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Estuarine Nitrifiers: New Players, Patterns and Processes

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Estuarine Nitrifiers: New Players, Patterns and Processes

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Abstract

Ever since the first descriptions of ammonia-oxidizing bacteria by Winogradsky in the late 1800's, the metabolic capability of aerobic ammonia oxidation has been restricted to a phylogenetically narrow group of bacteria. However, the recent discovery of ammonia-oxidizing Archaea has forced microbiologists and ecologists to re-evaluate long-held paradigms and the role of niche partitioning between bacterial and archaeal ammonia oxidizers. Much of the current research has been conducted in open ocean or terrestrial systems, where community patterns of archaeal and bacterial ammonia oxidizers are highly congruent. Studies of archaeal and bacterial ammonia oxidizers in estuarine systems, however, present a very different picture, with highly variable patterns of archaeal and bacterial ammonia oxidizer abundances. Although salinity is often identified as an important factor regulating abundance, distribution, and diversity of both archaeal and bacterial ammonia oxidizers, the data suggest that the variability in the observed patterns is likely not due to a simple salinity effect. Here we review current knowledge of ammonia oxidizers in estuaries and propose that because of their steep physico-chemical gradients, estuaries may serve as important natural laboratories in which to investigate the relationships between archaeal and bacterial ammonia oxidizers.

1. Introduction

Nitrification, the sequential oxidation of ammonia to nitrite and then nitrate, is a critical step in the nitrogen cycle, and is carried out by phylogenetically and physiologically distinct microorganisms. In coastal systems, nitrification is often coupled to denitrification (Jenkins and Kemp, 1984; Sebilo et al., 2006), resulting in the ultimate return of nitrogen to the atmosphere. Thus, the fate of ammonia plays a major role in the regulation of primary productivity, particularly in marine systems where nitrogen is often the limiting nutrient (Howarth, 1988). Despite the obvious ecological importance of nitrification, regulation of microbial populations that mediate it is poorly understood, particularly as new pathways of ammonia oxidation and new groups of ammonia-oxidizing organisms are discovered. Identifying environmental factors that regulate the diversity, distribution, and activity of nitrifiers is paramount to gain a more complete understanding of nitrogen-cycling, particularly in nitrogen-sensitive environments, such as estuaries and salt marshes.

The mixing of freshwater and saltwater in estuaries and salt marshes creates steep physico-chemical gradients that are accompanied by shifts in the resident microbial communities. Steep gradients of salinity, nitrogen, pH, oxygen, sulfide, and organic loading are common and some of these have been shown to correlate with shifts in microbial communities (del Giorgio and Bouvier, 2002; Crump et al., 2004; Hewson and Fuhrman, 2004; Bernhard et al., 2005). Many of the parameters known to shift along the estuarine gradient are also known to be important factors impacting ammonia oxidizers (e.g. Bernhard et al., 2005; Bernhard et al., 2007; Mosier and Francis, 2008; Santoro et al., 2008). Because of the steep gradients and the documented changes in activity and community composition of nitrifiers in estuaries and salt marshes, these habitats are ideal natural laboratories in which to study the dynamics of nitrifiers.

To date, there are no known microbes that can oxidize ammonia all the way to nitrate. Instead, ammonia-oxidizers convert ammonia to nitrite, while another group of organisms, nitrite-oxidizing bacteria, convert nitrite to nitrate. Until recently, the only known aerobic ammonia oxidizers belonged to two separate lineages within the domain *Bacteria*, the *Beta*- and

Gammaproteobacteria (Woese et al., 1984; Woese et al., 1985). Ammonia-oxidizing bacteria (AOB) use O_2 as their electron acceptor and NH_3 as their sole energy source, channeling some of the energy produced to fix CO_2. Although both ammonia- and nitrite-oxidizers are required for the complete oxidation of ammonia, most research has focused on ammonia oxidizers since they carry out the rate limiting step (Prosser, 1989).

What was once a fairly simple phylogenetic and physiological picture, however, is now known to be far more complex. The recent discovery of aerobic ammonia oxidation within the domain *Archaea* (Könneke et al., 2005; Treusch et al., 2005) has led to a dramatic shift in the current model of nitrification, and to new questions of niche differentiation between ammonia oxidizing *Archaea* (AOA) and the more well-studied AOB. Much of what we know about marine AOA has come from studies in open ocean environments, where patterns of AOA are generally similar, with AOA far outnumbering AOB (Wuchter et al., 2006; Mincer et al., 2007; Beman et al., 2008), suggesting that AOA are likely the predominant nitrifiers in the ocean. However, several recent studies of AOA and AOB in estuarine systems suggest a more complex picture of ammonia oxidizer ecology, so we focus here on the ammonia oxidizers in estuarine systems, where the patterns and processes appear to differ significantly from those in pelagic systems.

1. 1. Nitrification in estuaries and salt marshes

Effects of changes in physico-chemical conditions along an estuarine gradient on nitrification rates have been well documented, and indicate decreased nitrification as the salinity increases (Seitzinger, 1988; Rysgaard et al., 1999). However, the precise cause of the decrease in nitrification is less clear. It is known that 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). But salinity has also been shown to affect species composition of nitrifying communities (de Bie et al., 2001; Bollmann and Laanbroek, 2002), and functionally distinct communities of AOB along a salinity gradient have recently been reported

(Bernhard et al., 2007). Thus, decreases in nitrification may be due to substrate limitation or, alternatively, a shift in the nitrifying community.

Nitrification has received much attention in estuaries and other coastal systems, but relatively few studies have been conducted in salt marshes. Despite the paucity of measurements in salt marshes, reported values of nitrification vary widely, and may depend on the type of vegetation sampled or the methods used. Dollhopf et al. (2005) found an order of magnitude difference in nitrification rates in tall-form *Spartina alterniflora* compared to short-form *S. alterniflora* and unvegetated creek banks. However, Moin et al. (2009) reported no significant differences in potential rates among three different salt marsh grasses. Other studies have reported nitrification in only one type of vegetation (Anderson et al., 1997; Tobias et al., 2001) or have not provided detailed information on the vegetation types (Thompson et al., 1995). Because the roots help to oxygenate the sediments (Mendelssohn et al., 1981), it is thought that this may help to stimulate nitrification (An and Joye, 2001). Conversely, others have reported inhibition by the presence of microalgae, possibly due to competition for ammonium (Risgaard-Petersen et al., 2004). Therefore, our understanding of nitrification in salt marshes and the microorganisms responsible is severely lacking at present, despite the importance of nitrification in regulating nitrogen in these systems.

It is possible that much of the variability observed among nitrifying communities in estuaries and salt marshes is due at least in part to the difficulties of collecting comparable samples in these systems. Tidal cycles, type of vegetation or distance from vegetation are all critical factors that may significantly impact the communities that are present or active, but are often not adequately reported or accounted for in the studies. As tides ebb and flow, edaphic conditions may shift dramatically over only a few hours. The dynamic nature of estuaries and salt marshes may create a unique natural laboratory for studying nitrifying communities and the factors that regulate them, but it also creates inherent sampling difficulties and subsequent comparisons between studies, and may contribute to some of the variability in estuarine nitrifying communities.

1.2 Taxonomy of AOB

The majority of AOB form a monophyletic lineage within the *Betaproteobacteria* (Figure 1), comprised of two major genera *Nitrosomonas* and *Nitrosospira* (Head et al., 1993). Two other species, *Nitrosococcus oceani* and *N. halophilus*, are affiliated with the *Gammaproteobacteria* (Holmes et al., 1995; Purkhold et al., 2000), and appear to be restricted to marine or saline environments.

All autotrophic aerobic ammonia-oxidizing bacteria have ammonia monooxygenase (AMO), the enzyme responsible for the first step in ammonia oxidation. The gene encoding the alpha subunit of AMO (*amo*A) has been used extensively as a molecular marker for studies of ammonia oxidizer diversity and distribution (see review by Kowalchuk and Stephen, 2001). The forms of AMO differ significantly between the *Beta-* and *Gammaproteobacteria* AOB. In fact, analysis of *amo*A sequences (the gene encoding the subunit containing the active site) indicate that genes from *Gammaproteobacteria* AOB have higher similarities to sequences of *pmo*A, the gene coding for a subunit of the particulate methane monooxygenase, than to *amo*A genes from the *Betaproteobacteria* AOB (β-AOB) (Holmes et al., 1995). However, due to the apparent lack of gammaproteobacterial ammonia oxidizers in estuaries (Bernhard et al., 2005; Ward et al., 2007) or salt marshes (Moin et al., 2009), they will not be considered further in this review.

Although all aerobic AOB carry out the same basic metabolic processes, there exists great ecophysiological diversity among the cultivated strains. The β-AOB are represented by 14 cultivated strains (Koops and Pommerening-Roser, 2001), divided into two main clusters, *Nitrosomonas* and *Nitrosospira*. The greatest physiological and phylogenetic diversity exists within the *Nitrosomonas* cluster, which contains the type species, *Nitrosomonas europaea*. Several species (*Nitrosomonas marina*, *Nitrosomonas aestuarii*, and *Nitrosomonas cryotolerans*) are obligately halophilic, while the remaining species in the *Nitrosomonas* and *Nitrosospira* genera either have no salt requirement, are halotolerant or moderately halophilic (Koops and Pommerening-Roser, 2001). Among the 14 species, there are also widely differing substrate

affinities, with *Nitrosomonas oligotropha* and *Nitrosomonas ureae* displaying the lowest K_s values (1.9-4.2 µM NH_3), and *N. europaea* and related species having the highest K_s values (30-61 µM NH_3) (Koops and Pommerening-Roser, 2001). Based on the broad physiological differences among the AOB, one might expect that they will be differentially distributed in the environment.

Unfortunately, most cultured ammonia-oxidizing bacteria do not represent the majority of AOB found in natural environments based on sequence analysis of 16S rRNA genes and the functional gene, ammonia monooxygenase (see review by Kowalchuk and Stephen, 2001). The lack of environmentally-relevant AOB in culture collections represents a significant barrier to understanding their ecophysiology and is likely a reflection of substantial differences in their nutritional requirements and physiological limitations compared to cultivated AOB.

1.3 Discovery of Ammonia Oxidizing Archaea

Until recently, our understanding of the diversity of aerobic ammonia oxidizers was restricted to members of the *Proteobacteria*. However, the new taxonomy for ammonia oxidizers must now include members from the Domain *Archaea*. When *Archaea* were first described in the late 1970's, they were considered extremophiles, found primarily in environments such as hydrothermal vents, acidic hot springs, and anoxic environments. However, in the early 1990's DeLong (1992) and Fuhrman et al. (1992) independently documented the unexpected presence in temperate ocean samples of members of a group of *Archaea* known as the *Crenarchaeota*. The group of marine *Archaea* was named the Group I Crenarchaea (Figure 2) and have been found to account for up to 20% of the picoplankton and are estimated to account for up to 10^{28} cells in the world's oceans (Karner et al., 2001). Additional studies revealed that members of the *Crenarchaeota* closely related to marine Group I were also found in soils (Bintrim et al., 1997) and freshwater sediments (MacGregor et al., 1997). Despite their ubiquity and abundance, the physiology of the Group I *Crenarchaeota* was unknown since their laboratory cultivation remained elusive.

The only *Crenarchaeota* that had been grown in pure culture up until this point were thermophilic or hyperthermophilic *Archaea*, such those belonging to the *Sulfolobus* and *Desulfococcus* genera (Figure 2), with sulfur-based metabolisms. The first hint of the potential metabolic role of the Group I *Crenarchaeota* came from two independent metagenomic studies. Venter et al. (2004) reported the presence of a gene that appeared to be a homolog of the bacterial *amo*A gene on an archaeal scaffold obtained from the metagenome of the Sargasso Sea. Shortly thereafter, Treusch et al. (2005) reported finding a similar gene from a large DNA fragment from soil that also had an archaeal 16S rRNA gene belonging to the Group I.1b *Crenarchaeota*. The two studies provided independent evidence for the genetic potential of the Group I *Crenarchaeota* to oxidize ammonia, but further work would be necessary to definitively establish the nitrifying potential among members of the *Archaea*.

Fortunately, the definitive proof of the ability of an archaeon to oxidize ammonia was established soon thereafter by the cultivation of a saltwater aquarium isolate representing the Group I.1a *Crenarchaeota* (Könneke et al., 2005). The isolate, *Nitrosopumilus maritimus*, represents the first mesophilic crenarchaeal isolate. It grows autotrophically with bicarbonate as its sole carbon source and a stoichiometric conversion of ammonia to nitrite, thus firmly establishing the physiology of at least one member of the Group I *Crenarchaeota*. Since this discovery, putative genes for ammonia oxidation have also been reported in the sponge symbiont, *Cenarchaeum symbiosum*, along with genes for ammonia and urea transporters and urease, all consistent with the metabolic potential to utilize reduced nitrogen compounds (Hallam et al., 2006).

2. Autotrophy, heterotrophy, or mixotrophy among the AOA?

All aerobic AOB are known to be chemolithoautotrophs, although some have been recently shown to be facultative chemolithoautotrophs (Hommes et al., 2003). Initial tests of *N. maritimus* suggest that it, too, is inhibited by even small concentrations of organic carbon (Könneke et al., 2005), but it remains unclear whether the physiology of *N. maritimus* is

representative of all ammonia oxidizing *Archaea*. Previous studies using radiolabeled substrates indicate that at least some of the marine *Crenarchaeota* can take up organic compounds (Ingalls et al., 2006), particularly amino acids (Ouverney and Fuhrman, 2000; Teira et al., 2006). Additionally, genes for carbon fixation by a modified 3-hydroxypropionate pathway (indicative of autotrophy) and genes for a nearly complete TCA cycle (indicative of heterotrophy) in the genome of *Cenarchaeum symbiosum* suggest at least the genetic potential for mixotrophic metabolism (Hallam et al., 2006). And, finally, Agogue et al. (2008) report that most bathypelagic *Crenarchaeota* in the North Atlantic are not autotrophic, based on dark carbon fixation rates and *amo*A gene abundance.

Equally convincing data exists, however, for an autotrophic lifestyle of the marine *Crenarchaeota*. Pearson et al. (2001) reported δ-^{14}C signatures in archaeal isoprenoids that were consistent with chemoautotrophic growth below the euphotic zone. In a study in the North Sea, Wuchter et al. (2003) provided evidence that marine *Crenarchaeota* can take up bicarbonate in the absence of light by measuring incorporation of radiolabeled bicarbonate into crenarchaeol, a lipid specific to *Crenarchaeota*. Additionally, Herndl et al. (2005) also found increased bicarbonate uptake by *Crenarchaeota* with depth to 3000 m in the Atlantic Ocean. These seemingly conflicting data leave several unanswered questions for future research. First, are all the marine *Crenarchaeota* capable of ammonia oxidation, and second, are they heterotrophic, autotrophic or mixotrophic? The answers to these questions will likely only be adequately addressed by additional enrichment and cultivation work.

3. Diversity of AOA and AOB in estuaries

A recurring paradox in microbial ecology is high species diversity within a functionally similar group. Because all autotrophic ammonia oxidizers require NH_3, O_2, and CO_2, there is the potential for intense competition among ammonia oxidizers. Because estuaries experience high spatial and temporal heterogeneity of physico-chemical parameters, such as pH, NH_4^+ availability, O_2 tension, CO_2 availability, salinity, sulfide, and temperature, they are particularly

interesting habitats in which to study niche differentiation among ammonia oxidizers. Daily tidal fluctuations are typically accompanied by changes in a suite of physico-chemical properties, demanding physiological plasticity of the resident microbes or, alternatively, a community of microbes with varying tolerance to these conditions. Studies of AOB from natural environments have found differences in substrate affinity (Stehr et al., 1995; Suwa et al., 1997), differences in NH_3 oxidation rates (Bodelier et al., 1996) and distribution of nitrifying bacteria related to oxygen (Schramm et al., 2000; Gieseke et al., 2001) among different species, suggesting differences in the physiological tolerance. Diversity and distribution of AOA and AOB undoubtedly reflect differences in their environments, but because of a lack of cultured representative strains, much of the ecophysiology of estuarine ammonia oxidizers remains unresolved. Correlations of community composition with environmental variables, however, may provide some clues into physiological limits of estuarine AOA and AOB.

Studies of microbial diversity often focus on characterization of the 16S ribosomal RNA genes, which have universally conserved regions that are suitable targets for diversity studies. However, to study diversity within a functional group of microorganisms, the organisms must be monophyletic or, alternatively, one of the genes responsible for the function of interest may be targeted. Although the β–AOB form a monophyletic group, many of the primers targeting the 16S rRNA genes are selective rather than specific (Utaker and Nes, 1998), so diversity studies targeting this gene are more problematic. Distribution of ammonia oxidation among the *Archaea* is still under investigation, so targeting 16S rRNA genes may not necessarily correspond with metabolic potential. Therefore, diversity of AOA and β–AOB has been characterized primarily by analysis of the alpha subunit of ammonia monooxygenase gene (*amo*A) sequences. Although the archaeal and betaproteobacterial *amo*A genes are thought to be homologs, it is not possible to detect them both with the same set of PCR primers. However, primers for the two distinct *amo*A genes have previously been published (Rotthauwe et al., 1997; Francis et al., 2005; Koenneke et al., 2005) and used successfully in a variety of habitats.

Sequences of the *amo*A gene from different β−AOB indicate that although they are more variable than the 16S rRNA genes (Purkhold et al., 2000), they have phylogenetically conserved regions, providing a suitable target gene for primers (Rotthauwe et al., 1997; Nold et al., 2000). In fact, because of its greater diversity, the *amo*A gene may be a more effective marker for fine-scale diversity studies (Rotthauwe et al., 1997; Alzerreca et al., 1999; Oved et al., 2001). Analysis of 16S rRNA and *amo*A genes from most of the cultivated species of β−AOB suggests high congruence between the phylogenies based on each gene (Purkhold et al., 2000). Initial reports also suggest similar congruence of 16S rRNA and *amo*A genes among the Archaea (Nicol et al., 2008; Prosser and Nicol, 2008; Sahan and Muyzer, 2008).

3.1. Betaproteobacterial AOB phylogeny in estuaries

Phylogeny of the β−AOB appears to be quite consistent and predictable in estuaries. The majority of *amo*A sequences recovered from estuaries are affiliated with a group of nitrifiers that is currently unrepresented in culture collections, but is most closely related to the *Nitrosospira* group (Figure 3). Since there are no cultured representatives of these β−AOB, we can only infer their physiological tolerances. It is interesting to note, however, that the cluster of uncultured *Nitrosospira*-like β−AOB is comprised exclusively of sequences from marine or estuarine sites, suggesting specific adaptations to a saline environment. Despite their apparent numerical abundance, the ecological role of the uncultured *Nitrosospira*-like β−AOB remains uncertain. In a study of β−AOB richness and activity along a salinity gradient in the Ythan Estuary in Scotland, Freitag et al. (2006) found the usual β−AOB based on 16S rRNA gene sequencing, but were unable to confirm the activity of the *Nitrosospira* cluster 1-like AOB using a stable isotope probing approach with $^{13}C\text{-}HCO_3^-$. Instead, they identified sequences related to *Nitrosomonas cryotolerans* and *Nitrosospira* sp. Nm143 in the fraction of the labeled DNA, indicating active nitrifiers. Although the results of Freitag and colleagues may bring into question the ecological role of the *Nitrosospira*-like β−AOB found to be so prevalent in estuaries, their results may

simply indicate that these β−AOB do not respond well to laboratory manipulations, which would explain why they have evaded cultivation attempts to date.

Sequences related to *Nitrosomonas aestuarii*, *N. marina*, or *N. ureae* are also frequently recovered from estuarine sites. However, very few sequences have been found in estuaries that are closely related to the well-studied *Nitrosomonas europaea*, and most of these are from low salinity environments. In general, β−AOB communities vary along the salinity gradient, with communities at the freshwater end composed of sequences related to both the *Nitrosomonas* and *Nitrosospira* genera, while communities at the marine end of the gradient tend to be dominated by *Nitrosospira*-like sequences (de Bie et al., 2001; Francis et al., 2003; Bernhard et al., 2005; Ward et al., 2007). Finding similar β−AOB in geographically disparate estuaries suggests there are common factors regulating β−AOB distribution in estuaries. Although the primary factor may simply be the presence or absence of salt, other factors that covary with salinity, such as nitrogen or oxygen, may also be important. Controlled laboratory manipulations may be necessary to separate the effects of salinity from other potential regulating factors.

Temperature, pH, net primary productivity and organic loading have been implicated, along with salinity, as important factors regulating the distribution of β−AOB. Sahan and Muyzer (2008) investigated β−AOB diversity and distribution along a salinity gradient in the Westerschelde Estuary, and identified salinity and temperature as the most important factors regulating β−AOB distribution and reported higher β−AOB richness and diversity (measured by H') when salinity and temperature were low. Additionally, variability in net primary productivity was correlated with β−AOB community composition. In a study of eutrophic Tokyo Bay sediments, Urakawa et al. (2006) found shifts in the β−AOB communities that corresponded to changes in nutrients and organic inputs from river run-off. Interestingly, no sequences related to *Nitrosospira* spp. were detected, even in samples collected from the more marine end of the bay, suggesting that organic loading may be more important than increased salinity in regulating AOB communities. In other cases (O'Mullan and Ward, 2005), no correlation between β−AOB richness and environmental variables was detected.

Salinity also appears to affect the richness as well as the community composition of β−AOB. Several studies have reported a decrease in the number of AOB phylotypes as salinity increases (Bernhard et al., 2005; Ward et al., 2007; Sahan and Muyzer, 2008). The decrease in richness of β−AOB with increasing salinity may indicate a decrease in the importance of β−AOB in more saline environments, and may suggest a shift in the dominant nitrifiers. One popular hypothesis is that ammonia oxidizing *Archaea* may be the more important nitrifiers in marine systems.

3.2 Archaeal *amo*A phylogeny

Since the discovery of ammonia-oxidizing *Archaea*, the distribution and diversity of presumptive archaeal *amo*A genes has been investigated in various soils and sediments (Leininger et al., 2006; Chen et al., 2008; Jia and Conrad, 2009), oxic and suboxic marine layers (Wuchter et al., 2006; Coolen et al., 2007; Mincer et al., 2007; Beman et al., 2008), estuaries (Beman and Francis, 2006; Caffrey et al., 2007; Mosier and Francis, 2008; Sahan and Muyzer, 2008), salt marshes (Moin et al., 2009), subterranean environments (Weidler et al., 2007), wastewater sludge (Park et al., 2008), and corals (Beman et al., 2007). The similarity of putative AMO-encoding genes (subunits A, B, and C) to bacterial AMO genes is relatively low (38-51% amino acid sequence similarity) (Könneke et al., 2005), and whether the AMO-related genes are in fact homologs has not been confirmed. However, the presence of the archaeal *amo*A subunit has been correlated with the detection of nitrification activity (Könneke et al., 2005; Treusch et al., 2005; Leininger et al., 2006; Wuchter et al., 2006; Beman et al., 2008), and the wide distribution of archaeal *amo*A genes suggests a potentially important role in nitrification in many habitats.

Archaeal *amo*A sequences fall into one of two major clusters, a cluster dominated by sequences recovered from water column and sediments and a cluster dominated by sequences from soils and sediments (Figure 4). Sequences from both clusters have been recovered from

estuarine sediments, but in most estuaries, the majority of the sequences fall into the water column/sediment cluster (Table 1).

From the handful of studies in estuaries, AOA richness is generally much greater than β−AOB richness, based on numbers of different gene sequences (Table 1). Differences in the richness between AOA and β−AOB may reflect differences in metabolic activity. Although the only cultivated AOA is thought to be an obligate aerobic ammonia oxidizer (Könneke et al., 2005), it remains unknown whether all related *Crenarchaeota* are obligate ammonia oxidizers or even if they are capable of ammonia oxidation at all. As previously discussed, there is convincing evidence of heterotrophic metabolism among at least some of the group I *Crenarchaeota* (Ouverney and Fuhrman, 2000; Hallam et al., 2006; Ingalls et al., 2006). Alternatively, differences in richness of AOA and β−AOB may reflect differences in evolutionary history. The recent discoveries of thermophilic (de la Torre et al., 2008) and moderately thermophilic (Hatzenpichler et al., 2008) AOA raises interesting questions about the ancestry of AOA and ammonia oxidation.

Although factors that regulate AOA diversity have not yet been fully elucidated, Sahan and Muyzer (2008) found salinity, temperature, nitrite concentrations, and net primary productivity to produce major effects on the community structure. Dissolved oxygen has also been implicated as an important factor affecting AOA communities, leading to a shift in the AOA to β−AOB ratios (Santoro et al., 2008), as well as selecting for specific AOA phylotypes (Lam et al., 2007; Park et al., 2008). Temperature was found to be a key factor in regulating AOA and β−AOB richness in an aquarium biofilter system (Urakawa et al., 2008). Not surprisingly, many of the same factors have been shown to have significant effects on β−AOB communities (Francis et al., 2003; Bernhard et al., 2005; Ward et al., 2007).

The vast majority of AOA *amo*A sequences recovered from other marine systems, such as pelagic waters and coral reefs, affiliate primarily with the water column/sediment cluster. Only in coral reefs (Beman et al., 2007) and coastal marine sediments (Park et al., 2008) were sequences recovered that were related to the soils/sediment cluster. Within the water

column/sediment cluster, however, there appear to be distinct clades of archaeal *amo*A genes that roughly correlate with habitat. For example, Hallam et al. (2006) found shallow (0-130 m) and deep (500-4000 m) ecotypes of archaeal *amo*A genes recovered from the Central Pacific, Monterey Bay, and the Antarctic peninsula. A similar distinction with depth was reported by Mincer et al. (2007), who suggested the separation may reflect differences in responses to photoinhibition, although photoinhibition has not been reported in cultures of *N. maritimus*. Such clear habitat-specific AOA sequence types have not emerged from estuarine studies. Mosier and Francis (2008) observed a cluster of AOA sequences that is dominated by sequences from low salinity environments, but several new sequences from a salt marsh also fall into this cluster (Moin et al., 2009), thus bringing into question the low-salinity phylotype hypothesis. It is likely that the current data set does not yet cover the entire depth and breadth of archaeal ammonia oxidizer diversity.

4. Distribution and abundance of AOA and AOB

Although AOA and β−AOB are both present in estuarine systems, the proportion of archaeal versus bacterial ammonia oxidation still remains under investigation. Currently, only a small number of studies are available that have investigated AOA and β−AOB at the same time in the same environment. Most studies have measured abundance of AOA and β−AOB by measuring the abundance of the *amo*A gene using real-time or quantitative PCR (QPCR). Archaeal *amo*A genes range in abundance from 10^4 to 10^9 gene copies per gram of sediment (Mosier and Francis, 2008; Santoro et al., 2008; Moin et al., 2009). The magnitude of this variation may simply reflect the extraordinary variation in AOA abundance among different sites, but it may also be an artifact of methodology since the primers used in the QPCR varied in different studies. In a review of the current literature, we have found at least six different PCR primers and/or primer combinations used for quantification. It is unknown at this point how differences in primer specificity may affect the absolute numbers of AOA detected. However, in our analyses of salt marsh and estuarine AOA, abundance varied over 4 orders of magnitude

within the same system (Moin et al., 2009), using the same primers and cycle parameters, so we think it is unlikely that the variability in AOA abundance in estuarine systems is due to methodology differences alone.

Abundance of betaproteobacterial *amo*A genes in estuaries is also quite variable, ranging from 10^4 to 10^8 copies per gram of sediment (Bernhard et al., 2007; Mosier and Francis, 2008; Santoro et al., 2008; Moin et al., 2009). Unlike quantification methods for archaeal *amo*A, most studies have used the PCR primers for betaproteobacterial *amo*A published by Rotthauwe et al. (Rotthauwe et al., 1997), although some short-comings of these commonly-used primers have recently been identified (Hornek et al., 2006). Part of the difference in primer development for archaeal and bacterial *amo*A genes undoubtedly stems from a more complete characterization of β−AOB in estuaries compared with the current, and ever-changing, diversity of archaeal *amo*A.

4.1 Relationship of AOA and AOB to estuarine gradients

Although the richness of archaeal *amo*A is much higher than the richness of betaproteobacterial *amo*A based on the phylotypes detected (Table 1), it is clear that the absolute abundance differs among systems, and likely reflects differences in physico-chemical properties that change along the estuarine gradient. Several studies have focused on AOA and β−AOB in coastal areas of California (Beman and Francis, 2006; Mosier and Francis, 2008; Santoro et al., 2008). Two of these studies found a correlation between increasing salinity and increasing number/proportion of β−AOB (Mosier and Francis, 2008; Santoro et al., 2008). Additional factors that appear to influence the ratio between the AOA and β−AOB in those systems are oxygen availability and C/N ratio, in which increasing oxygen availability and decreasing C/N ratio led to an increase of the number of AOA (Beman and Francis, 2006; Mosier and Francis, 2008). In the Westerschelde estuary (between Belgium and The Netherlands) AOA and β−AOB were present at all sampling points, but the community composition shifted with changing salinity and temperature (Sahan and Muyzer, 2008).

Although salinity is often identified as a key factor in regulating nitrifier community composition and abundance, it is likely that salinity is not the only factor involved, since many other factors may covary with salinity. AOA abundance has also been strongly correlated with lead concentrations and percent clay (Mosier and Francis, 2008) pore water sulfide (Caffrey et al., 2007), and pH (Moin et al., 2009). However, because salinity often covaries with many of these variables in estuaries, it is difficult to identify causative factors. Interestingly, strong correlations between dissolved inorganic nitrogen species and AOA abundance have not been detected in estuarine studies, but enrichment studies in soils have reported increased AOA abundance when ammonium is added (Leininger et al., 2006; Chen et al., 2008).

4.2 Ratios of AOA to AOB

Unlike most studies in pelagic or terrestrial systems where AOA always outnumber β−AOB, often by orders of magnitude, ratios of AOA to β−AOB in estuaries are more variable, with β−AOB often outnumbering AOA. Ratios of AOA to β−AOB within studies typically show lower ratios at sites with higher salinity, but the pattern is certainly far from robust (Figure 5). Mosier and Francis (2008) and Santoro et al. (2008) found a shift in the AOA to β−AOB ratios related to changes in salinity, with β−AOB outnumbering AOA as salinity increased, due primarily to changes in the abundance of β−AOB. Caffrey et al. (2007) also found β−AOB to outnumber AOA at estuarine sites in Weeks Bay. In Plum Island samples, the direction of the change in the ratio of AOA to β−AOB followed the same pattern as in Mosier and Francis (2008), with ratios generally decreasing with increasing salinity, but AOA were always more abundant than β−AOB (Bernhard et al., 2010). In the marsh samples, salinity is not significantly different among sites, but the ratios ($\log_{10}$) range from -0.03 to 2.91 in surface (0-2 cm) sediments, indicating a shift from β−AOB dominance at some sites to AOA dominance at other sites. These patterns suggest a strong spatial structure within estuaries, perhaps driven by changes in salinity, but the evidence so far does not indicate a simple salinity effect. Interestingly, from most oceanic studies of AOA and β−AOB, AOA are thought to be the

dominant nitrifiers. Based on this, one would predict that as salinity increases in the estuary, ratios of AOA to β−AOB would also increase, yet the reverse appears to be true. The driving force behind this shift in numerical dominance, and possibly metabolic dominance, in estuaries versus oceanic systems has yet to be explained. Possible niche differentiating factors could include ammonium availability, oxygen partial pressure, sulfide concentrations or organic loading.

5. Correlation of abundance patterns with rates

One of the primary questions about AOA is how much do they actually contribute to nitrification? Wuchter et al. (2006) provide evidence via enrichment cultures and a correlation between ammonia oxidation rates and abundance of the marine *Crenarchaeota*, supporting the hypothesis that marine *Crenarchaeota* are nitrifying in pelagic waters. In the Gulf of California, Beman et al. (2008) also provide convincing evidence that the marine *Crenarchaeota* are actively oxidizing ammonia and are far more abundant than β−AOB. Additionally, Mincer et al. (2007) reported distribution patterns of the *Crenarchaeota* and nitrite-oxidizing *Nitrospina* species that suggests a metabolic link. Thus, it is clear from studies in pelagic systems that AOA are abundant and often outnumber β−AOB, and the current evidence suggests that at least some of the group I *Crenarchaeota* are involved in nitrification. However, the evidence from estuarine systems suggests a more complex story.

In a previous study of the abundance of β -AOB and potential nitrification rates along an estuarine salinity gradient, strong site-specific correlations between ammonia oxidation rates and β -AOB abundance were detected that suggested functionally distinct β -AOB communities along the gradient (Bernhard et al., 2007). Highest rates, but lowest β -AOB abundances, 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. It was hypothesized that the distribution of AOA could explain the varying correlations between rates and β -AOB abundance along the salinity gradient. However, inclusion of AOA

abundance with β-AOB abundance caused the correlation with rates to collapse completely at the low and mid salinity sites, and decreased by more than 10% at the high salinity site (Bernhard et al., 2010), suggesting that AOA are not actively nitrifying in the Plum Island estuary, despite relatively high numbers.

In a recent study of AOA abundance in several southeastern estuaries (Caffrey et al., 2007), AOA abundance positively and significantly correlated with potential nitrification rates at only 2 of 6 sites. A similar lack of correlation between potential rates and AOA abundance was also reported in a Connecticut salt marsh system (Moin et al., 2009). Interestingly, β–AOB abundance did not correlate with potential rates in either of these studies, suggesting that either our understanding of the relationship between nitrification activity and the organisms responsible is incomplete or that the methodology is flawed.

Potential rates are typically measured by adding ammonium to sediment slurries and incubating them with shaking for hours to days, thus disrupting any sediment structure, and rates are calculated by following the generation of nitrate. Potential nitrification rates are typically much higher than in situ rates, but whether it is the addition of ammonium or non-limiting oxygen conditions that is responsible for stimulating nitrification is not clear. It is thought that potential nitrification rates should correlate with the abundance of ammonia oxidizers (Henriksen, 1980). Thus, inaccurate measurements of either the potential rates or the abundance of ammonia oxidizers will lead to a collapse in the expected relationship. Physiological diversity among cultivated isolates of AOB has been well-documented (see review by Koops and Pommerening-Roser, 2001), and because of the great genetic diversity of archaeal *amo*A genes, it is also likely that there is great diversity in physiological optima among AOA. A recent study reported extremely high affinity for ammonium of the cultivated AOA, *N. maritimus* (Martens-Habbena et al., 2009), but did not show inhibition at ammonium concentrations typical of potential rate experiments (i.e. 0.3-0.5 mM). However, due to vastly different oxidation kinetics among AOA and AOB and the recently-reported sensitivity to agitation of *N. maritimus* (Martens-Habbena et al., 2009), it is unlikely that potential nitrification rates reflect the

contribution of all nitrifiers present. It is also possible that some nitrifiers are not active at the time of sampling, and thus might show a lag in activity upon the addition of ammonium or oxygen. Significant differences in recovery after starvation have been demonstrated in some cultivated AOB (Bollmann et al., 2002). It would be useful, in future studies, to measure in situ rates using stable isotopes, instead of relying on the simpler, but likely biased, measures of potential rates.

In most of the recent studies, AOA and β−AOB abundances are measured by quantitative PCR, which is vulnerable to a variety of potential biases. For instance, the primers may not target all the nitrifiers. Primers are generally designed to target previously determined sequences, but the sequence database for AOA is constantly expanding, with new sequence types discovered in almost every study. Biases associated with DNA extractions and PCR amplification have also been well-documented (e. g. Suzuki et al., 1998; Acinas et al., 2005), and are often not fully explored or controlled for in most studies due to time and financial constraints. Additionally, since *amo*A gene abundance does not necessarily indicate activity (e.g. Jia and Conrad, 2009), we suggest that measures of gene expression in addition to gene abundance may be helpful to determine which populations are contributing to nitrification under different conditions.

6. Future directions

Since the first hint of AOA from metagenomic data sets, followed shortly by confirmation in a pure culture, evidence on the distribution and abundance of AOA in a variety of environments continues to mount. Molecular surveys of 16S rRNA and archaeal *amo*A genes indicate that AOA are widely distributed, and quantitative analyses from soils, open ocean, coastal sediments, and even wastewater treatment plants suggest AOA are abundant members of the microbial community. Furthermore, enrichment cultures from hot springs, estuarine sediments, and soils indicate that they are active, and probably play a role in both the nitrogen and carbon cycles. Although it appears that AOA are ubiquitous, abundant, and undoubtedly

important to global nutrient cycles, many questions remain unanswered about their ecology and physiology.

6.1. Estuaries as a natural laboratory for AOA and AOB ecology

Estuarine and salt marsh sediments appear to be suitable habitat for members of both major clusters of AOA (water column and soil) and may serve as ideal natural laboratories to explore niche differentiation between AOA and β-AOB. Although in most cases, AOA and β-AOB coexist, their relative contributions to nitrification remains unclear. Based on changing ratios of AOA to β-AOB along salinity gradients, it is likely that the contribution to nitrification of the two groups also changes. Unfortunately, there are currently no methods available that can discriminate archaeal from bacterial ammonia oxidation, but several approaches may provide some clues. For example, Schauss et al. (2009) reported differential responses of soil communities of AOA and β-AOB to additions of sulfadiazine (an antibiotic that inhibits the growth of most Gram-positive and many Gram-negative bacteria), and provided strong evidence for a significant archaeal contribution to nitrification. Conversely, using stable isotope probing, Jia and Conrad (2009) showed that β-AOB were functionally more important in agricultural soils than AOA. Conducting similar experiments with estuarine AOA and β-AOB under different salinity, substrate, and redox regimes will be critical to understanding how AOA and β-AOB utilize resources.

6.2. Importance of continued cultivation of AOA and AOB

In the past two decades, the field of microbial ecology has moved away from cultivation and relied very heavily on molecular surveys of microbial genes to study populations and communities (Pace et al., 1986; Amann et al., 1995). The lack of cultivation work, however, has now become a deficit to progress in understanding the role of different microbes in their natural environment. Molecular techniques must be ground-truthed with pure cultures and enrichment experiments. Without being able to grow a microbe in the lab, it is difficult to ever really

understand their physiology and metabolism. The presence of a gene (or even the expression of it) tells only of the metabolic potential. The lack of AOA pure cultures is a perfect example. Until we have more isolates in culture, we will not be able to definitively answer some of the most pressing questions. For example, although genes for AMO A, B, and C subunits have been identified in *Archaea* (Könneke et al., 2005; Hallam et al., 2006), no gene for the oxidation of hydroxylamine (the intermediate produced by AOB) has been identified in the genomes studied to date (Hallam et al., 2006). The lack of a complete pathway for ammonia oxidation as found in AOB raises the possibility that AOA may oxidize ammonia with a different metabolic pathway. The answer to this will be best addressed by directed biochemical and genetic studies of pure cultures.

Carbon utilization remains another question plaguing the AOA research community. Understanding the trophic status is paramount to understanding the impact of *Archaea* on global systems. Evidence for heterotrophy, or at least mixotrophy, among marine *Crenarchaeota* is mounting (Ouverney and Fuhrman, 2000; Ingalls et al., 2006; Teira et al., 2006; Agogue et al., 2008), and it is clear that some marine *Crenarchaeota* are not obligate autotrophs. However, what is still unclear is whether the *Crenarchaeota* that can take up organic carbon also have *amo*A and can nitrify. Cultures obtained so far indicate only obligate autotrophy. Until heterotrophic or mixotrophic *Crenarchaeota* can be isolated, the evidence will likely remain ambiguous.

The depth and breadth of ammonia oxidation within *Archaea* also remains unresolved. Recent studies suggest that ammonia oxidation may be much more deeply distributed throughout the *Archaea* and is not restricted to the Group I *Crenarchaeota*. Two distinct AOA enriched from hot springs, one that is related to other AOA from soils (Hatzenpichler et al., 2008), the other forming a distinct cluster that does not fall within either the water column/sediment or the soil/sediment clusters (de la Torre et al., 2008), have recently been identified, and Mincer et al. (2007) provide evidence that another group of Archaea known as pSL12 (currently unrepresented

in the culture collection) may also harbor *amo*A genes. As more cultures of putative AOA are obtained, we will gain a more complete picture of their distribution and phylogeny.

Conclusions

Over four decades ago, Stanley Watson calculated that the number of AOB in the ocean did not account for the estimated rates of nitrification (Watson, 1965) and predicted that there must be other ammonia oxidizers in the ocean, but until now, none had been discovered. Thus, the discovery of ammonia oxidation among *Archaea* has helped solve a forty-year old mystery, but it has also led to an entirely new set of questions about the ecology and physiology of ammonia oxidizers. Although much of the evidence in the last few years has come from ocean and soil environments, we think the steep environmental gradients found in estuaries provide an ideal natural laboratory in which to study AOA and AOB dynamics and ecology.

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Table 1. Distribution of archaeal *amo*A (AOA) sequences and number of archaeal and betaproteobacterial *amo*A (β−AOB) operational taxonomic units (OTU), using a 95% nucleotide identity cutoff, recovered from different estuaries. Total number of sequences in each study are indicated parenthetically.

	% of clones in cluster		No. of OTUs (no. of clones sequence)		
Estuary	Water column/ sediment	soils/ sediment	AOA	β−AOB	Reference
Bahi'a del To'bari, Mexico	83	17	42 (282)	9 (61)	Beman and Francis 2006
San Francisco Bay, CA	85	15	67 (415)	41 (378)	Mosier and Francis 2008
Barn Island salt marsh, CT	55	45	21 (120)	7 (83)	Moin et al. 2009
Westerschelde estuary, The Netherlands	60	40	10 (10)	4 (9)	Sahan and Muyzer 2008
Huntington Beach, CA	100	0	52 (338)	2 (200)	Santoro et al. 2008
Changjiang estuary, China	72	28	31 (47)	NA	Dang et al. 2008
Plum Island Sound estuary, MA	94	6	33 (451)	13 (55)	Bernhard et al. 2010; Bernhard et al. 2005
Average	74	26	37	10	

Figure captions.

Figure 1. Phylogenetic relationships among ammonia oxidizing bacterial 16S ribosomal RNA genes and some methane oxidizing bacteria (MOB) for reference. The unrooted neighbor-joining tree was inferred from an alignment of sequences with 1144 nucleotide positions. Bootstrap values (≥ 50) are indicated above the nodes. The gray boxes indicate representative sequences from uncultured AOB affiliated with the *Nitrosospira*-like cluster 1 and *Nitrosomonas*-like cluster 5 as designated by Stephen et al. (1996) that are currently unrepresented in the culture collection.

Figure 2. Phylogenetic relationships among archaeal 16S ribosomal RNA genes. The unrooted neighbor-joining tree was inferred from an alignment of sequences with 1204 nucleotide positions. Bootstrap values (≥ 50) are indicated above the nodes. Asterisks indicate *Archaea* known to have an ammonia monooxygenase gene.

Figure 3. Phylogenetic relationships among betaproteobacterial *amo*A nucleotide sequences. The unrooted neighbor-joining tree was inferred from an alignment of sequences with 329 nucleotide positions. Bootstrap values (≥ 50) are indicated above the nodes. Only sequences from estuarine studies and cultivated representative β–AOB are included in the tree. Numbers inside each polygon indicate the number of sequences in the cluster.

Figure 4. Phylogenetic relationships among deduced archaeal AmoA protein sequences recovered from the three sites. The unrooted neighbor-joining tree was inferred from an alignment of protein sequences with 181 amino acid residues. Bootstrap support (≥50) is indicated above the nodes. Only sequences from estuaries and representative *Archaea* known to have *amo*A genes are included in the tree. Colored circles by each cluster indicate the locations where the sequences in that cluster were recovered. Numbers inside each polygon indicate the number of sequences in the cluster.

Figure 5. Relationship between AOA to β−AOB ratios (log10) and salinity from four different estuaries: Plum Island Sound (Bernhard et al., 2007; Bernhard et al., 2010), Barn Island salt marsh (Moin et al., 2009), San Francisco Bay (Mosier and Francis, 2008), and a subterranean estuary in Huntington Beach, CA (Santoro et al., 2008).

Figure 1

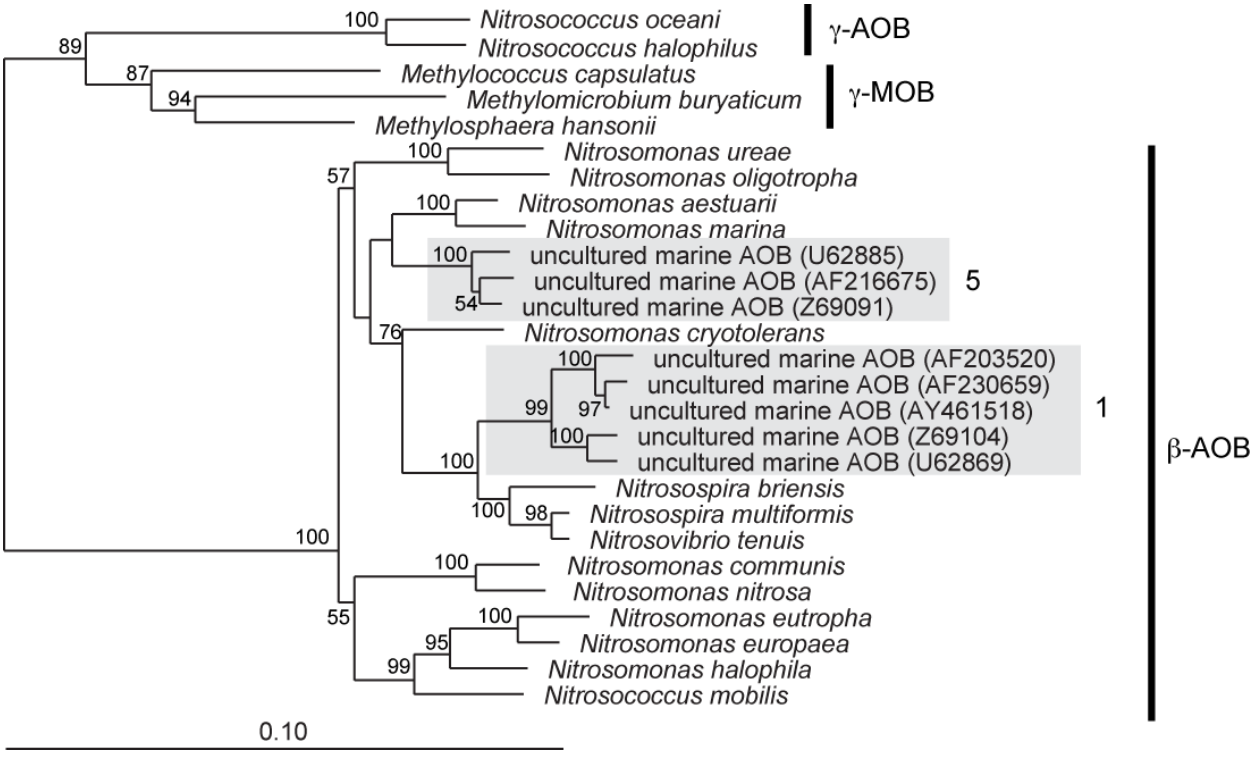
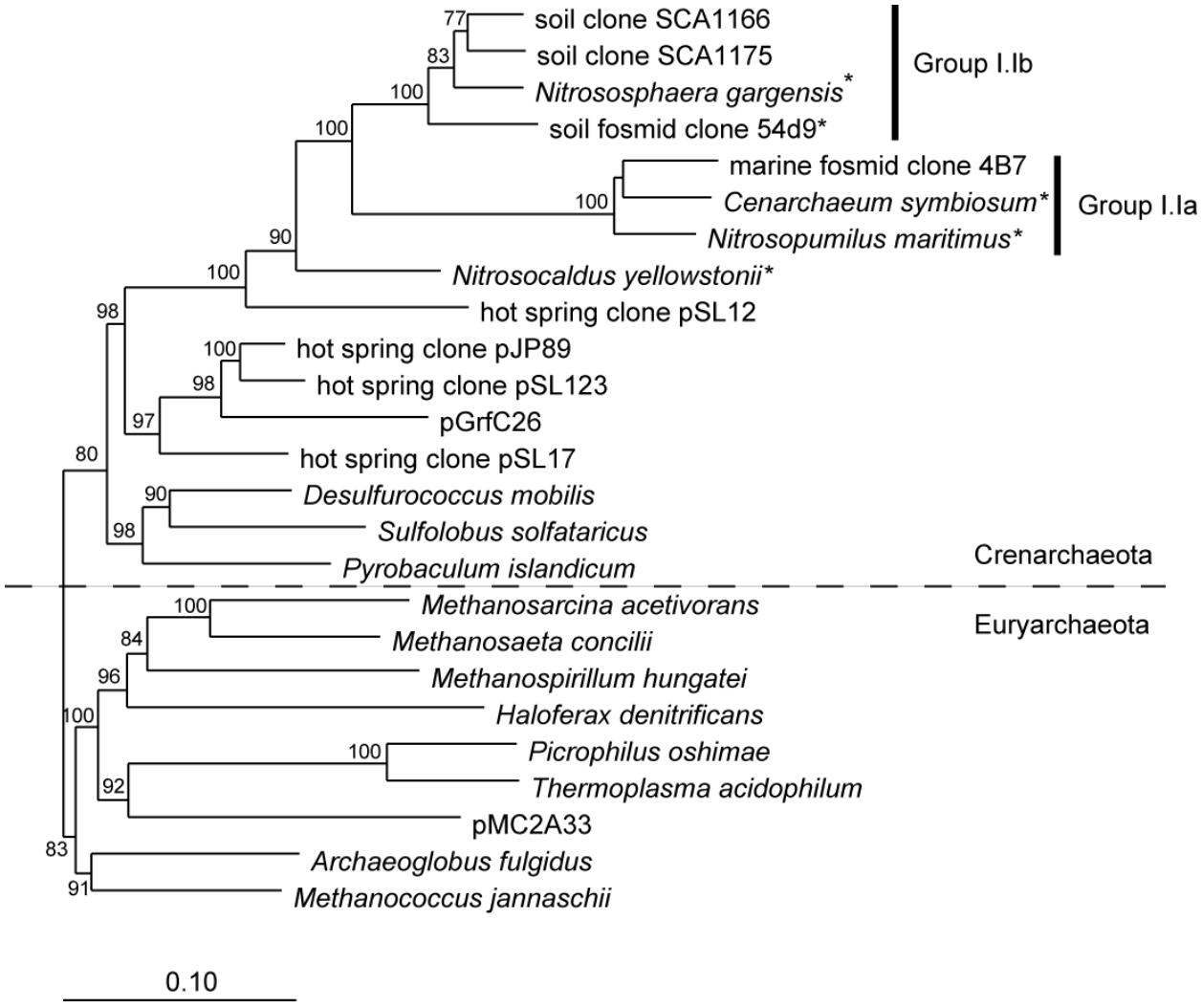

Nitrosococcus oceani
Nitrosococcus halophilus
γ-AOB
Methylococcus capsulatus
Methylomicrobium buryaticum
γ-MOB
Methylosphaera hansonii
Nitrosomonas ureae
Nitrosomonas oligotropha
Nitrosomonas aestuarii
Nitrosomonas marina
uncultured marine AOB (U62885)
uncultured marine AOB (AF216675)
uncultured marine AOB (Z69091)
5
Nitrosomonas cryotolerans
uncultured marine AOB (AF203520)
uncultured marine AOB (AF230659)
uncultured marine AOB (AY461518)
uncultured marine AOB (Z69104)
uncultured marine AOB (U62869)
1
β-AOB
Nitrosospira briensis
Nitrosospira multiformis
Nitrosovibrio tenuis
Nitrosomonas communis
Nitrosomonas nitrosa
Nitrosomonas eutropha
Nitrosomonas europaea
Nitrosomonas halophila
Nitrosococcus mobilis
0.10

Figure 2


soil clone SCA1166
soil clone SCA1175
Nitrososphaera gargensis*
soil fosmid clone 54d9*
Group I.Ib
marine fosmid clone 4B7
Cenarchaeum symbiosum*
Nitrosopumilus maritimus*
Group I.Ia
Nitrosocaldus yellowstonii*
hot spring clone pSL12
hot spring clone pJP89
hot spring clone pSL123
pGrfC26
hot spring clone pSL17
Desulfurococcus mobilis
Sulfolobus solfataricus
Pyrobaculum islandicum
Crenarchaeota
Euryarchaeota
Methanosarcina acetivorans
Methanosaeta concilii
Methanospirillum hungatei
Haloferax denitrificans
Picrophilus oshimae
Thermoplasma acidophilum
pMC2A33
Archaeoglobus fulgidus
Methanococcus jannaschii
0.10

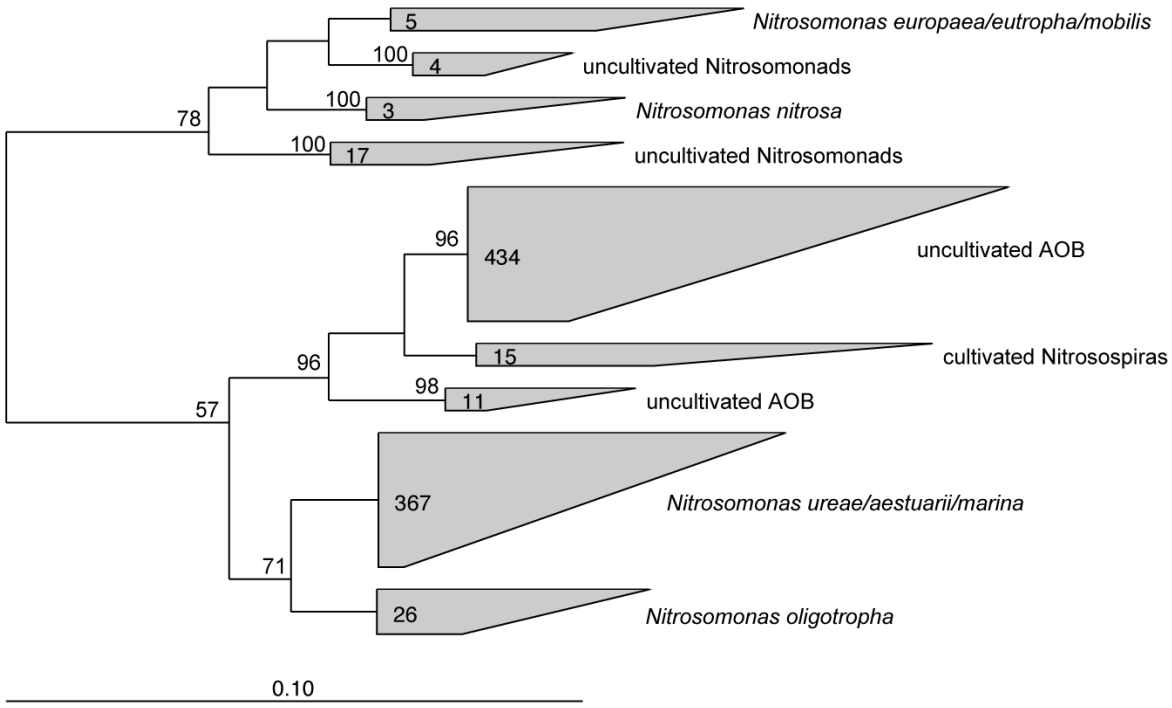

5
Nitrosomonas europaea/eutropha/mobilis
100
4
uncultivated Nitrosomonads
100
3
Nitrosomonas nitrosa
78
100
17
uncultivated Nitrosomonads
96
434
uncultivated AOB
15
cultivated Nitrosospiras
96
98
11
uncultivated AOB
57
367
Nitrosomonas ureae/aestuarii/marina
71
26
Nitrosomonas oligotropha
0.10

Figure 3

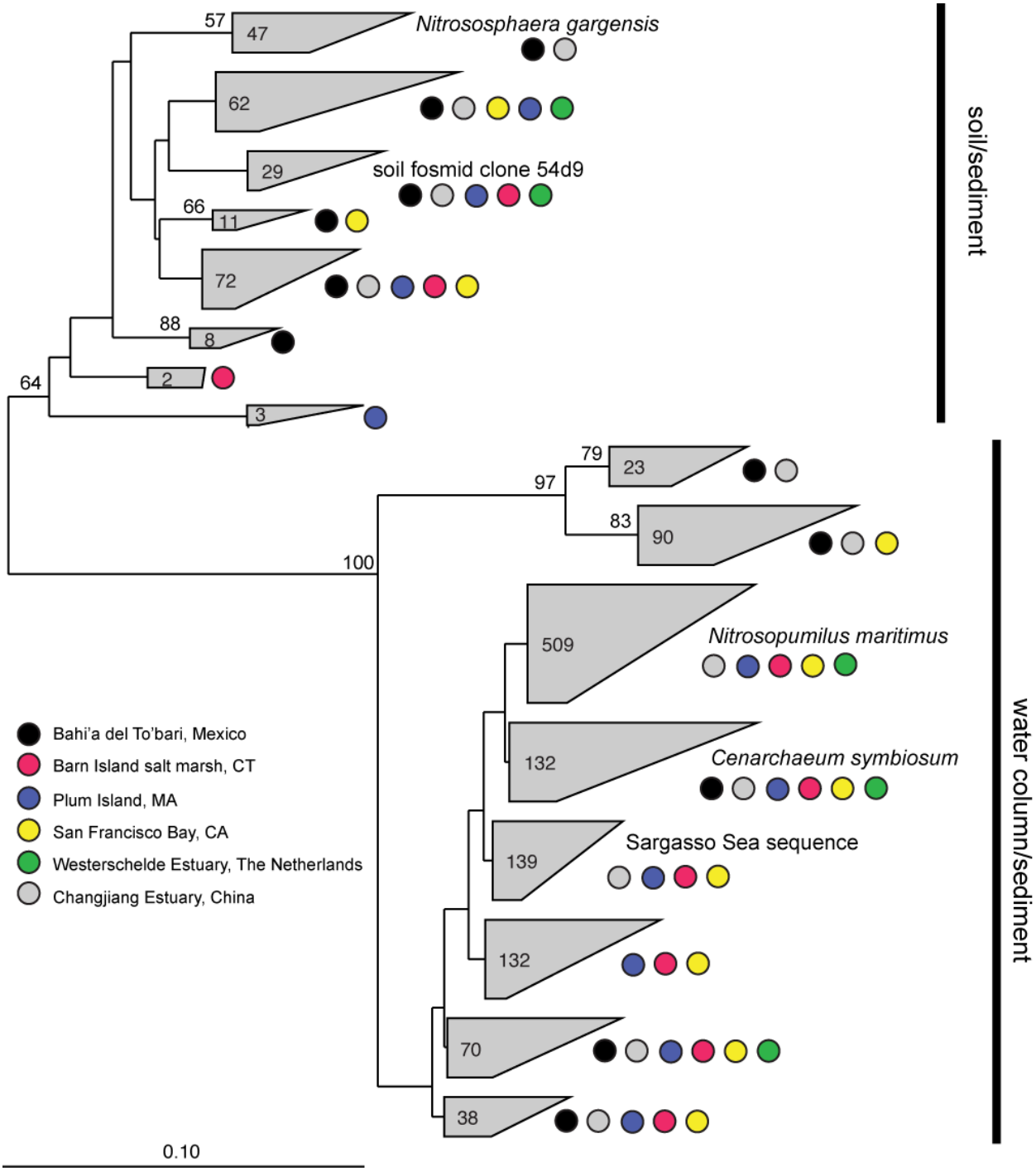

Nitrososphaera gargensis
soil fosmid clone 54d9
Nitrosopumilus maritimus
Cenarchaeum symbiosum
Sargasso Sea sequence
soil/sediment
water column/sediment
57
47
62
29
66
11
72
88
8
64
2
3
79
23
97
83
90
100
509
132
139
132
70
38
Bahi'a del To'bari, Mexico
Barn Island salt marsh, CT
Plum Island, MA
San Francisco Bay, CA
Westerschelde Estuary, The Netherlands
Changjiang Estuary, China
0.10

Figure 4

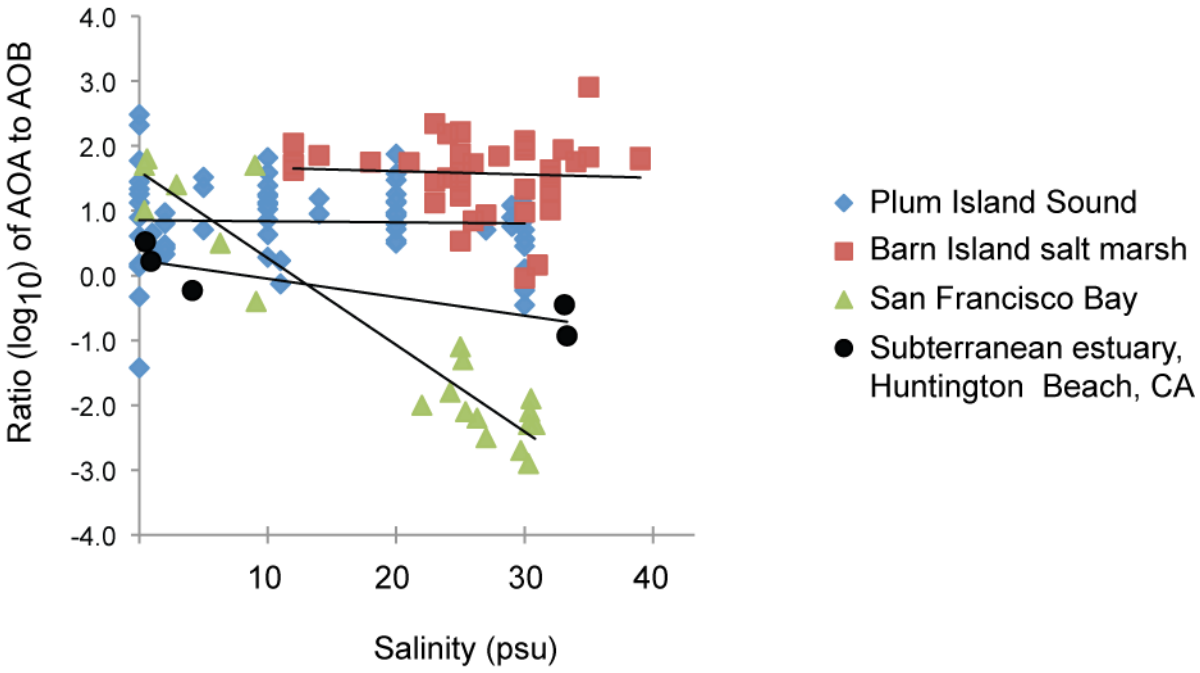

4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
Ratio (log_{10}) of AOA to AOB
10
20
30
40
Salinity (psu)
Plum Island Sound
Barn Island salt marsh
San Francisco Bay
Subterranean estuary, Huntington Beach, CA

Figure 5