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Abundance of Ammonia-Oxidizing *Archaea* and *Bacteria* along an Estuarine Salinity Gradient in Relation to Potential Nitrification Rates[▽]

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Abundance of ammonia-oxidizing *Archaea* (AOA) was found to be always greater than that of ammonia-oxidizing *Bacteria* along an estuarine salinity gradient, and AOA abundance was highest at intermediate salinity. However, AOA abundance did not correlate with potential nitrification rates. This lack of correlation may be due to methodological limitations or alternative energy sources.

Nitrification, the sequential oxidation of ammonia to nitrite and nitrate, is a critical step in the nitrogen cycle and is mediated by a suite of phylogenetically and physiologically distinct microorganisms. However, recent discoveries, such as anammox bacteria (33) and ammonia-oxidizing *Archaea* (20), have illustrated the fact that our current understanding of the depth and breadth of ammonia-oxidizing microorganisms is incomplete. Since their discovery several years ago, ammonia-oxidizing *Archaea* (AOA) have been reported to be present in a variety of environments, including various soils and sediments (13, 22, 34), oxic and suboxic marine layers (5, 10, 25, 36), estuaries (4, 9, 26, 31), subterranean environments (35), wastewater sludge (30), corals (6), and sponges (14). Much of the evidence for the presence of AOA in the environment comes from molecular studies of the distribution of presumptive archaeal ammonia monooxygenase genes thought to be homologs of the betaproteobacterial genes encoding the alpha subunit of ammonia monooxygenase (*amoA*). However, what remains less certain is the contribution to nitrification of AOA relative to ammonia-oxidizing *Betaproteobacteria* (β-AOB). In pelagic systems where AOA consistently outnumber β-AOB (10, 21, 25), there is strong evidence for archaeal nitrification based on molecular and biogeochemical data (5, 36). In estuarine systems, however, the data are more ambiguous (9, 27, 31), suggesting a more complex relationship in these systems.

In a previous study of the abundance of β-AOB and potential nitrification rates along a salinity gradient in the Plum Island Sound estuary (8), we found site-specific ammonia oxidation kinetics that correlated with salinity and β-AOB community structure. The highest rates, but lowest β-AOB abundance, were detected at the low-salinity site, suggesting that there may be other ammonia oxidizers contributing to nitrification or that there are significant differences in cell-specific oxidation kinetics of the resident β-AOB. Since the previous study did not include *Archaea*, we have now investigated whether the distribution of AOA could explain the various correlations between potential nitrification rates and β-AOB abundance along the salinity gradient.

Diversity and abundance of AOA were measured from three sites representing low (0.5 to 8.7 practical salinity units [psu]), mid (6.3 to 24.7 psu), and high (20.5 to 31.7 psu) salinity in the Plum Island Sound estuary in northeastern Massachusetts. Site descriptions and sampling methods are described elsewhere (7, 8, 16). Archaeal *amoA* gene sequences were amplified, cloned, and analyzed as previously described (11, 20). A total of 14 clone libraries were generated from samples taken on at least three separate dates from each site (Table 1). Neighbor-joining and parsimony analyses were performed by using ARB software (23). Most of the sequences were affiliated with the water column/sediment cluster first described by Francis et al. (13) and were closely related to sequences recovered from other marine and estuarine environments (Fig. 1). The remaining sequences were affiliated with the soil/sediment cluster. Sequences most closely related to the cultivated marine archaeon, *Nitrosopumilus maritimus*, were the most frequently recovered sequence type from all three sites. Additionally, sequences from all three sites were distributed throughout the tree and showed little site specificity.

Abundances of AOA were measured from the same samples collected previously and were compared to the reported β-AOB abundances and potential nitrification rates (8). Archaeal *amoA* gene abundance, measured by real-time PCR as described by Moin et al. (26), ranged from 3.8×10^4 to 2.4×10^8 copies g (dry weight) of sediment^{-1} (Fig. 2A). The forward primer used in the real-time PCR assay is internal to the forward primer used for cloning and was a perfect match to 441 of the 451 archaeal *amoA* sequences recovered in this study. The other 10 sequences had a single mismatch that was either A to G or T to C, which are the most common *Taq* polymerase errors (12). Archaeal *amoA* gene abundance was greatest in

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TABLE 1. Number of archaeal *amoA* gene clones from each site and sampling date

Site	No. of clones from date indicated							Total no. of clones
	April 2001	August 2001	April 2002	September 2002	April 2003	September 2003	Multiple[a]	
P22	7	7	7				72	93
P14	40	43	36		47		45	211
R8C	42	43		8		8	46	147
Total	89	93	43	8	47	8	163	451

[a] Clones were generated from pooled DNA samples from multiple dates.

April at the high-salinity site, and there were significant seasonal differences at the mid- and high-salinity sites. In general, archaeal *amoA* abundances in Plum Island are similar to values reported in other estuaries (26, 27, 31). Our highest values (10^8 gene copies g sediment^{-1}) are quite high, but Moin et al. (26) reported archaeal *amoA* gene abundances as high as 10^9 gene copies g sediment^{-1} in a salt marsh. Additionally, Nelson et al. (28) reported abundances of 16S rRNA genes related to *N. maritimus* as high as 10^9 gene copies g sediment^{-1} in a salt marsh.

Ratios of AOA to β-AOB ranged from 1 to greater than 100 and were most variable at the low-salinity site (Fig. 2B). Significant seasonal differences in ratios were detected only at the mid-salinity site. Despite significantly different archaeal *amoA*

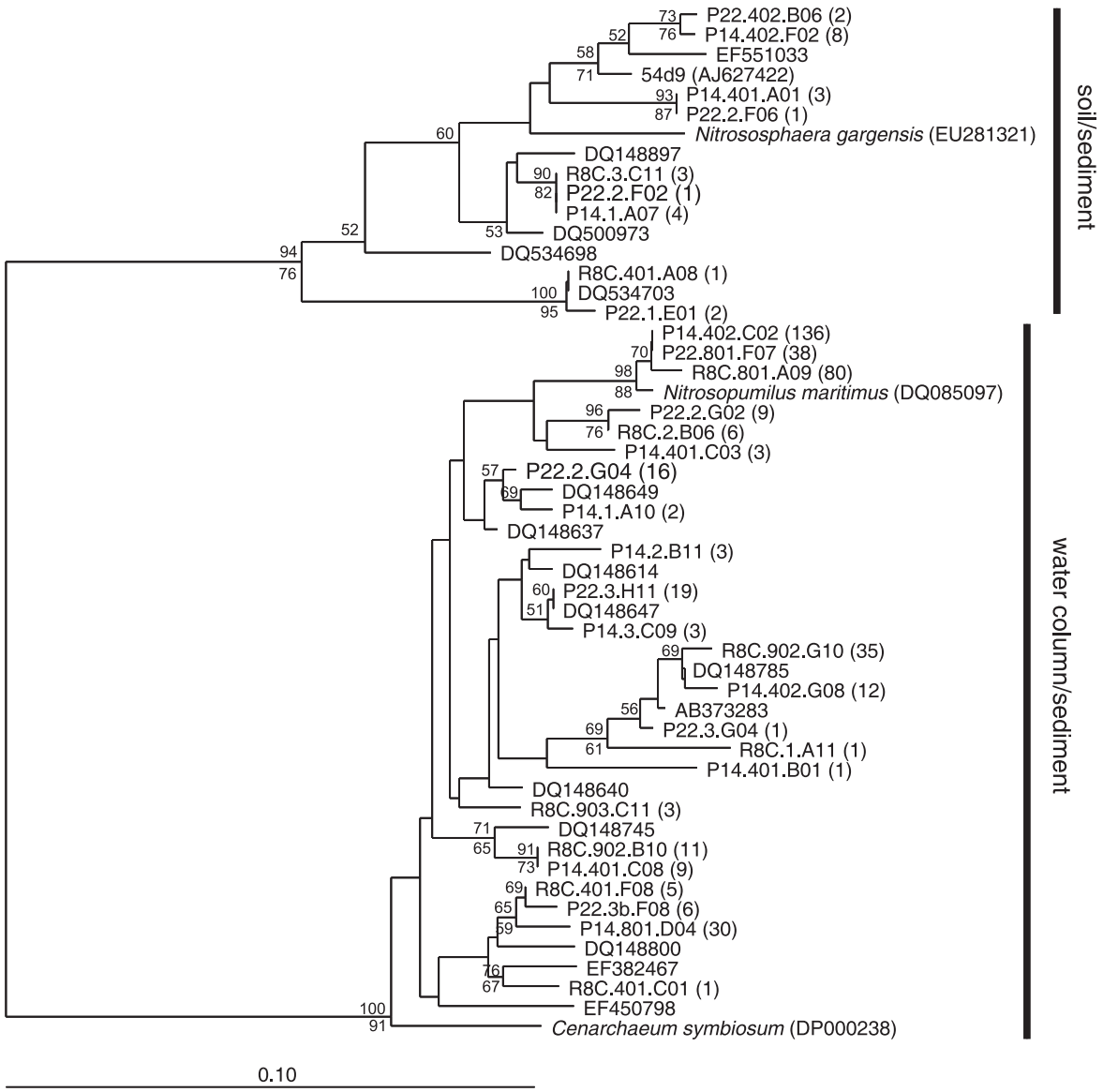

FIG. 1. Phylogenetic relationships of representative deduced archaeal *amoA* protein sequences recovered from Plum Island Sound sediments representing low (P22 prefix), mid (P14 prefix), and high (R8C prefix) *in situ* salinity. The tree was inferred from 181 amino acid residues. Bootstrap values (≥50) based on 100 replicates for neighbor-joining (above the node) and parsimony (below the node) analyses are indicated. Numbers in parentheses indicate the number of similar sequences recovered from the same site. Water column/sediment and soil/sediment clusters are as designated by Francis et al. (13).

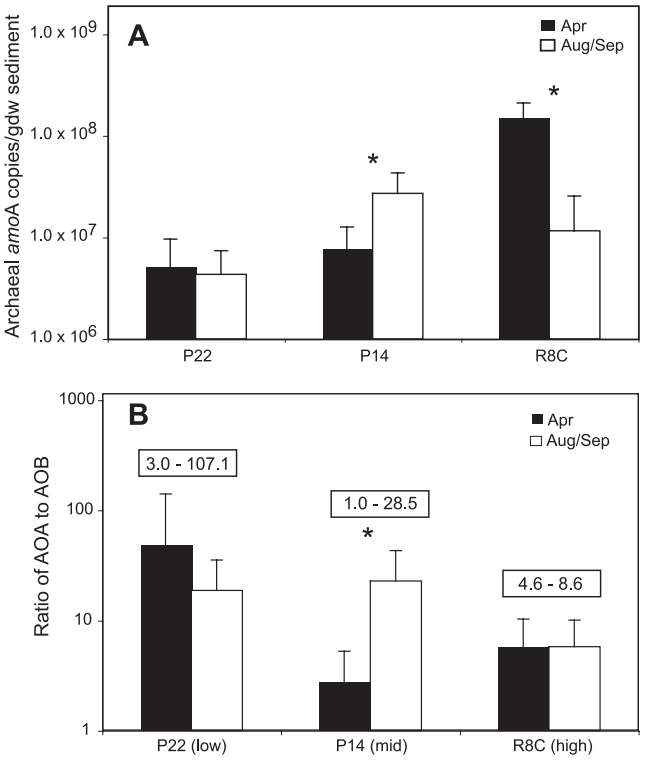

FIG. 2. Average (±1 standard deviation) gene copy number of archaeal ammonia monooxygenase genes (*amoA*) in Plum Island Sound sediments (panel A) and the ratio of AOA to β-AOB abundance (panel B) in April and August-September averaged from duplicate (2001) or triplicate (2002 and 2003) cores from each site over 3 years. The range of ratios for each site is indicated in the box above the columns in panel B. Asterisks indicate a statistically significant difference between April and August-September values (determined by Student's *t* test using $\alpha = 0.05$). Values of *amoA* abundance at R8C in April are from 2001 only. All other data are the averages of values from 3 years.

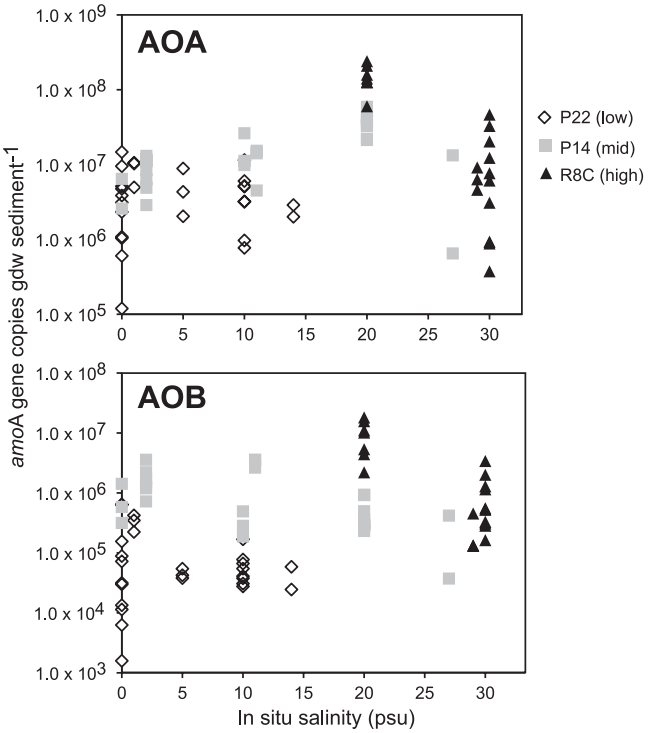

FIG. 3. Abundance of *amoA* genes from ammonia-oxidizing *Archaea* (AOA) and ammonia-oxidizing *Betaproteobacteria* (AOB) in relation to *in situ* salinity (psu). Symbols represent individual P22 (low-salinity), P14 (mid-salinity), and R8C (high-salinity) samples collected on different dates.

abundances in April and August at the high-salinity site, ratios of AOA to β-AOB were relatively constant, suggesting similar responses of AOA and β-AOB communities to seasonal changes in the environment at this site. Ratios reported from other estuarine studies range from less than 1 to greater than 80 (9, 27, 31) and have been correlated to changes in salinity (27, 31). Similar to results of other estuary studies, the ratios were lower at higher salinities, but unlike results of other estuary studies (9, 27, 31), β-AOB were never more abundant than AOA.

Although we did not detect a linear relationship between AOA abundance and salinity, there was a pattern of highest AOA abundance at 20 psu (Fig. 3, upper panel). The pattern was similar for β-AOB, but only in samples from the high-salinity site (Fig. 3, lower panel). Whether salinity or some other factor that covaries with salinity is a controlling variable remains unclear. Previous researchers have found the highest nitrifying activity at intermediate salinities with high turbidity (18, 32), and it has been hypothesized that the high turbidity provides the optimal substrate concentrations and habitat for estuarine nitrifiers (2). However, additional studies with cultivated isolates or enrichment cultures are necessary to more fully elucidate the ecophysiology of estuarine AOA and β-AOB.

We also investigated relationships among AOA, β-AOB, and potential nitrification rates. AOA and β-AOB abundances were significantly and positively correlated when samples from all sites were included (Table 2). However, the correlation appears to be driven primarily by the strong relationship between AOA and β-AOB abundances at the high-salinity site. We observed a similar pattern of correlation between AOA and potential nitrification rates. Although the correlation coefficients for AOA abundance versus potential nitrification rates and AOA versus β-AOB abundances were significant,

TABLE 2. Pearson's correlation coefficients describing the relationships between AOA and β-AOB abundance and potential nitrification rates[a]

Comparison	Pearson's correlation coefficient (*r*) for indicated samples			
	All	P22 (low salinity)	P14 (mid salinity)	R8C (high salinity)
AOA versus β-AOB	0.78*	0.26	−0.13	0.79*
AOA versus rates[b]	0.50*	0.34	−0.22	0.81*
β-AOB[b] versus rates	0.70*	0.81*	0.87*	0.96*
AOP versus rates	0.53*	0.42*	−0.17	0.83*

[a] AOA and β-AOB abundances were measured by real-time PCR quantification of the archaeal or betaproteobacterial *amoA* gene, respectively. AOP represents the combined abundance of AOA and β-AOB. Asterisks indicate statistically significant regressions using an α value of 0.05.

[b] Data are from Bernhard et al. (7). rates, potential nitrification rates.

they were lower than the correlation coefficient for β-AOB abundance versus potential nitrification rates. Additionally, when AOA and β-AOB abundances were combined (as ammonia-oxidizing prokaryotes [AOP] in Table 2) and correlated with potential nitrification rates, the correlation was much lower than when only β-AOB abundance was included in the correlation. The high-salinity site was the only site showing a strong positive correlation between AOA abundance and potential rates, but the relationship between β-AOB abundance only and potential rates was still much stronger.

Contrary to our initial hypothesis, the inclusion of AOA as part of the ammonia-oxidizing community does not fully explain previously reported differences in ammonia oxidation kinetics related to changes in salinity. In a previous work, there was a significant decrease in potential nitrification rates and β-AOB abundance from spring to late summer repeatedly over 3 years (8). However, in the current study, AOA abundance decreased in late summer only at the high-salinity site, while abundance remained constant at the low-salinity site and was significantly greater in late summer at the mid-salinity site. Thus, there is no consistent relationship between AOA abundance and either potential nitrification rates or β-AOB abundance patterns. In a previous study of estuarine AOA and β-AOB, Caffrey et al. (9) also found no correlation between potential nitrification rates and AOA abundance at four of six sites. Although further studies are necessary to confirm AOA nitrification activity in the estuary, our data do not support a significant AOA contribution, particularly at the low- or mid-salinity sites, despite relatively high AOA abundance.

The lack of correlation between AOA and potential nitrification rates has several possible explanations. It may be that AOA are inactive, perhaps transient, members of the estuarine community, and the high rates are due to very active AOB. It is also possible that AOA use alternative energy sources and thus are not actively nitrifying in the estuary. Several studies have reported evidence of heterotrophic metabolism among at least some marine *Crenarchaeota* (1, 17, 29), suggesting the possibility of either mixed populations of autotrophic and heterotrophic *Crenarchaeota* or a community of mixotrophic *Crenarchaeota*. Chemoautotrophic growth on ammonia oxidation has been demonstrated for *Nitrosopumilus maritimus* (20), but there is only circumstantial evidence of archaeal nitrification activity in marine environments, with the most convincing data being from open ocean studies (5, 36). Additionally, although increased archaeal *amoA* gene expression after ammonium additions has been reported for soil microcosms (34), the specific role of the putative archaeal ammonia monooxygenase remains to be confirmed.

Since potential nitrification rates are typically measured under nonlimiting oxygen and substrate conditions, an alternative explanation for the observed lack of correlation is that high substrate concentrations or other conditions of the potential rate experiments may inhibit AOA, particularly since we now know that *N. maritimus* has a very high affinity for ammonium (24). The ammonium added to our incubations (300 μM) was within the ranges found *in situ* (7) and is considerably lower than the 2 to 3 mM concentrations of ammonium that have been shown to at least partially inhibit AOA from hot springs (15) and pure cultures of *N. maritimus* (24). However, it is still possible that the ammonium additions were too high for activity of the resident AOA in our study. Additionally, AOA activity may also be inhibited by agitation (24), which was used in our potential rate measurements to maintain nonlimiting oxygen conditions. Alternatively, archaeal *amoA* gene abundance may not be an appropriate indicator of their nitrifying activity, based on a recent study of AOA and β-AOB in nitrogen-rich agricultural soils, in which β-AOB were primarily responsible for the oxidation of ammonia despite a high abundance of archaeal *amoA* genes (19).

We must also consider the possibility that we still have not accounted for the presence of all organisms capable of oxidizing ammonia, including methane oxidizers and heterotrophic nitrifiers. Methane has been reported to be a significant component of the C cycle at the low-salinity site (16) and may support populations of methane oxidizers that are capable of ammonia oxidation under certain environmental conditions (3). To our knowledge, the role of heterotrophic nitrifiers in estuarine sediments has not been investigated. Additionally, previous attempts to amplify genes representing the gammaproteobacterial ammonia oxidizers in Plum Island Sound were unsuccessful (7).

In summary, AOA outnumbered β-AOB along the salinity gradient and were most abundant at intermediate salinity. Additionally, our results do not support a major contribution of AOA to nitrification in the Plum Island Sound estuarine sediments. The lack of correlation between AOA abundance and potential nitrification rates may be a methodological artifact or may reflect alternative energy sources used by AOA. *In situ* rate measurements in future studies will be important to resolve the role of archaeal contributions to nitrification in estuarine systems.

Nucleotide sequence accession numbers. Sequences were deposited in GenBank under accession numbers GQ499386 to GQ499836.

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