ABUNDANCE, DIVERSITY, AND ACTIVITY OF AMMONIA-OXIDIZING PROKARYOTES IN THE COASTAL ARCTIC OCEAN IN SUMMER AND WINTER

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Marine Studies

Fall, 2009

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ABSTRACT

Ammonia oxidation, the first step in nitrification, is performed by certain *Beta*- and *Gammaproteobacteria* and mesophilic *Crenarchaea* to generate metabolic energy. Ammonia monooxygenase (*amoA*) genes from both Bacteria and Crenarchaea have been found in a variety of marine ecosystems, but the relative importance of Bacteria versus Crenarchaea in ammonia oxidation is unresolved, and seasonal comparisons are rare. In this study, we compare the abundance of betaproteobacterial and crenarchaeal *amoA* genes in the coastal Artic Ocean during summer and winter seasons over two years. Betaproteobacterial and crenarchaeal *amoA* genes were present in both seasons, but were more abundant during the winter. Archaeal *amoA* genes were more abundant than betaproteobacterial *amoA* genes in the first year, but betaproteobacterial *amoA* was more abundant than archaeal *amoA* the following year. Summer and winter betaproteobacterial *amoA* clone libraries were significantly different. Gene sequences of *amoA* were similar to those found in temperate and polar environments. The ratio of archaeal *amoA* gene copies to Marine Group I crenarchaeal 16S rRNA genes averaged 2.9 over both seasons, implying that ammonia oxidation was common in Crenarchaea at this location. Nitrification rates were highest in the winter when ammonia oxidizer abundance was greatest, suggesting that ammonia oxidation plays

an important role in coastal arctic waters during the winter when the ocean is ice covered and photosynthesis is at a minimum.

Chapter 1

INTRODUCTION

Ammonia oxidation is the first, rate limiting step in nitrification, the microbially mediated process where NH_4^+ is oxidized to NO_2^- and then to NO_3^- (Thamdrup and Dalsgaard 2008). Ammonia oxidation is suggested by the presence of a subunit of ammonia monooxygenase (*amoA*), which catalyzes the initial oxidation of ammonia to hydroxylamine (Arp and Stein 2003). Previously, only certain groups within the *Beta-* and *Gammaproteobacteria* were believed to perform ammonia oxidation, but then *amoA* was detected in mesophilic *Crenarchaea* (Francis et al. 2005, Wuchter et al. 2006). Archaeal *amoA* has been found in a variety of environments, including soils (Leininger et al. 2006), marine sediments (Beman and Francis 2006), and in the water column in both temperate (Mincer et al. 2007) and polar seas (Galand et al. 2009). Archaeal *amoA* is much more abundant than bacterial *amoA* in many marine environments, including the Monterey Bay and North Pacific (Mincer at al. 2007), the Gulf of California (Beman et al. 2008), coastal Antarctic and central Arctic Oceans (Kalanetra et al. 2009), and estuarine marine sediments (Beman and Francis 2006). In the eastern Mediterranean Sea archaeal *amoA* was abundant,

whereas betaproteobacterial *amoA* was not detected (De Corte et al. 2009). However, in San Francisco Bay sediments, betaproteobacterial *amoA* outnumbered archaeal *amoA* in higher salinity waters (Mosier and Francis 2008).

The biochemical process used by Crenarchaea to oxidize ammonia is unclear. Hydroxylamine oxidoreductase and cytochromes c_{554} and cm_{552}, which operate downstream of ammonia monooxygenase in bacteria, have not been found in the genome of the one mesophilic Crenarchaea genome fully sequenced to date, *Cenarchaeum symbiosum* (Hallam et al. 2006). However, *Nitrosopumilis maritimus*, a cultured marine crenarchaeote isolated from a marine aquarium, contains *amoA*, oxidizes ammonia and fixes CO_2 autotrophically in cultures lacking organic carbon (Könnecke et al. 2005). Indirect evidence for crenarchaeal ammonia oxidation includes a North Sea enrichment culture where crenarchaeal cell numbers and nitrite concentrations increased while ammonium concentrations decreased (Wuchter et al. 2006). In addition, crenarchaeal *amoA* genes were strongly expressed in the suboxic zone in the Black Sea where nitrification was occurring (Lam et al. 2007). Microradiography and fluorescence *in situ* hybridization were also used to show that at least some crenarachaea were using CO_2 as a carbon source (Herndl et al. 2005).

The fraction of marine mesophilic Crenarchaea that are ammonia oxidizers is also not clear, particularly in deep waters. The ratio of archaeal *amoA* genes to 16S rRNA genes from Marine Group I crenarchaeal 16S rRNA genes is often used as an

indicator of the potential for ammonia oxidation in environmental samples (for example, Wuchter et al. 2006, Mincer et al. 2007, and De Cotre et al. 2009). Most mesophilic Crenarchaea have been found in two phylogentic groups, the Marine Group I archaea and the pSL-12 like archaea (Mincer et al. 2007). The *Cenarchaeum symbiosum* genome contains one *amoA* gene and one 16S rRNA gene (Hallam et al. 2006). Agogue et al. (2008) found that the ratio of archaea *amoA* gene copies to Marine Group I Crenarchaeal 16S rRNA genes (*amo*A:16S rRNA ratio) was about 1:1 in subsurface waters (100-150m). However, this ratio declined by one to two orders of magnitude in water layers at 200-1,000m and below 1000m. Crenarchaeal *amoA*:16S rRNA ratios also declined with depth in the eastern Mediterranean Sea, from 0.49 at 100m to 0.12 at 400 m, and below 0.05 at depths greater than 750m (De Corte et al. 2009). However, a metagenomic analysis of a whole genome shotgun library from the North Pacific found a 1:1 ratio of crenarchaeal *amoA*:16S rRNA genes even at depths of 4000m (Konstantinidis et al. 2009). Crenarchaeal *amoA*:16S rRNA ratios were about 1:1 and did not vary with depth in the central Arctic Ocean (Kalanetra et al. 2009). Ratios of 1:1 *amoA*:16S rRNA were also reported in a North Sea enrichment culture (Wuchter et al. 2006), and Monterey Bay and North Pacific water samples (Mincer et al. 2007). However, higher ratios of crenarchaeal *amoA*:16S rRNA genes have also been found, such as 2.8 in the North Sea (Wuchter et al. 2006), 2.5 in the Gulf of California (Beman et al. 2008), 2.0 in Circumpolar Deep

Water west of the Antarctic Peninsula (Kalanetra et al. 2009), and 8 in the eastern Canadian Arctic (Galand et al. 2009). More information is needed to establish the extent of ammonia oxidation among mesophilic marine archaea.

The few available estimates of nitrification rates vary widely (Yool et al. 2007), and measurements of both *amoA* abundance and nitrification rates from the same samples are rare. In one study, ammonium oxidation rates were correlated with *amoA* and Marine Group I Crenarchaeal 16S rRNA gene copies throughout the water column of the Gulf of California (Beman et al. 2008). Nitrification rates range from 3.1 nmol l^{-1} d^{-1} in the oligotrophic Atlantic (Clark et al. 2008) to as high as 45 nmol l^{-1} d^{-1} in Monterey Bay and Southern California Bight (Ward et al. 1987, Ward et al. 2005), but *amoA* abundance was not examined in these studies. No estimates of nitrification are available from polar waters.

Summer and winter conditions differ greatly in polar regions, so nitrification rates and ammonia oxidizer abundance can be expected to differ seasonally in this environment. Chemoautotrophy may become more important during the winter when primary productivity is low. Ammonia oxidation is inhibited by light (Guerrero and Jones 1996) and can be limited by competition with phytoplankton for assimilation of ammonium (Canfield et al. 2005). If most marine Crenarchaea oxidize ammonia, their abundance may increase when darkness limits primary productivity. Archaeal abundance was higher during the winter than summer in Antarctica coastal waters

where archaeal rRNA levels negatively correlated with chlorophyll *a* concentrations (Murray et al. 1998) and in the Southern Ocean west of the Antarctic Peninsula where crenarchaeal populations were about 1% of prokaryotes during the summer, but increased to 10% in surface waters during the winter (Church et al. 2003). In Franklin Bay in the western Canadian Arctic, archaea were 16% of total prokaryotes in surface waters during winter, but decreased to less than 10% in spring and summer (Alonso-Saez et al. 2008).

In this study we examined winter and summer communities of ammonia oxidizing prokaryotes in the coastal Arctic Ocean near Barrow, Alaska. Surface waters were sampled to determine the abundance of betaproteobacterial and archaeal *amoA* genes and Marine Group I *Crenarchaea* 16S rRNA genes in the summer and winter over two years. Clone libraries were prepared to examine partial *amoA* gene sequences and to compare coastal arctic ammonia-oxidizing communities with those found elsewhere. Nitrification rates and other physical data were examined to understand how these communities vary with such seasonal extremes and provide more information about an understudied ocean. Nitrification rates and the abundance of betaproteobacterial and crenarchaeal *amoA* varied seasonally, reaching their highest levels in winter.

Chapter 2

METHODS AND MATERIALS

Sample Collection

Seawater samples were obtained from the coastal Arctic Ocean near Barrow, Alaska, over a two year period (Table 1). Sample sites were located in nearshore waters of the Chukchi and Beaufort Seas on either side of Barrow Point. A total of ten summer samples were obtained from surface waters (~2 m depth) via a manual pump from small boats in July 2007, and July 2008. Seven winter samples were extracted by drilling a hole in the sea ice and manually pumping water from directly below the ice (~2m) in January 2007 and January 2008. Between 1.3 and 1.8 l of seawater were filtered through 0.22 µm Durapore membrane filters (Millipore) on the same day as collection at a land based facility. Filters were then preserved in a cetyltrimethyl ammonium bromide (CTAB) buffer and stored –80º C until DNA extractions were performed.

Environmental Parameters

Nutrient concentrations, chlorophyll *a* concentrations, bacterial production, and microscopic direct counts of bacterial productions were obtained as in Cottrell and Kirchman (2009). Briefly, nutrient concentrations were determined using the sodium nitroprusside method for ammonium (Solorzano 1969) or an automated, segmented flow colorimetric analysis for nitrite + nitrate, phosphate, or silicate. Chlorophyll *a* concentrations were obtained by extraction of filtered particulates captured by GF/F filters (Whatman) in 90% acetone and subsequent measurement of fluorescence (Parsons et al. 1984). Prokaryote abundance was measured microscopically from 0.2 μm polycarbonate filters stained with 4', 6-diamidino-2-phenylindole and semiautomated image analysis (Cottrell and Kirchman 2003). Bacterial production was determined by 1.5 to 2.5 hour incubations with ^{3}H-leucine at *in situ* temperatures in the dark, and subsequent radioassay to quantify the incorporation of ^{3}H-leucine into cells (Kirchman 2001).

DNA Extraction and PCR screening

DNA was extracted from the filters using a modified CTAB protocol, involving two chloroform extractions and a high-salt isopropanol precipitation (Dempster et al. 1999). The Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) was used to measure DNA concentrations via fluorescence. Samples were initially

screened for presence of betaproteobacterial, gammaproteobacterial, and archaeal *amoA* genes by PCR followed by examination of PCR products on 1.5% agarose gels. Primers used are listed in Table 2. Approximately 10 ng of extracted DNA was screened in 20 µl PCR reactions.

Betaproteobacterial *amoA* PCR products from positive tests from one summer 2007 Chukchi Sea sample (Chuk-1 in Table-1) and three pooled winter 2008 Chukchi Sea samples (Chuk-3, Chuk-4, and Chuk-5) were cloned using the TOPO TA Cloning Kit (Invitrogen). Archaeal *amoA* products from two pooled winter 2008 Chukchi Sea samples (Chuk-4 and Chuk-5) were also cloned; however, summer archaeal *amoA* was not. The clones were sequenced via the Sanger method by the High Throughput Genomics Unit of the University of Washington, Seattle, Washington. Initial betaproteobacterial *amoA* sequences were 492 bp long, and the library contained 92 clones from summer 2007 and 91 clones from winter 2008. The archaeal *amoA* sequences were 624 bp long. and the library contained 45 sequences from winter 2008 samples. Sequences of poor quality or short reads and primer sequences were excluded.

Phylogenetic analysis

Sequences were aligned via BioEdit (Hall et al. 1999) or Mega Version 4 (Tamura et al. 2007) using CLUSTALW. Clustering and operational taxonomic unit

(OTU) analysis was by DOTUR (Schloss and Handelsman 2005). OTUs were chosen so as to contain all sequences that are less than 5% divergent in nucleotides. Coverage ratios for each library are calculated as 1 minus the quotient of the number of OTUs containing only one sequence divided by the total number of sequences. Summer and winter betaproteobacterial libraries were compared using ∫-Libshuff (Schloss et al. 2004). Phylogenetic trees were prepared using Mega 4. Nucleotide sequences including all three codon positions were analyzed with 500 bootstrap replicates, and were based on the Jukes-Cantor model of DNA substitution. Sequences from Barrow and other environments were first trimmed to 383 bp in the case of betaproteobacterial *amoA* and 591 bp in the case of archaeal *amoA* to exclude primer sites and so that the analysis was performed on sequences of equal lengths. The amino acid based tree for betaproteobacterial *amoA* was based on distances adjusted by the Poisson correction.

Quantification of *amo*A and 16S rRNA Genes by Quantitative PCR (qPCR)

qPCR reactions were performed on a Rotor-Gene 6000 real time rotary qPCR analyzer (Corbett Research) using SYBR GREEN PCR Master Mix (Applied Biosystems). Primers used for each quantified gene are shown in Table 2. The primer concentrations was 0.16 pM in each 12.5 µl reaction, and the following cycling times were used: 95 ºC for 10 minutes, followed by individual cycles of 95 ºC for 15s, 50 ºC for 15s, and 72ºC for 15s. Plasmids made by cloning environmental samples were

used as standards. Standards and samples were quantified via Pico Green analysis using the Rotor-Gene 6000. Standard curves were developed plotting C_t values against log gene copies. The C_t or threshold cycle represents the first PCR cycle where the fluorescent signal exceeds background. Efficiencies were calculated by raising 10 to an exponent equal to -1 divided by the slope of the standard curve. At an efficiency of 100%, a difference of one C_t unit represents a doubling of DNA quantity. Standard curve correlation coefficients and efficiencies were as follows: betaproteobacterial *amoA* ($r^2 = 0.994$, efficiency = 83%), archaeal *amoA* Group A ($r^2 = 0.998$, efficiency = 77%), Crenarchaea Marine Group I 16S rRNA ($r^2 = 0.999$, efficiency = 71%), and pSL12 Group 16S rRNA ($r^2 = 0.999$, efficiency = 86%). Melting curve and agarose gel electrophoresis analyses of qPCR products were used to test for non-specific amplification. Gene abundance was normalized both per ng of extracted DNA and per ml of filtered seawater.

Nitrification Rates

^{15}N-labeled NH_4^+ was used to measure nitrification rates using the dinitrifier method of Sigman et al. (2001). This nitrification assay was performed on four samples from summer 2008 (Beau-5, Beau-6, Chuk-6, Chuk-7) and four samples from winter 2009 (Chuk-8, Beau-7, Beau-8, Beau-9). Briefly, ^{15}N-labeled NH_4^+ was added to 200 ml of seawater to reach a final concentration of 1 μM. Three replicates were

incubated in the dark for 24 hr. Initial samples and samples taken after 24 hours were filtered through 0.22 µm polycarbonate filters, and the filtrate was frozen at -80 ºC until later analysis. A denitrifier, *Pseudomonas aureofaciens,* modified to lack nitrous oxide reductase, was used to convert NO_2^- or NO_3^- in the filtrate to nitrous oxide (N_2O), which was trapped. The $\partial^{15}N$ values for the trapped N_2O were obtained via isotope ratio mass spectrometry and converted into nitrification rates. The mass spectrometry work was performed by the Isotope Biogeochemistry Laboratory at the University of Hawaii.

Chapter 3

RESULTS

The summer and winter environments in the coastal Arctic Ocean near Barrow, Alaska are very different (Table 3). The ocean is ice covered during much of the winter, and polar darkness persists for two months. Water temperatures varied between –1.7 ºC in winter to as high as 5.1 ºC in summer. Nutrient concentrations also varied with the seasons and were highest in the winter. Nutrient levels decline each summer once sunlight and photosynthesis return. Chlorophyll *a* concentrations, while never reaching zero during the winter, were about 10 times higher during summer. Both prokaryotic abundance and production were also highest during the summer months. Prokaryote abundance ranged between 0.22 and 0.55 x 10^6 cells per ml during the winter, increasing to a range of 0.93 – 1.91 x 10^6 cells per ml during the summer. Bacterial production followed a similar pattern and was 19 – 103 nmol C L^{-1} hr^{-1} in winter, increasing to 156 – 280 nmol C L^{-1} hr^{-1} in the summer. Although there were significant differences between seasons for each environmental parameter ($p< 0.05$, Student t-test), differences between the Chukchi Sea and Beaufort Sea values were not significant ($p> 0.05$, Student t-test).

Phylogeny of Bacterial and Archaeal *amoA*

Ammonia oxidizing bacteria and archaea were present in the coastal Arctic Ocean during both seasons; however, partial *amoA* gene sequences from these organisms found in clone libraries were not very diverse. Of 183 betaproteobacterial *amoA* sequences from both seasons, only six operational taxonomic groups (OTUs) were found (Table 4), defined by <5% divergence in nucleotides. The Chao1 statistic, a nonparametric estimate of species richness, was seven for the entire set of summer and winter betaproteobacterial *amoA*. Individual betaproteobacterial *amoA* libraries contained only three and five OTUs in summer and winter respectively. The summer and winter betaproteobacterial *amoA* sequences were different from each other ($p < 0.01$, ∫-Libshuff test). Archaeal *amoA* sequences were less diverse than the betaproteobacterial sequences. In a library containing 45 clones from winter, only four OTUs of archaeal *amoA* were present. The *amoA* gene from Gammaproteobacteria was not detected.

Most betaproteobacterial *amoA* sequences from this study separated into three large clades, one group containing mostly summer sequences and two groups containing mostly winter sequences (Figure 1). The large summer clade contained 84 summer sequences and 21 winter sequences. Another more distant summer clade

contained 4 sequences. There are four predominantly winter clades, with the largest containing 40 winter sequences and no summer sequences. The second largest winter clade contained 28 winter sequences and four summer sequences. Sequences in each of these groups were similar to sequences in other marine environments, including water column and sediments in both polar and temperate seas (e.g. Kalanetra et al. 2009, O'Mullan et al. 2005). The large group containing 40 winter sequences forms a unique clade with other polar sequences with a > 97% identity in nucleotides. However, sequences from other more temperate environments become a part of this clade with these sequences if the cutoff is widened to a level of > 95% identity. Most of the Barrow betaproteobacterial *amoA* sequences are more closely related to cultured *Nitrosomonas* species such as *Nitrosomonas cryotolerans* than to the *Nitrosospiras* species.

Betaproteobacterial *amoA* amino acid sequences are even less diverse than the nucleotide sequences (Figure 2). While the two large winter nucleotide OTUs are different by 30 or 50 nucleotides from the large summer group, most of these substitutions are anonymous. The winter group with 28 winter and 4 summer sequences is nearly identical to the large summer group on an amino acid basis. The largest winter group with 40 sequences differs from the large summer group by only 3 to 5 amino acids.

Archaeal *amoA* sequences (Figure 3) form two distinct groups similar to the shallow water Group A and deep water Group B clades reported by Beman et al. (2008). These sequences were also similar to other reported sequences from a variety of non-polar environments including the Black Sea, North Pacific, and Gulf of California. Although *amoA* sequences from both Group A and Group B clades were found in clone libraries, only Group A *amoA* was quantifiable via quantitative PCR, so that all abundance data discussed below includes only Group A archaeal *amoA*.

Abundance of *amoA* Genes

Both betaproteobacterial and archaeal Group A *amoA* genes were more abundant during the winter than the summer (Figures 4 and 5). When normalized to ng of extracted DNA, betaproteobacterial *amoA* copies increased from 94 in summer 2007 to 4,600 copies in winter 2008 (Figure 4). In the following year, betaproteobacterial *amoA* again increased from 207 in the summer to over 6,600 copies per ng of DNA in the winter. Archaeal *amoA* genes from the Group A (shallow water) clade were detected via qPCR; however, archaeal *amoA* genes from Group B (deeper water) were not. Group A archaeal *amoA* increased from 103 in summer 2007 to 17,872 copies per ng of DNA in winter 2008 and from 47 in summer 2008 to 1,297 in winter 2009. In winter 2008, archaeal *amoA* copies were more abundant than betaproteobacterial *amoA* copies (17,872 versus 4,600 copies per ng of DNA).

However, during the following winter, betaproteobacterial *amoA* copies were five-fold greater than archaeal *amoA*. Similar results were obtained when *amoA* abundance was normalized per ml of filtered seawater (Figure 5). Again, both betaproteobacterial and archaea *amoA* was more abundant during the winter than in the summer.

Crenarchaea within Marine Group I were also more abundant during the winter than in the summer (Table 5), following a pattern similar to that of archaeal *amoA* genes. Marine Group I 16S ribosomal RNA genes increased from 29 in summer 2007 to 5,546 copies per ng of DNA in winter 2008. The following year, 16S rRNA from this archaeal group increased from 30 copies in summer 2008 to 455 in winter 2009. Marine archaea in the pSL12 group were not common in our samples, with 16S rRNA copies ranging from 44 – 311 copies per ng DNA in winter. Copies of pSL12 16S rRNA genes were higher during summer months, but nonspecific DNA amplification occurred in the summer samples. Cloning and sequencing of the nonspecific DNA revealed that the PCR products were not from 16S rRNA genes (data not shown). As a result, pSL12 data were excluded from all ratio calculations below.

While *amoA* gene copies were only weakly correlated with ammonium concentrations ($r = 0.40$, $p > 0.05$), they were more strongly correlated with nitrate/nitrite concentrations ($r = 0.88$, $p < 0.01$). Nitrate and nitrite are the products of the nitrification process. Total *amoA* gene copies were also negatively correlated

with chlorophyll *a* concentrations ($r = -0.60$, $p < 0.05$) and bacterial production ($r = -0.72$, $p < 0.01$).

Even during winter 2008, ammonia oxidizers did not make up a large proportion of the prokaryotic population. Summer ratios of *amoA* to total 16S rRNA averaged 0.04% versus an overall winter mean of 3.8%. Archaeal Marine Group I 16S rRNA copies averaged 0.6% of bacterial 16S copies during the winter, but were less than 0.01% of bacterial 16S copies during the summer (Table 5).

Ratios of *amo*A Genes to Marine Group I 16S rRNA Genes

The ratio of archaea *amoA* genes to Marine Group I 16S rRNA genes averaged 2.87 over all samples (Figure 6). The *amoA*:16S rRNA ratio was similar in both seasons, with a summer mean of 2.57 and a winter mean of 3.31 *amo*A:16S rRNA ($p > 0.05$, Student t-test). Archaea *amoA* gene copies correlated with Marine Group I 16S rRNA copies ($r = 0.96$, $p < 0.01$).

Nitrification Rates

Nitrification rates were over 20-fold higher in the winter than in the summer (Figure 7). Summer 2008 nitrification rates from four locations averaged 0.15 nmol l^{-1} day^{-1}. Winter 2009 rates, again from four samples, ranged from 2.82 to 4.48 nmol l^{-1} day^{-1}, with a mean of 3.59 nmol l^{-1} day^{-1}. Total *amoA* gene copies from the same

locations averaged 300 copies per ng of DNA in summer 2008 and nearly 8,000 in winter 2009. When nitrification rates are plotted against total betaproteobacterial and archaeal *amoA* (Figure 7), separate winter and summer groups appear. Nitrification rates correlate with total *amoA* copies per ng of DNA ($r = 0.74$, $p < 0$ .05). Correlation of nitrification rates to *amoA* copies is similar for betaproteobacterial *amoA* ($r = 0.75$, $p < 0.05$) and archaeal *amoA* ($r = 0.70$, $p > 0.05$) separately; however, the correlation with archaeal *amoA* is not significant at the 0.05 level.

Chapter 4

DISCUSSION

The coastal Arctic Ocean is ice-covered during the winter, and the sun stays below the horizon for two months each year. Photosynthesis by phytoplankton in the water column or algae in sea ice can fuel food webs during the sunlit months, but photosynthesis is drastically reduced and ceases altogether during the dark, winter months. Chemoautotrophy, such as ammonia oxidation, may become important during the winter when primary production is low. Our objectives in this study were to examine ammonia oxidizing prokaryotes in the coastal Arctic Ocean, in particular comparing their abundance and activity in summer versus winter, and relating these data to environmental parameters such as ammonium concentrations.

Our samples were taken from nearshore surface waters of the Arctic Ocean on both the Chukchi Sea and Beaufort Sea sides of Barrow Point. Summer and winter environments differed in physical conditions, prokaryotic abundance and production, and in chlorophyll *a* and nutrient concentrations. Both ammonium and nitrite + nitrate concentrations were higher in the winter than in the summer. The higher nutrient concentrations reflected the winter reduction in photosynthesis, as indicated

by the 10-fold reduction in chlorophyll a concentrations from summer to winter. Ammonium concentrations were twice as high in winter 2009 compared to winter 2008. However, corresponding nitrite + nitrate concentrations declined by half from the first winter to the second, but both were well above summer levels. Lower winter primary production was also reflected in lower prokaryotic abundance and bacterial production. Abundance declined 3-fold on average from summer to winter; the corresponding drop in bacterial production was almost 7-fold. However, nitrification rates increased by a factor of 24, implying a greater role for chemoautotrophy during the winter. In the Monterey Bay, nitrification rates from all depths varied 4.5-fold in bimonthly measurements over a two year period; however, ammonium concentrations were always low, with a maximim of 0.08 μM (Ward 2005).

Abundance of both betaproteobacterial and archaeal ammonia oxidizers was higher in the winter than in the summer, tracking the higher winter ammonium concentrations and nitrification rates, although the strongest correlation was between *amoA* gene copies and nitrite/nitrate concentrations ($r = 0.88$, $p < 0.01$). Abundance of Marine Group I Crenarchaea, as measured by 16S rRNA genes, also increased during the winter. If both ammonia oxidizer abundance and nitrification rates are indicators of the importance of this process to the ecosystem, ammonia oxidation is more important in coastal Arctic waters during the winter when photosynthesis is at a minimum and heterotrophic bacterial production declines. Crenarchaeal abundance is

also higher in the winter than in the summer in Antarctic waters (Murray et al. 1998, Church et al. 2003). Similarly, crenarchaeal abundance is higher in the winter in the North Sea and positively correlates with crenarchaeal *amoA* gene copy numbers (Wuchter et al. 2006, Herfort et al. 2007).

In Alaska coastal waters, copies of *amoA* genes correlated with nitrification rates and nitrite + nitrate concentrations, but were negatively correlated with chlorophyll *a* concentrations and bacterial production, suggesting that ammonia oxidation and nitrification become a greater factor in the environment when photosynthetic primary production is low. Archaeal abundance was also negatively correlated with chlorophyll *a* concentrations in nearshore Antarctic waters (Murray et al. 1998). In the Baltic Sea, *amoA* copy numbers were positively correlated with concentrations of nutrients, such as ammonium, nitrite, and nitrate (Herfort et al. 2007). Nitrification rates were correlated with both crenarchaeal 16S rRNA copies and *amoA* gene copies in the Gulf of California (Beman et al. 2008). Low summer nitrification rates could result from competition or inhibition by phytoplankton (Canfield et al. 2005, Herfort et al. 2007) or light inhibition (Guerrero and Jones 1996). Nitrification rates during winter may increase once these controls are removed.

Partial *amoA* gene sequences from our samples were not very diverse, with only six OTUs found in betaproteobacterial *amoA* and four OTUs found in archaeal *amoA* sequences, with each OTU representing <5% nucleotide divergence. Low

diversity in *amoA* genes has also been reported for other environments. In sediment samples from an eutrophic estuary in Mexico, Beman and Francis (2006) found 6 – 7 betaproteobacterial *amoA* OTUs (defined as 5% nucleotide difference) and between 4 and 10 archaeal *amoA* OTUs in libraries containing 22 – 32 clones. In the San Francisco Bay, Mosier and Francis (2008) found 4 – 11 archaeal *amoA* OTUs and 3 – 11 betaproteobacterial OTUs in individual locations, but from all sites in salinities ranging from 0 – 31, a total of 67 unique archaeal and 41 unique bacterial OTUs were found. Kim et al (2008) detected six OTUs in a lake and five OTUs at a 10m depth in the Baltic Sea. In the eastern Canadian Arctic, Galand et al. (2009) reported between three and five phylotypes in archaeal 16S rRNA clone libraries (cutoff at 97% identity). Some studies have suggested that diversity may be driven by differences in salinity (Bernhard et al. 2005, Mosier and Francis 2008). However, Barrow salinities ranged from only 29.3 to 34.4.

Sequences from our Arctic samples were closely related to sequences found in other environments. Both winter and summer Betaproteobacteria *amoA* sequences were similar to sequences obtained from the central Arctic Ocean (Kalanetra et al 2009), but they were also similar to water column sequences from the Baltic Sea (Kim et al. 2008), the Monterey Bay (O'Mullan et al. 2005), oxygen minimum zones off Peru (Lam et al. 2009) and Chile (Molina et al. 2007), and to sediment sequences from the Chesapeake Bay (Francis et al. 2003), San Francisco Bay (Mosier and Francis

2008), and Plum Island Sound Estuary in Massachusetts (Bernhard et al. 2005). One group of winter betaproteobacterial *amoA* sequences from Barrow form a clade with other polar sequences (Kalenetra et al. 2009) at > 97% similarity, hinting that a uniquely polar form of *amoA* exists; however, sequences from other more temperate regions enter this group if the cutoff is relaxed to > 95% similarity. Betaproteobacterial *amoA* amino acid sequences were even less diverse than nucleotide sequences.

Archaeal *amoA* genes were less diverse than betaproteobacterial *amoA*, and were similar to other sequences from the Arctic and Antarctic (Kalanetra et al. 2009) and elsewhere (e. g. Francis et al. 2003, Bernhard et al. 2005, Kim et al. 2008). Crenarchaeal 16S rRNA sequences from both the central Arctic Ocean and Antarctic waters also had low diversity and were similar to those from temperate regions (Bano et al 2004, Kalanetra et al. 2009). Both shallow water clade Group A archaeal *amoA* and deeper water Group B sequences were detected in our clone libraries, as was the case in the Gulf of California (Beman et al. 2008), although Group B *amoA* was not quantifiable by qPCR.

The question as to whether all marine Crenarchaea are ammonia oxidizers remains open. The two genomes of ammonia oxidizing Crenarchaea at least partially sequenced to date, *Cenarchaeum symbiosum* and *Nitrosopumillus maritimis*, both contain one *amoA* and one 16S rRNA gene (Könneke et al. 2005, Hallam et al. 2006,

De Corte et al. 2009). *N. maritimus* appears to be an obligate chemoautotroph, able to grow in culture with only ammonium as an energy source and bicarbonate as a carbon source, with even small amounts of organic compounds inhibiting its growth (Könneke et al. 2005). However, *C. symbiosum* may be capable of obtaining carbon both by autotrophic fixation of CO_2 or via organic inputs based on the presence of genes in its genome that suggest that it may be capable of mixtotrophy (Hallam et al, 2006). A wide range of *amoA*:16S rRNA ratios have been reported in studies of uncultured Marine Group I Crenarchaea. The ratio was about 1:1 in an enrichment culture experiment in the North Sea (Wuchter et al 2006). Ratios varied between 0.01 to 2.8 in the Black Sea (Lam et al. 2007), and as high as 3.4 in the North Sea (Wuchter et al. 2006, Herfort at al. 2007). The highest ratio detected to date was an *amo*A:16S ratio of 8 in eastern Canadian Arctic waters (Galand et al. 2009). Mincer et al. (2007) found an approximate 1:1 ratio in the Monterey Bay, but the *amo*A:16S rRNA ratio varied with depth at station ALOHA in Hawaii, with a peak much greater than 1:1 at 200m (Mincer et al. 2007). A ratio of archaeal *amoA* genes to Marine Group I crenarchaeal 16S rRNA greater than one suggests that all mesophilic marine Crenarchaea can oxidize ammonia. However, there are other possible explanations for ratios greater than 1:1. Some marine Crenarchaea may possess multiple copies of the *amoA* gene as do some bacteria (Klotz and Norton 1998, Holmes et al. 2001), and

marine crenarchaeal 16S rRNA may be underestimated due to problems with the PCR primers.

Some studies suggest that ammonia oxidation by marine Crenarchaea may be common in upper water layers but that archaea in deeper waters with low ammonium concentrations may survive heterotrophically (Agogue et al. 2008, De Corte et al. 2009). Low *amoA* to Marine Group I crenarchaeal 16S ratios in the eastern Mediterranean (De Corte et al. 2009) and North Atlantic Ocean (Agogue et al. 2008) support this view. Other studies including a metagenomic analysis of a whole genome library from the North Pacific (Konstantinidis et al. 2009) and qPCR results from the Central Arctic Ocean (Kalanetra et al. 2009) found 1:1 ratios of *amoA* to 16S rRNA throughout the water column, suggesting that ammonia oxidation may be widespread at all depths. The Northern Pacific metagenomic analysis suggests that current qPCR primer sets may not capture all of the *amoA* genes found at greater depths, leading to underestimates of *amoA*:16S rRNA ratios in deeper waters (Agogue et al. 2008). Abundance of Marine Group I cenarchaeal rRNA genes may also be underestimated by commonly used primer sets. Mincer et al. (2007) developed a primer set for the pSL12-like clade, which was fairly abundant in the North Pacific, in an effort to explain variation in *amoA*:16S rRNA ratios. The ratio of *amoA* to 16S rRNA was close to 1:1 when rRNA gene copies from both Marine Group I and pSL12like archaea were combined (Mincer at al. 2007). However, pSL12-like archaea were not

common in our samples, with 16S rRNA copies ranging from 44 – 311 copies per ng DNA in winter. The pSL12 primer set produced non-specific amplification in summer samples (Table 5), so pSL12-like archaea were excluded from all of our copy numbers and ratios.

We have shown that betaproteobacterial and archaeaa *amoA* genes are more abundant in the winter than in the summer. Although ammonium was present at low levels and some nitrification occurred during the summer, nitrification rates and *amoA* gene abundance both increase substantially in winter when sunlight and photosynthesis were low. Both betaproteobacterial and crenarchaeal *amoA* genes are present and are similar to *amoA* genes found in other marine water column and sediment ecosystems, but they are not especially diverse. Ratios of Crenarchaea *amoA* genes to Marine Group I archaeal 16S rRNA genes were over 2:1 in both summer and winter periods, implying that most or all Marine Group I *Crenarchaea* were capable of ammonia oxidation.

However, the presence of archaeal *amoA* genes and increases in their abundance during the winter when nitrification rates are higher does not necessarily demonstrate that marine Crenarchaea are actually performing chemoautotrophic ammonia oxidation. This study did not measure mRNA expression level or functional activity of bacterial or archaeal *amoA* genes. In one recent study in an agricultural soil, bacterial ammonia oxidation was more important than archaeal ammonia

oxidation, even though archaeal *amoA* was more abundant than bacterial *amoA* (Jia and Conrad 2009). Other experiments that could be performed include ^{14}C-bicarbonate incubations in the dark combined with microradiography and fluorescence *in situ* hybridization to show that Crenarchaea actually fix CO_2 into organic material, and inhibition experiments involving acetylene, which irreversibly binds to *amoA* inactivating ammonia oxidation (Hyman and Wood 1985).

Table 1. Sample locations, dates, and times. Locations refer to coastal Arctic Ocean sites on the Chuckchi or Beaufort Sea side of Barrow Point.

Expedition	Site	Location	Latitude (N)	Longitude (W)	Date	Local Time
Summer, 2007	Chuk 1	Chukchi	71°25'21.60"	156°51'38.88"	7/11/07	8:12
	Chuk 2	Chukchi	71°23'4.80"	156°48'43.08"	7/11/07	9:06
	Beau 1	Beaufort	71°26'52.20"	156º 3’22.80”	7/13/07	8:48
	Beau 2	Beaufort	71°23'27.00"	156° 9'3.24"	7/13/07	9:22
Winter, 2008	Chuk 3	Chukchi	71°21'9.90"	156°40'59.50"	1/26/08	13:30
	Chuk 4	Chukchi	71°21'9.90"	156°40'59.50"	1/28/08	14:46
	Chuk 5	Chukchi	71°21'9.90"	156°40'59.50"	1/30/08	15:14
Summer, 2008	Beau 3	Beaufort	71° 24.338'	156° 8.026'	8/2/08	15:01
	Beau 4	Beaufort	71° 23.491'	156° 9.01'	8/2/08	15:47
	Chuk 6	Chukchi	71° 22.447'	156° 45.306'	8/4/08	13:44
	Chuk 7	Chukchi	71° 21.158'	156° 40.962'	8/4/08	16:23
	Beau 5	Beaufort	71° 26.900'	156° 3.300'	8/8/08	11:23
	Beau 6	Beaufort	71° 23.500'	156° 9.050'	8/8/08	11:55
January, 2009	Chuk 8	Chukchi	71° 21.693'	156° 38.125'	1/9/09	11:50
	Beau 7	Beaufort	71° 21.831'	156° 15.396'	1/12/09	12:25
	Beau 8	Beaufort	71° 21.827'	156° 15.389'	1/13/09	12:56
	Beau 9	Beaufort	71° 21.827'	156° 15.389'	1/14/09	12:00

Table 2. PCR primers used for screening and cloning of environmental DNA and for determining gene abundance using quantitative PCR.

Gene	Group	Primer Name	Primer Sequence (5' – 3')	Purpose	Ref
amoA	Betaproteobacteria	AmoA-1F* AmoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	Screening qPCR	(1) (2)
amoA	Crenarchaea	AmoA-19F AmoA-643R	ATGGTCTGGCTWAGACG TCCCACTTWGACCARGCGGC CATCCA	Screening	(3)
amoA	Crenarchaea Group A	Arch-amoAFA Arch-amoAR	ACACCAGTTTGGYTACCWTCDGC GCGGCCATCCATCTGTATGT	qPCR	(4) (5)
amoA	Crenarchaea Group B	Arch-amoAFB Arch-amoAR	CATCCRATGTGGATTCCATCDTG GCGGCCATCCATCTGTATGT	qPCR	(4) (5)
16S rRNA	Total Bacteria	w49dir w34rev	CGGTCCAGACTCCTACGGG TTACCGCGGCTGCTGGCAC	qPCR	(6) (7)
16S rRNA	Crenarchaea Marine Group I	GI_751F GI_956R	AAGGTTARTCCGAGTGRTTTC TGACCACTTGAGGTGCTG	qPCR	(8)
16S rRNA	Crenarchaea PSL12 Group	pSL12_750F pSL12_876R	GTACTCCCCAGGCGGCAA GGTCCRCCAGAACGCGC	qPCR	(8)

(1) Rotthauwe et al. 1997
(2) Stephen et al. 1999
(3) Leininger et al. 2006
(4) Beman et al. 2008
(5) Francis et al 2005
(6) Delbès et al. 1998
(7) Lee et al. 1996
(8) Mincer at al 2007

Table 3. Environmental parameters in the coastal Chukchi and Beaufort Seas near Barrow, Alaska. Mean values and standard deviations (sd) for individuals sample sites are listed.

	Summer 2007				Winter 2008		Summer 2008				Winter 2009		
	Chukchi		Beaufort		Chukchi[a]		Chukchi		Beaufort			Beaufort	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	Chukchi[b]	mean	sd
Number of Samples	2		2		3		2		4		1	3	
Prokaryotes (10^6 cells per ml)	1.75	0.25	1.91	0.25	0.55	0.18	0.93	–	1.21	0.11	0.22	0.52	0.05
Water Temperature (ºC)	5.1	0.6	3.2	1.0	–0.5	2.2	4.2	2.9	2.5	1.5	–1.7	–1.7	–
Salinity	32.0	–	22.0	2.8	35.3	0.6	32.0	–	30.3	0.5	30.0	34.7	0.6
Chl a (µg/l)	0.55	0.06	0.41	0.11	0.06	0.01	1.62	0.38	1.03	0.12	0.14	0.04	–
Ammonium (µM)	0.39	0.02	0.25	0.01	1.88	0.23	0.74	0.35	0.66	0.48	4.72	3.60	0.14
Nitrate/Nitrite (µM)	0.90	0.26	0.25	0.02	11.35	0.19	0.63	0.29	1.47	1.21	6.86	6.98	0.58
Phosphate (µM)	0.53	–	0.22	0.06	1.95	0.08	0.72	0.14	0.72	0.07	1.30	0.99	0.09
Silica (µM)	9.92	1.06	4.65	0.21	22.97	0.85	9.61	0.27	9.01	0.40	18.20	15.87	0.40
Bacterial Production (nmol C L^{-1} hr^{-1})	237	39	280	35	20	4	156	97	180	22	103	19	1

[a] Beaufort Sea was not sampled in winter 2008.
[b] Only one Chukchi Sea site was sampled in winter.

Table 4. Clone library composition and diversity statistics for *amoA* libraries. Groups are defined at 5% nucleotide acid divergence.

Library	Season	Total number of clones	Number of groups	% Coverage	Chao 1	95% Confidence for Chao 1
Betaproteobacteria	Summer/ Winter	183	6	98.9%	7	(6.1 – 19.7)
Betaproteobacteria	Summer	92	3	100.0%	3	(3 – 3)
Betaproteobacteria	Winter	91	5	97.8%	6	(5.1 – 18.5)
Crenarchaea	Winter	45	4	100.0%	4	(4 – 4)

Table 5. Abundance of Bacteria and Crenarchaea from the coastal Chukchi Sea (Chuk) or Beaufort Sea (Beau) near Barrow, Alaska. Total prokaryotes counts based on microscopically enumerated direct counts. All other counts based on qPCR quantification of 16S rRNA genes.

Sample Location	Total Prokaryotes (10^3 cells/ml)		Total Bacteria (10^3 copies/ng)		Total Marine Group I (10^3 copies/ng)		pSL12 Group (10^3 copies/ng)	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd
Summer, 2007								
Chuk – 1	1,576	432	481	33	0.01	0.00	0.25	0.03
Chuk – 2	1,923	152	519	35	0.01	0.01	0.14	0.05
Beau – 1	2,088	302	482	66	0.02	0.01	0.38	0.04
Beau – 2	1,737	193	987	16	0.06	0.01	0.79	0.12
Winter, 2008								
Chuk – 3	422	77	687	65	9.94	1.85	0.15	0.04
Chuk – 4	467	132	151	14	1.71	0.50	0.04	0.01
Chuk – 5	748	159	460	36	4.99	0.19	0.17	0.06
Summer, 2008								
Beau – 3	1,066	268	538	11	0.05	0.01	0.16	0.14
Beau – 4	1,271	305	720	58	0.05	0.00	0.34	0.01
Chuk – 6	929	133	662	22	0.04	0.01	0.25	0.03
Chuk – 7	935	233	607	57	0.02	0.00	0.19	0.03
Beau – 5	1,183	417	425	17	0.01	0.00	0.18	0.02
Beau – 6	1,331	210	544	131	0.01	0.00	0.28	0.06
Winter, 2009								
Chuk – 8	220	77	212	3	0.15	0.01	0.10	0.01
Beau – 7	524	106	409	21	0.58	0.07	0.19	0.02
Beau – 8	568	156	399	50	0.53	0.04	0.21	0.05
Beau – 9	475	55	366	31	0.56	0.07	0.31	0.03

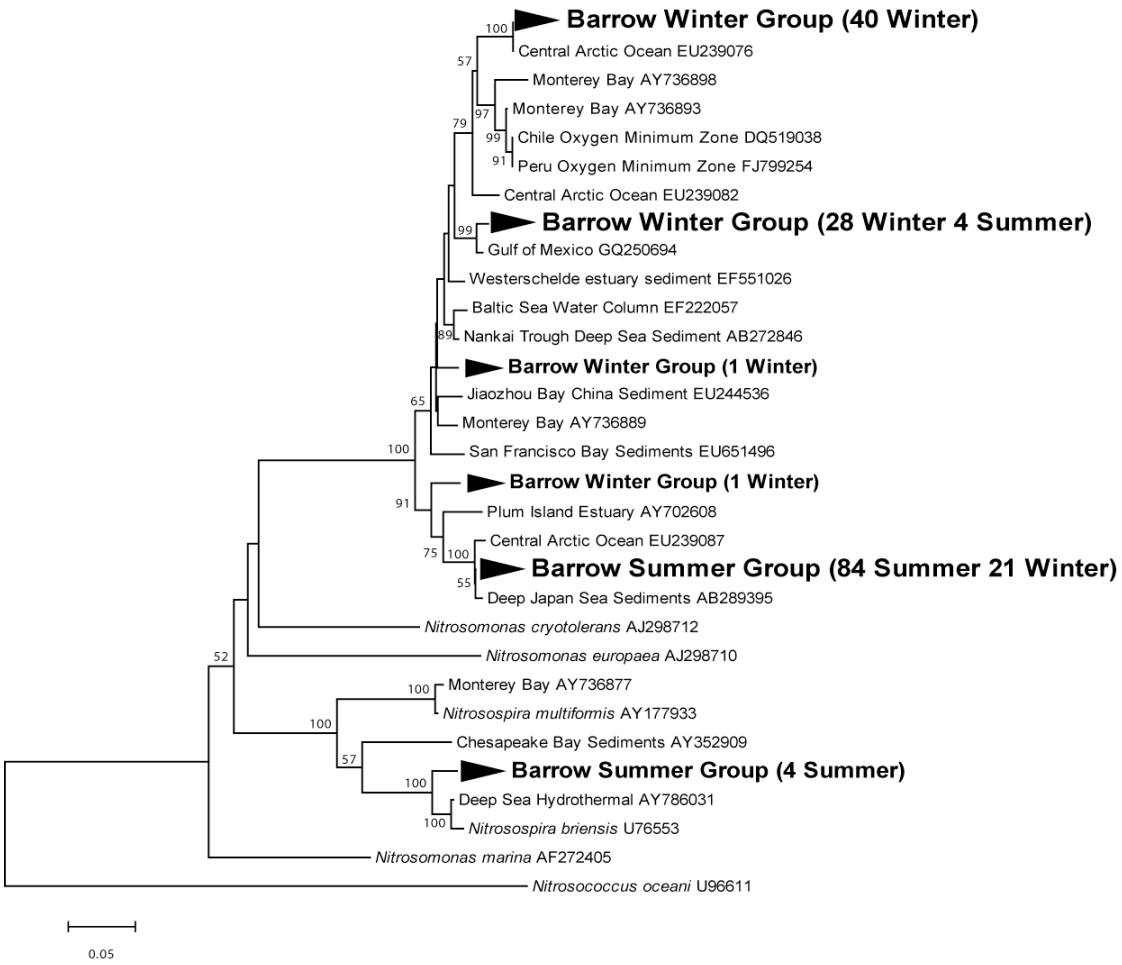

Figure 1. Neighbor Joining Phylogenetic Tree of Betaproteobacterial *amoA*. The phylogeny is based on nucleotide sequences. Bootstrap values lower than 50% are omitted.

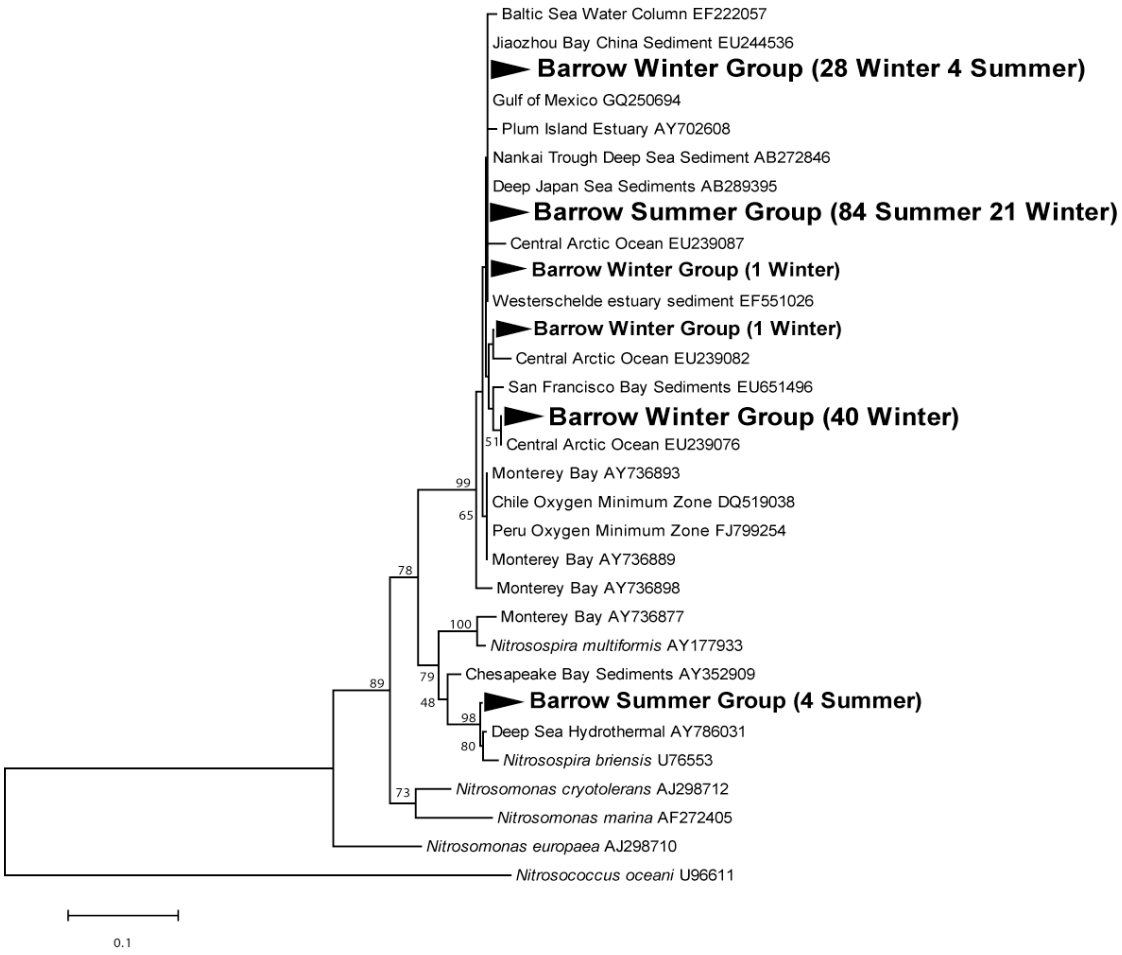

Figure 2. Neighbor Joining Phylogenetic Tree of Betaproteobacterial *amoA*. The phylogeny is based on amino acid sequences. Bootstrap values lower than 50% are omitted.

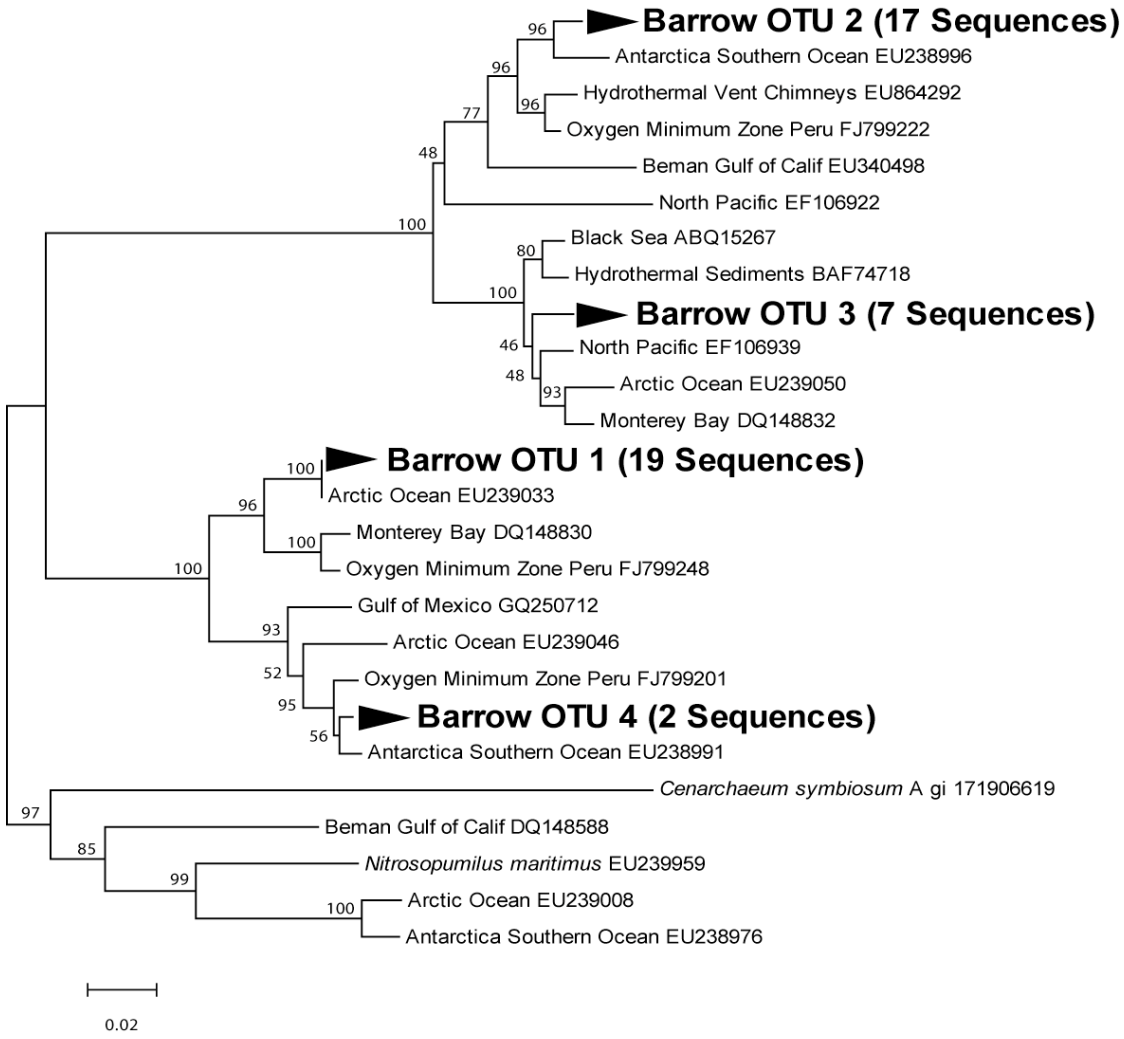

Figure 3. Neighbor Joining Phylogenetic Tree of Archaeal *amoA*. The phylogeny is based on nucleotide sequences. Bootstrap values lower than 50% are omitted.

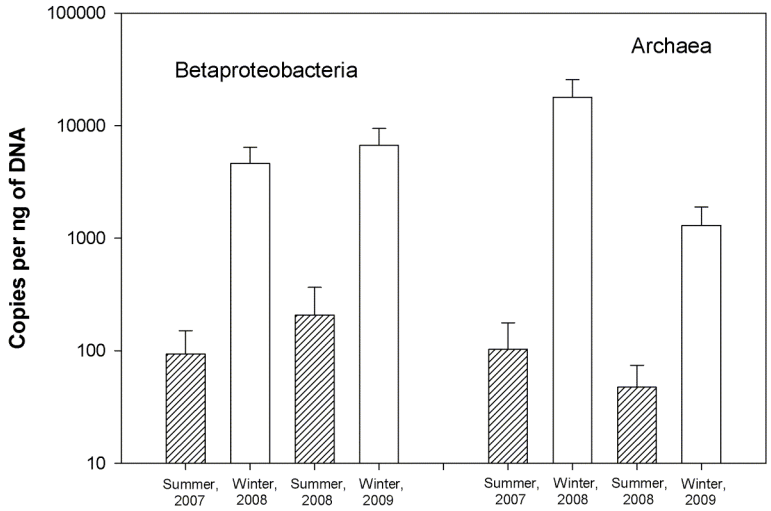

Figure 4 – *amoA* gene copies per ng of DNA – betaproteobacteria versus archaea (log scale). The number of sample locations in each season was summer 2007 (4), winter 2008 (3), summer 2008 (6), and winter 2009 (4).

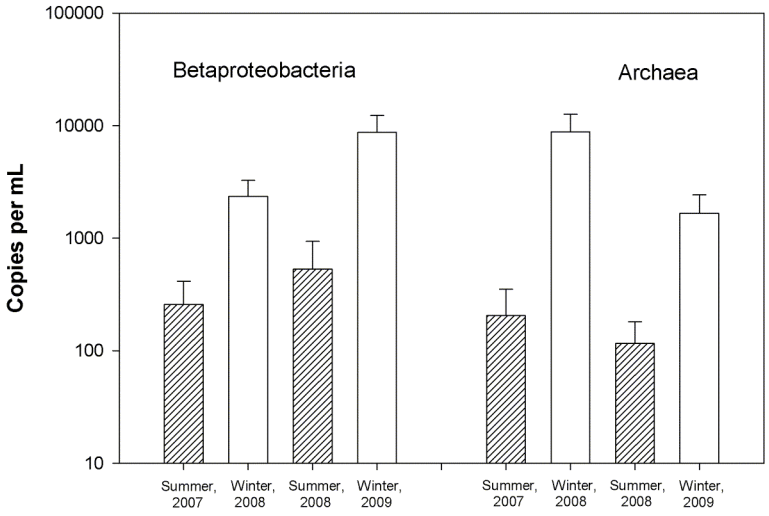

Figure 5 – *amoA* gene copies per ml of seawater – betaproteobacteria versus archaea (log scale). The number of sample locations in each season was summer 2007 (4), winter 2008 (3), summer 2008 (6), and winter 2009 (4).

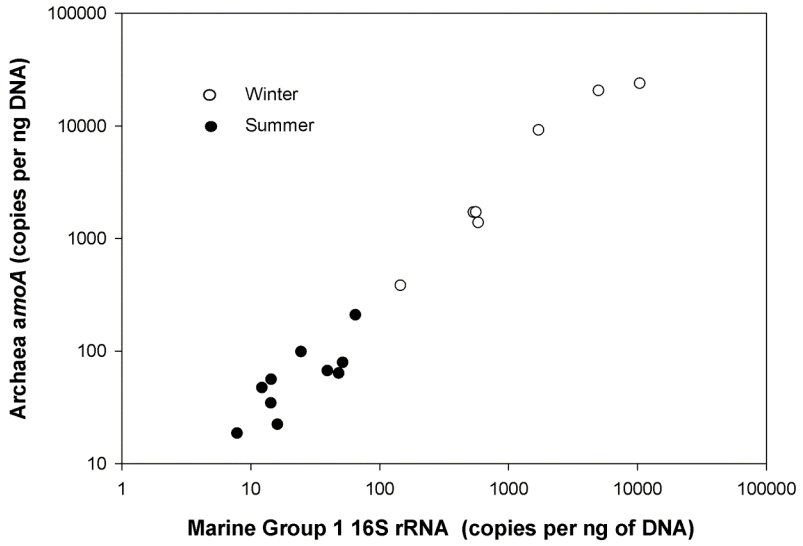

Figure 6. Ratio of Archaea *amoA* copies to Marine Group I copies of the 16S rRNA gene. The 16S rRNA gene data do not include archae*a* in the pSL12 group.

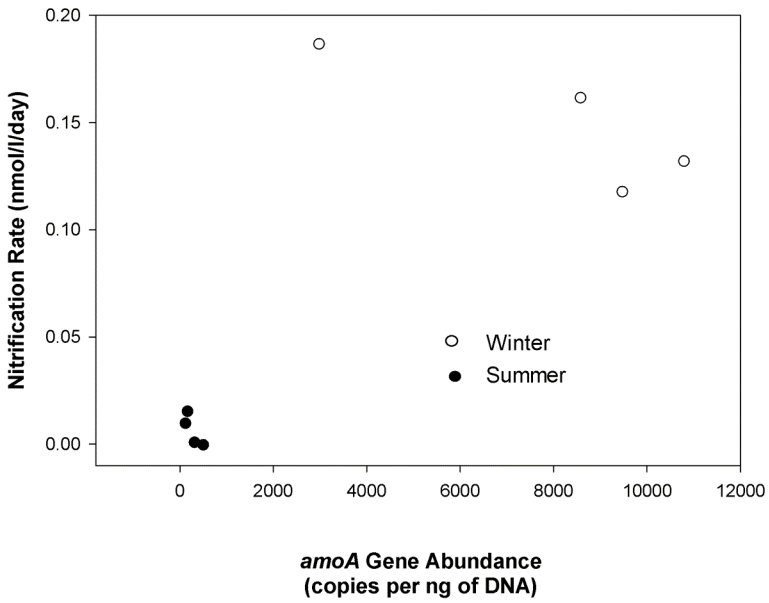

Figure 7. Nitrification rate versus *amoA* gene copies. Gene copies of *amoA* include both betaproteobacterial and archaea *amoA*.

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