 2.5 *amo*A copies per cell (Norton *et al.*, 1996) and that AOB are the only ammonia oxidizers in the system, average cell-specific ammonia oxidation rates differed by more than two orders of magnitude ($1.6 \pm 0.7 \times 10^{-2}$, $2.0 \pm 0.5 \times 10^{-3}$, $2.9 \pm 0.6 \times 10^{-4}$ fmols N cell^{-1} h^{-1} at the low-, mid- and high-salinity sites respectively).

We also investigated the relationship between AOB abundance and activity at the three sites. Correlations between potential nitrification rates and *amo*A copy number varied among sites, with the highest correlation detected at the high-salinity site ($r = 0.96$) (Fig. 3). The slopes of the regressions were also significantly different (ANCOVA, $P < 0.0005$).

To assess the correlation of community structure and potential nitrification rates, we re-analysed the terminal restriction fragment length polymorphism (TRFLP) data reported in the study by Bernhard and colleagues (2005) using non-metric multidimensional scaling (NMS). The ordination of the samples explained 95% of the variability in the data, with 74% attributed to axis 1 (Fig. 4). Three conspicuous clusters (A, B and C in Fig. 4) included all samples with rates > 40 nmol N gdw^{-1} day^{-1}, although some samples within the clusters had lower rates. Additionally, mean rates among the three clusters were not significantly different (ANOVA, $F = 0.52$, $P = 0.59$, d.f. = 37), but diversity (Shannon's Index) was significantly different for all three clusters (ANOVA, $F = 56.9$, $P < 5 \times 10^{-12}$, d.f. = 37). Closer inspection of the AOB communities revealed that samples within each cluster were always comprised of the same *amo*A TRFs, with samples in clusters A, B and C comprised of five, four and three TRFs respectively. TRFs 128, 279 and 343 represent *Nitrosospira*-like sequences primarily, while TRFs 197 and 330 represent *Nitrosomonas*-related sequences (Bernhard *et al.*, 2005). Samples not included in clusters A, B, or C were comprised of many of the same TRFs, but with different relative abundance patterns, and often included additional TRFs not included in samples in clusters A, B and C. Sequence identification of all *amo*A TRFs can be found in the study by Bernhard and colleagues (2005).

Discussion

Understanding the link between microbial diversity and ecosystem processes is a fundamental goal of microbial ecologists. It is paramount to understand how changes in diversity affect nutrient cycling and energy transfer in ecosystems, yet it remains poorly understood. Although a variety of researchers have attempted to address the issue (e.g. Naeem and Li, 1997; Garland and Lehman,

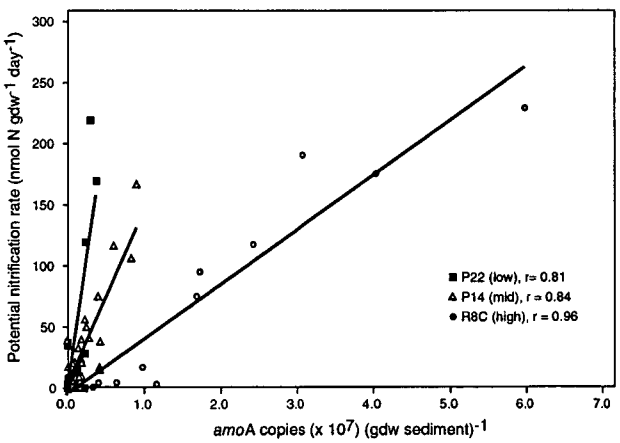

Fig. 3. Correlation between potential nitrification rates and *amo*A gene copy number at sites along a salinity gradient in Plum Island Sound, MA. Correlation coefficients (r) are shown for regression lines for samples collected at low- (P22), mid- (P14) and high-salinity (R8C) sites.

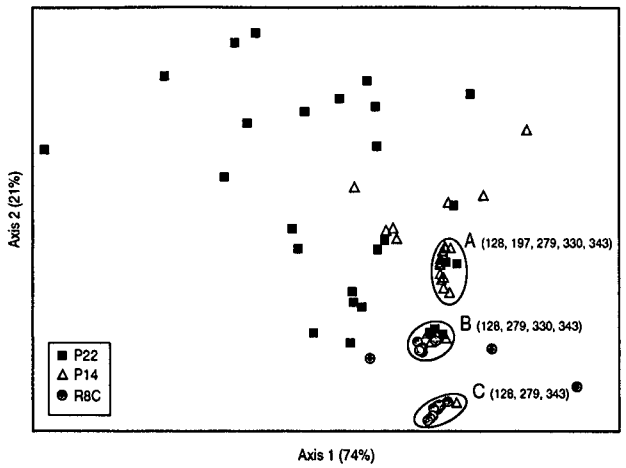

Fig. 4. Non-metric multidimensional scaling ordination of sediment samples based on TRFLP analysis of the *amo*A gene. Each symbol represents the ammonia-oxidizing community based on relative abundance of TRFs of the *amo*A genes. The distance between points is proportional to the dissimilarity of the communities. All samples with potential nitrification rates > 40 nmol N (gdw sediment)$^{-1}$ day^{-1} are included in clusters marked A, B and C. Terminal restriction fragments that comprise the AOB communities of samples in each cluster are indicated parenthetically. Sequence identification of *amo*A TRFs is described in the study by Bernhard and colleagues (2005). Sites represent low (P22), mid (P14) and high (R8C) salinity.

1999; Bell *et al.*, 2005), most have not been able to reach conclusive results that relate directly to natural systems. Using soil AOB as a model in microcosm (Webster *et al.*, 2005) and manipulated systems (Horz *et al.*, 2004), researchers have recently found evidence for a relationship between AOB community structure and ecosystem functioning. By examining the population structure of estuarine sediment AOB in a natural system in relation to salinity and potential activity, we observed site-specific differences in nitrification rates that were correlated with community composition and abundance of AOB. These studies suggest that changes in AOB community structure may lead to alterations in ecosystem functioning.

Potential nitrification rates in our study were highly variable, but were similar to potential rates from other studies in estuarine (Rysgaard *et al.*, 1999; Caffrey *et al.*, 2003; Dollhopf *et al.*, 2005) and freshwater sediments (Bodelier *et al.*, 1996). Potential rates are often much higher than *in situ* rates possibly because of non-limiting oxygen and substrate concentrations, while rates measured *in situ* may be substrate-limited. In some studies, potential rates have been found to correlate well with *in situ* nitrification rates (Henriksen *et al.*, 1981; Kemp *et al.*, 1990), although other studies have not found consistent correlations (Caffrey *et al.*, 2003). Similarly, our estimates of AOB abundance, measured by real-time PCR quantification of *amo*A genes, were comparable to estimates of AOB in other marine or estuarine environments using competitive PCR (Dollhopf *et al.*, 2005) and real-time PCR of the *amo*A gene (Risgaard-Petersen *et al.*, 2004).

Correlation between rates and amo*A abundance*

Because potential nitrification rates are thought to roughly represent the abundance of AOB (Henriksen, 1980), we expected these two parameters to show a strong correlation. Although we did find strong correlations, the relationships were site-specific, suggesting functionally distinct ammonia-oxidizing communities along the salinity gradient. Interestingly, potential rates at R8C were generally lower than rates at P14 or P22, but *amo*A abundance was often higher and better correlated with the rates. These results have several possible explanations: (i) AOB activity at R8C was inhibited during the potential rate incubations; (ii) there are other ammonia oxidizers at P22 and P14 contributing to nitrification that are not detected by the *amo*A primers; or (iii) AOB at R8C have lower cell-specific ammonia oxidation rates.

Inhibition of nitrifiers by ammonium has been reported previously (Stehr *et al.*, 1995; Suwa *et al.*, 1997). In fact, some nitrifiers are inhibited at ammonium levels as low as 0.2 mM (Magalhaes *et al.*, 2005), which is below the ammonium concentration used in our study (0.3 mM). Because the AOB communities at R8C are significantly different from those at P22 and P14 (Bernhard *et al.*, 2005), it is possible that the AOB at R8C were more sensitive to ammonium. However, we used ammonium concentrations that were similar to *in situ* ammonium concentrations (Hopkinson *et al.*, 1999). Additionally, the high correlation coefficient between potential rates and *amo*A abundance at R8C makes this explanation seem unlikely.

Although other studies have found good correlations between *amo*A abundance (measured by real-time PCR) and potential nitrification activity in marine sediment microcosms (Risgaard-Petersen *et al.*, 2004) and salt marsh sediment (Dollhopf *et al.*, 2005), a second explanation for the observed varying relationships between rates and AOB abundance in our study is the presence of ammonia-oxidizing microorganisms not targeted by the *amo*A assay. Our estimation of AOB abundance based on *amo*A gene copy number targets only the AOB within the Betaproteobacteria, which are thought to be most abundant in estuarine systems (Francis *et al.*, 2003; Bernhard *et al.*, 2005). However, there may be other microorganisms contributing to nitrification, such as methane-oxidizing bacteria, which are known to be capable of oxidizing ammonia under certain conditions (Bedard and Knowles, 1989). It is also possible that the recently discovered ammonia-oxidizing Archaea (Könneke *et al.*, 2005) are active in this system and differentially distributed relative to the AOB. 16S rRNA gene sequences related to ammonia-oxidizing Archaea have been

detected in Plum Island Sound sediment (Könneke *et al.*, 2005); however, their abundance and distribution have not been determined.

Thirdly, site-specific relationships between potential nitrification rates and *amo*A abundance may reflect differences in cellular ammonia oxidation rates. Differences in substrate affinity, growth rates, recovery after starvation and nitrification kinetics among AOB isolates have been well-characterized in laboratory studies (Prosser, 1989; Koops and Pommerening-Roser, 2001; Bollmann and Laanbroek, 2002; Webster *et al.*, 2005). Oxidation rates among cultivated AOB are known to vary considerably, often by an order of magnitude or more (see Prosser, 1989 for review). Although rates determined in our study are two to three orders of magnitude lower than cellular oxidation rates of cultivated (Prosser, 1989) and soil (Okano *et al.*, 2004) AOB, they are comparable to cell-specific rates determined in another estuarine environment (Dollhopf *et al.*, 2005). Because the dominant AOB previously identified in Plum Island Sound (Bernhard *et al.*, 2005) are not represented in the culture collection and vary between sites, population-specific differences may also contribute to variable cellular oxidation rates between sites and relative to cultivated and soil AOB.

Site differences in potential rates, amo*A abundance and community structure*

It has been suggested that community composition may effect significant impacts on ecosystem functioning (see Loreau *et al.*, 2001 for review). In support of this hypothesis, we observed clear site-specific differences between potential rates and *amo*A abundance that were correlated with AOB community composition, but not with porewater ammonium concentrations (Bernhard *et al.*, 2005). Ordination analysis identified population clusters associated with high nitrification rates. Furthermore, high rates were never detected in samples having a community composition different from those in clusters A, B and C. Interestingly, clusters A, B and C differed significantly in diversity, but not in nitrification potential. These results indicate a direct and quantitative link between AOB community composition and ecosystem function.

Direct salinity effects on potential nitrification rates

Salinity has long been implicated as a major factor affecting nitrification rates in estuaries, with rates generally decreasing as salinity increases. However, potential rates in our study do not indicate a consistent adverse salinity effect on activity. This is in contrast to previous studies that found consistent, significant inverse relationships between salinity and nitrification rates among cultivated nitrifiers (Stehr *et al.*, 1995; Bollmann and Laanbroek, 2002) and estuarine samples (Rysgaard *et al.*, 1999). Negative correlations between salinity and nitrification in these studies have been attributed to increased ammonium efflux from the sediment (Seitzinger *et al.*, 1991; Rysgaard *et al.*, 1999), salinity-induced changes in community composition of ammonia oxidizers (Bollmann and Laanbroek, 2002), or adverse salinity effects on the metabolism of the microbes (Stehr *et al.*, 1995; Rysgaard *et al.*, 1999). Because AOB community composition did not change (as measured by TRFLP analysis of the *amo*A gene) over the course of the incubations (data not shown), we believe our short-term potential nitrification measurements under different salinity regimes are a valid representation of salinity tolerance of the natural community. Additionally, because ammonium efflux should not be a factor in continuously shaken containers, it is likely that the observed differences reflect physiological adaptations of the resident AOB.

Although it might be anticipated that AOB would exhibit highest nitrification rates under conditions closest to *in situ* salinities, other studies do not support this relationship. Some studies have shown maximum nitrification rates at 0 psu for AOB collected from brackish or marine sites (Rysgaard *et al.*, 1999; Bollmann and Laanbroek, 2002). Others have demonstrated optimum nitrification rates of estuarine isolates between 5 and 10 psu (Jones and Hood, 1980), between 15 and 30 psu (Magalhaes *et al.*, 2005), or between 0 and 20 psu (MacFarlane and Herbert, 1984), with subsequent reductions in activity or inactivation at higher salinities. In most cases, we also observed maximal rates at intermediate salinities. This response might reflect an intrinsic characteristic of ammonia oxidizer physiology, and suggests that salinity may not be the primary factor that determines population distribution. However, the communities from the three sites did exhibit different responses to the highest- and lowest-salinity treatments, suggesting that they are optimized, at least to some degree, to ambient salinity. The broad salinity tolerance exhibited at all of the stations may reflect the highly dynamic salinity regime that these bacteria experience in the field over both tidal and seasonal time scales. When held under constant conditions for 70 days, AOB from site P22 had maximal ammonium oxidation rates over a much narrower salinity range than we found for field populations (Mondrup, 2001).

Seasonal differences in potential rates and amo*A abundance*

Potential nitrification rates and *amo*A abundance data also showed a significant seasonal effect, with both rates and abundance decreasing by up to an order of magnitude from April to early autumn. The differences in activity most likely reflect a seasonal change in abundance based

on the correlation between potential rates and *amo*A abundance. Seasonal differences in nitrification have been observed in previous estuarine studies (Berounsky and Nixon, 1993; Thompson *et al.*, 1995; Eriksson *et al.*, 2003), but the cause remains unclear. It has been suggested that AOB may compete with members of the microphytobenthos for ammonium (Risgaard-Petersen *et al.*, 2004) and this competition could become more intense in autumn. However, in our study, porewater ammonium concentrations increased at two of the stations later in the year (Bernhard *et al.*, 2005), suggesting other mechanisms are more important. All of the sites experience increased average salinity over the season, but the dramatic decline in rates is not consistent with our potential nitrification data that suggest AOB have a fairly wide salinity tolerance. It is possible that AOB experience greater oxygen limitation and/or increased sulfide toxicity in summer when sediment oxygen demand peaks.

Conclusions

Most studies of the relationship between microbial diversity and system processes have been primarily in artificial or manipulated systems (e.g. Naeem and Li, 1997; Ayala-del-Rio *et al.*, 2004; Bell *et al.*, 2005), and have not focused on a single functional group. One exception is the study by Cavigelli and Robertson (2000), demonstrating that the community composition of denitrifying bacteria has a significant impact on denitrification and N_2O production. Our observation of structurally and functionally distinct ammonia-oxidizing communities at different sites in Plum Island Sound is another demonstration of a direct and quantitative link between biodiversity and process in a natural system. These studies indicate strong associations between the physical/chemical features of a site, the community composition and its activity. The robust association between specific constellations of AOB populations within a site and high potential rates is particularly notable, suggesting that community response to added ammonium can in some instances be predicted with good confidence. However, the varying correlations between rates and community structure show that this relationship is still not fully understood. A more complete accounting of all contributing populations, now known to include Archaea, is needed to better constrain relationships among environment, diversity and activity.

Experimental procedures

Sample collection

Detailed site descriptions and sample collection procedures are described elsewhere (Bernhard *et al.*, 2005). Briefly, replicate sediment cores were collected in spring and late summer for 3 years (2001–2003) from sites in Plum Island Sound, MA estuary. At two sites, representing low (P22) and mid (P14) salinity, samples were collected at all time points. Samples from the high-salinity (R8C) site were collected at all time points except Spring 2002. Average salinity (psu) at each site during our experiments ranged from 0.5 to 8.7 (P22), 6.3 to 24.7 (P14) and 20.5 to 31.7 (R8C) (Bernhard *et al.*, 2005).

Potential nitrification rates

Potential nitrification was measured at all sites under four salinity conditions: 0, 5, 10 and 30 psu. Media for each salinity condition were made by diluting artificial seawater, the '0' salinity was actually a 1/500 dilution of artificial seawater to provide essential salts. All samples were amended with 300 μM ammonium (as NH_4Cl) and 60 μM phosphate (as KH_2PO_4). For each site replicate, at each depth and salinity, small sediment samples were placed into three 50 ml centrifuge tubes. The nutrient/salinity stock solution was added, and the samples were kept in the dark at 22°C and continually shaken. In 2001 we used 45 ml of stock solution and 1.7 g (wet weight) of sample, in subsequent years we used 30 ml of solution and 1 g (wet weight) of samples to eliminate the possibility of oxygen limitation during the incubation by increasing the headspace. An additional subsample from each replicate and depth was weighed, dried and re-weighed to obtain wet/dry conversion factors. One incubated subsample from a treatment replicate was harvested at 24, 48 and 72 h. Samples were centrifuged, filtered and immediately frozen for nitrate analysis. (Although AOB only oxidize ammonium to nitrite, nitrite is quickly oxidized to nitrate. Tests showed that nitrite concentrations were negligible in our incubations.) NO_3^- concentrations were determined by colorimetric analysis using the cadmium reduction method on a Lachat QuickChem flow injection analyser (Johnson and Petty, 1983). Nitrification rates were calculated based upon changes in nitrate concentration per gram of dry weight of sediments over time. Obvious outliers were omitted from the regression (largely some of the final points in 2001 where oxygen limitation may have occurred).

Some changes were made in the sampling depth and the replication over the course of the study. In April and August of 2001, four 6 cm cores were taken from each site. All cores were sectioned into four depths: 0–0.5, 0.5–1.0, 1.0–1.5, 1.5–2.0 cm (in April 2001) or 0–0.5, 0.5–1.0, 1.0–2.0, 2.0–4.0 cm (in August 2001). In order to have enough material for the experimental treatments, cores were paired and sections were combined and homogenized to create duplicate samples from each site. In subsequent years, three cores were collected. Each core was analysed separately, but the entire 0–2 cm depth was homogenized and incubated.

Potential rates measured in salinity treatments closest to *in situ* salinities were used in correlation analyses between potential rates and AOB abundance. To measure the effect of salinity on potential rates, we calculated per cent nitrification potential response by scaling the rate data based on the maximum response for each site for each year. We then calculated the average per cent response for all years combined.

DNA extraction

DNA extractions were carried out as previously described (Bernhard *et al.*, 2005). Briefly, DNA was extracted from approximately 0.5 g of sediment using the FastDNA kit for Soil (Qbiogene, Carlsbad, CA) following the manufacturer's directions with slight modifications.

AOB abundance

Ammonia-oxidizing bacteria abundance was estimated by quantifying the number of *amo*A copies per gram of sediment (dry weight) using real-time PCR for the *amo*A gene with the primer set *amo*A-1F (Rotthauwe *et al.*, 1997) and *amo*A-2R-TC (Nicolaisen and Ramsing, 2002). Reaction conditions were as previously described (Risgaard-Petersen *et al.*, 2004) with the following modifications: each 20 µl PCR contained 1× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.5 µM each primer and approximately 10 ng of DNA. All reactions were carried out using an iQ iCycler (Bio-Rad). All samples were run in duplicate at minimum and compared with standard curves generated in each experimental run using at least four standards. Standards were purified plasmid DNAs representing *amo*A genes previously recovered from Plum Island Sound, MA (Bernhard *et al.*, 2005). Amplification of standards was linear over seven orders of magnitude (1×10^{-9}–1×10^{-15} g DNA). DNA concentrations of sediment samples were estimated by spectroscopic analysis at 260 nm and by agarose gel electrophoresis by comparing the density of the bands with those of a DNA High Mass Ladder (Fermentas), using NIH Image (http://rsb.info.nih.gov/nih-image/) to measure band densities. Concentrations measured by the two methods were in good agreement ($r = 0.90$).

Statistical analysis

Differences in *amo*A abundance among sites for each sampling date were determined by ANOVA (data from Apr01, Aug01, Sep02 and Sep03) or Student's *t*-test (Apr02 and Apr03), using $\alpha = 0.05$. Differences among the slopes of the regression of potential nitrification rates and *amo*A abundance were calculated by ANCOVA analysis (Zar, 1984). Data from TRFLP analysis of the *amo*A gene (Bernhard *et al.*, 2005) were used to assess the relationship between community composition and nitrification rates. Non-metric multidimensional scaling was used to ordinate the samples using Sørenson's distance measure. The ordination was based on relative abundances of TRFs of the *amo*A gene by calculating the area under each peak. Data were transformed by an arcsin square root function to reduce skew. PC-ORD v.4 (McCune and Mefford, 1999) was used to perform the NMS. The proportion of variance explained by each axis was determined by calculating the coefficient of determination between distances in ordination space and distances in the original sample space.

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