Ammonia oxidation rates and nitrification in the Arabian Sea

Silvia E. Newell,[1,2] Andrew R. Babbin,[2] Amal Jayakumar,[2] and Bess B. Ward[2]

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[1] Nitrification rates, as well as the relationships between rates and ammonia oxidizer abundance (both archaeal and bacterial), were investigated in the Arabian Sea. Ammonia oxidation rates were measured directly using $^{15}N\text{-}NH_4^+$ stable isotope additions in gas-impermeable, trace metal clean trilaminate bags (500 mL) at in situ temperature. Tracer incubations were performed at three stations at depths above, below, and within the oxycline of the open-ocean oxygen minimum zone (OMZ). Ammonia oxidation rates were similar to previous open-ocean measurements, ranging from undetectable to 21.6 ± 0.1 nmol L^{-1} d^{-1}. The highest rates at each station occurred at the primary nitrite maximum (above the OMZ), and rates were very low at depths greater than 900 m. The abundances of both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) were estimated using the *amoA* gene by quantitative polymerase chain reaction (qPCR). Both AOA and AOB *amoA* were detected above, within, and below the OMZ, although the AOA were always more abundant than the AOB, by a factor of 35–216. Nitrification rates were not directly correlated to AOA or AOB *amoA* abundance. These rates offer new insight into the role of nitrification in the mesopelagic zone. The abundance of AOA *amoA* genes at 1000 m suggests that ~50% of the microbial biomass could be autotrophic. Additionally, the integrated nitrification rate at depth implies that nitrification could consume most of the ammonium produced by the flux of organic carbon in the mesopelagic zone.

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1. Introduction

[2] Nitrogen is the limiting nutrient in most of the world's oceans, and the new production paradigm defines the input of new nitrogen as nitrate from the deep ocean [*Dugdale and Goering,* 1967], as well as nitrogen derived via N fixation and atmospheric deposition. However, oxidation of ammonia (which is regenerated from reduced organic matter) through nitrification also produces nitrate in the euphotic zone, supporting 12%–32% of marine primary production globally [*Yool et al.*, 2007] as well as the reservoir of nitrate in the deep ocean [*Ward,* 2008]. Recent studies have inspired a resurgence of interest in nitrification [e.g., *Clark et al.*, 2008; *Karl,* 2000; *Yool et al.*, 2007], highlighting the small number of direct measurements of nitrification as a source of nitrate available to incorporate into models of primary production.

[3] Ammonia oxidation to nitrite is the first step of nitrification, followed by nitrite oxidation, performed by the mutually exclusive groups of ammonia-oxidizing organisms (AOO) and nitrite-oxidizing bacteria (NOB). Ammonia oxidation involves at least two primary enzymatic reactions. The first step, oxidation of ammonia to hydroxylamine, is considered the rate-limiting step and is catalyzed by the ammonia monooxygenase enzyme (AMO). The second step, conversion of hydroxylamine to nitrite, is catalyzed by the hydroxylamine oxidoreductase (HAO) in ammonia-oxidizing bacteria (AOB).

[4] One of the most exciting discoveries in nitrogen cycling in the last decade is a clade of crenarchaeota containing the *amoA* gene (which encodes for the AMO enzyme), now called ammonia oxidizing archaea (AOA) [*Schleper et al.*, 2005; *Könneke et al.*, 2005]. Previously, it was thought that autotrophic ammonia oxidation was restricted to groups of betaproteobacteria and gammaproteobacteria (the AOB), but recent studies in the open ocean have detected AOA *amoA* in quantities that are orders of magnitude higher than AOB *amoA* [*Wuchter et al.*, 2006; *Mincer et al.*, 2007; *Agogué et al.*, 2008; *Beman et al.*, 2008; *Lam et al.*, 2009; *Beman et al.*, 2010; *Santoro et al.*, 2010]. However, the rates of archaeal and bacterial ammonia oxidation in the ocean have not been quantified separately, and there is often no relationship between AOA or AOB abundance and rates of ammonia oxidation [*Bernhard et al.*, 2010; *Santoro et al.*, 2010]. As currently understood, AOA and AOB are autotrophs that make a living by oxidizing ammonia. Thus, it is surprising that a strong correlation between *amoA* gene abundance and nitrification rate is not

[1]Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey, USA.
[2]Department of Geosciences, Princeton University, Princeton, New Jersey, USA.

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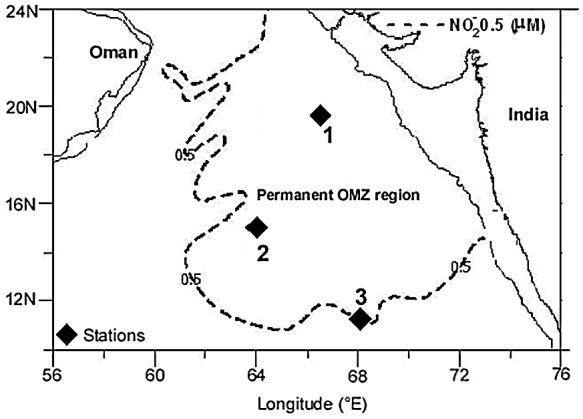

Figure 1. Map of the Arabian Sea. Stations are marked with diamonds, and the dashed line demarcates the region in which nitrite in the OMZ exceeds 0.5 μM, the permanent OMZ. Adapted from *Bulow et al.* [2010], copyright 2010, with permission from Elsevier.

always observed, suggesting that further research into the regulation of nitrification and the metabolism of AOO is warranted.

[5] *amoA* gene abundance suggests correspondingly high abundances of AOA below the euphotic zone between 400 and 1000 m [*Mincer et al.*, 2007; *Beman et al.*, 2010; *Santoro et al.*, 2010], but it is unclear whether these archaea are actively oxidizing ammonia. Preliminary data suggest that a significant fraction of crenarchaeota within this depth range is autotrophic [*Ingalls et al.*, 2006; *Hansman et al.*, 2009], but there is some debate as to what proportion oxidize ammonia [*Agogué et al.*, 2008; *Beman et al.*, 2010; *Church et al.*, 2010; *Santoro et al.*, 2010]. Archaeal metabolism in the mesopelagic ocean has not been studied closely, but if it is dominantly autotrophic, a shift in our understanding of the paradigm governing mesopelagic nutrient cycling may be required.

[6] Nitrogen cycling within and around oxygen minimum zones (OMZs) has been the focus of much recent research, and the Arabian Sea OMZ is the largest in the world and is responsible for nearly half of the fixed N loss due to water column denitrification [*Devol,* 2008]. The goal of this study was to explore the role of nitrification and ammonia-oxidizing organisms at three stations in the Arabian Sea, not only in the surface ocean but also under low-oxygen conditions and in the mesopelagic zone below the OMZ, in order to explore the controls on rates of ammonia oxidation and the abundance and metabolism of AOO. We measured rates of ammonia oxidation in the Arabian Sea OMZ using an improved $^{15}NH_4^+$ isotope tracer method to minimize the perturbations to ambient conditions that incubations necessarily involve. Additionally, quantitative polymerase chain reaction (qPCR) was used to quantify the relative abundance of the *amoA* gene from both groups of organisms at the same stations.

2. Methods

2.1. Sample Collection

[7] Experiments were conducted in the Arabian Sea during the late monsoon/intermonsoon transition in September and October 2007 on the KNOX08 leg of the R/V *Roger Revelle*. All three stations were in water depth > 3000 m. Stations 1 (19°N 66°E) and 2 (15.15°N 64°E) were located within the region historically documented [*Naqvi et al.*, 1982] as the permanent OMZ (Figure 1). Oxygen concentration was <1 μM between 100 or 140 m and 750 m at Stations 1 and 2, respectively (Figure 2). Station 3 (11°N 68°E) was located on the periphery of the permanent OMZ (Figure 1), and the depleted oxygen layer was less than 200 m thick at this location. Samples were collected from 10 L Niskin bottles on a conductivity–temperature–depth (CTD) rosette system. A Seabird O_2 sensor (calibrated to shipboard Winkler assays [*Carpenter,* 1965]) was used to determine the depth and location of the OMZ. Nitrite concentrations were measured on a Shimadzu UV-Visible spectrometer following *Strickland and Parsons* [1972], and ammonium was usually below detection. Oxygen, nitrate, nitrite and ammonium concentrations have been reported previously [*Bulow et al.*, 2010].

[8] Water samples for nitrification rates were collected from three depths at Stations 1 and 2 and four depths at Station 3, while samples for gene abundance were taken at three similar depths from different casts at Stations 1 and 3 and four depths at Station 2. The depths were matched by features in the CTD profile in order to sample the same water for both assays, and they corresponded to specific features of the NO_2^- depth profile: (1) the Primary Nitrite Maximum (PNM), (2) the oxycline at the top of the OMZ (112–126 m depth), and (3) the area below the lower boundary of the OMZ, where the O_2 concentration was above 10 μM (Table 1). The additional depth at Station 2, 200 m, was within the heart of the OMZ.

2.2. Rate Measurements

[9] Ammonia oxidation rates were measured directly using a sensitive tracer method with novel improvements: ^{15}N stable isotope additions of $^{15}NH_4^+$ were made to water incubated in gas-impermeable, trace metal clean trilaminate bags (~500 mL) at near in situ temperature. Duplicate experiments with 0.5 μM $^{15}NH_4^+$ tracer (final concentration) were performed at each of three or four depths, along with a second set of duplicate experiments to which both 0.5 μM $^{15}NH_4^+$ tracer and 100 mg/mL ampicillin (final concentrations) were added. Ampicillin is a betalactam antibiotic and should target only bacteria—not archaea—as it targets cell wall production during cell growth in both Gram positive and Gram negative bacteria by acting as a competitive inhibitor of transpeptidase.

[10] Seawater collected at each depth was transferred directly from the Niskin bottles into the 500 mL trilaminate bags with no bubbles and no headspace. Tracer and antibiotic were added during the transfer. The bags were weighed to allow volume estimation and incubated for 24 h at near in situ temperatures (room temp or 26°C for the PNM and oxycline depths and 4°C for the deep samples). At time zero and after 24 h, 30 and 120 mL of water from each bag, respectively, was filtered and immediately frozen at −80°C for storage until later analysis in the laboratory.

2.3. Analysis of $^{15}NO_2^-$

[11] The sodium azide method of *McIlvin and Altabet* [2005] was used to convert NO_2^- into N_2O. In brief, the

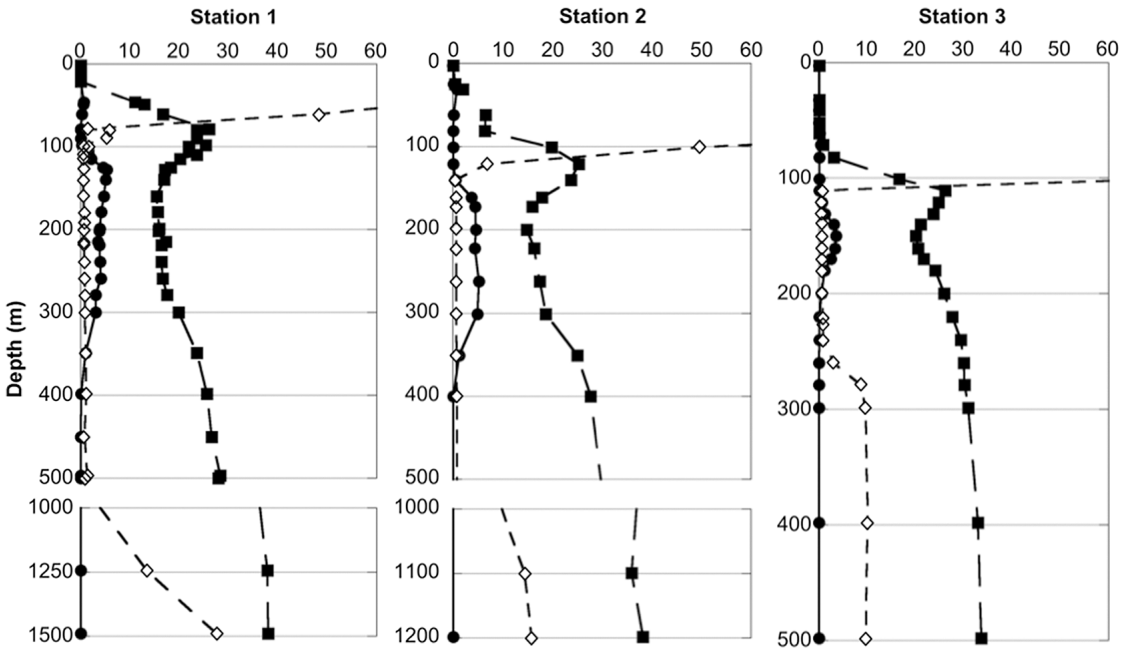

Figure 2. Depth profiles for three stations in the AS OMZ. Closed squares represent nitrate concentration (μM), closed circles are nitrite concentration (μM), and open diamonds are oxygen concentration (μM). Adapted from *Bulow et al.* [2010], copyright 2010, with permission from Elsevier.

azide in acetic acid buffer was prepared on the day it was to be used by combining a 1:1 by volume mixture of 20% acetic acid and 2 M sodium azide. The buffer was then purged with He at 20 psi for 10 min to remove any N_2O produced from NO_2^- contained in the reagents. The 7.5 mL sample aliquots were placed in 12 mL Exetainer vials (LabCo, UK). The vials were capped tightly, and 0.30 mL of the azide/acetic acid buffer was added to each vial via a syringe and shaken vigorously. After 15 min, the sample aliquot was made alkaline with a syringe addition of 0.15 mL of 6 M NaOH and shaken. All reactions were performed at room temperature and in a fume hood. The sensitivity of this method is appropriate for natural abundance measurements with a standard deviation of 0.2‰ [*McIlvin and Altabet*, 2005], so the limit of detection for the resulting rates depends on the replication between incubations more than the absolute sensitivity of the method.

[12] Samples were shipped inverted to the UC Davis Stable Isotope Facility, where N_2O concentration and $^{45/44}N_2O$ isotope ratios were measured using a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., UK). The detection limit for isotopic measurements was 1 nanomole N_2O, and blanks were air N_2. All samples with a rate above zero had at least 1.5 nanomoles NO_2^- converted to N_2O. $^{46}N_2O$ was not detected at levels significantly above background in any sample. Rate measurements were calculated from the production of $^{45}N_2O$ (converted from $^{15}NO_2^-$) from the $^{15}NH_4^+$ tracer additions, after subtracting out any $^{15}NO_2^-$ in the T_0 sample. Any $^{15}NO_2^-$ produced during the 24 h

Table 1. Sample Depths and Nitrite and Oxygen Concentrations at Stations 1 (19°N 66°E), 2 (15.15°N 64°E), and 3 (11°N 68°E), Grouped by the Target Water Column Feature

Target Feature	Station	Ammonia Oxidation Rate Measurement Depths (m)	DNA Sample Depth (m)	Oxygen[a] (μmol l^{-1})	Nitrite[a] (μmol l^{-1})
PNM	1	50	60	67, 48	0.5, 0.24
	2	40	30	142, 131	–, 0.85
	3	70	70	183	0.41
	3	80	80	166	0.11
Oxycline	1	95	102	1.7, 0.63	0.26, 0.40
	2	136	150	0.52, 0.52	0.51, 3.0
	3	99	–	71	0.07
OMZ	2	–	200	0.68	4.62
Mesopelagic	1	1500	–	27	N.D.
	2	1041	1100	14, 15	N.D., N.D.
	3	900	916	19	N.D.

[a]Where samples for measuring ammonia oxidation rates and collecting DNA were made at different depths, the first oxygen and nitrite value corresponds to the depth for the ammonia oxidation rates, and the second value corresponds to DNA sample depth. N.D. indicates that the value was below the limit of detection.

incubation was assumed to have resulted from ammonia oxidation. The nitrification rate was calculated using the equations of *Ward and Kilpatrick* [1990] without accounting for ammonium regeneration. This likely resulted in an underestimate of the rate, because regeneration would dilute the substrate pool. The degree of error introduced by not accounting for regeneration is estimated to be on the order of 10%–30%.

2.4. DNA Extraction

[13] Particulate material from approximately 12 L of seawater from each depth was filtered onto Sterivex capsule filters (0.2 μm filter, Millipore, Inc., Bedford, MA) using a peristaltic pump. Filters were frozen immediately and transported in liquid nitrogen and stored at −80°C until extraction. DNA was extracted from the filters using the PureGene DNA kit (Gentra, Minneapolis, MN), following a slight modification of the manufacturer's protocol: lysis buffer (0.9 mL) was added to the filter and incubated with gentle rotation at 55°C for 1 h, and then at 65°C for 1 h. The solution was removed and the incubation repeated with fresh solution.

2.5. Quantitative PCR Analysis

[14] Primers Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) [*Francis et al.*, 2005] were used to amplify a 635 bp region of archaeal *amoA* for qPCR. Primers amoAF and amoA2R [*Rotthauwe et al.*, 1997] were used to amplify a 491 bp region of β-AOB *amoA*. Standards for both AOB and AOA *amoA* quantification were prepared by amplifying a constructed plasmid containing the respective *amoA* gene fragment, followed by quantification and serial dilution. Vector assimilation into the plasmid was confirmed by restriction digest analysis.

[15] All AOA or AOB *amoA* assays for all depths were carried out within a single assay plate to maximize internal consistency for comparison of gene abundance between environmental DNA samples and between primer combinations [*Smith et al.*, 2006]. Each assay included triplicate no template controls (NTC) and triplicates for each of the three standards. Environmental DNA samples were also assayed in triplicate. For the qPCR amplification mixture, the Quantitect SYBR Green PCR kit (Qiagen Inc.) was used. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and 30 ng of template were amplified in each reaction carried out on a Stratagene MX3000P (Agilent Technologies, La Jolla, CA). AOA *amoA* amplification followed the protocol of *Francis et al.* [2005], and β-AOB *amoA* amplification followed the protocol of *Beman et al.* [2008]. Automatic analysis settings were used to determine the threshold cycle (Ct) values, and efficiencies were between 92% and 108%. The gene abundances were calculated according to the following equation:

$$\text{Gene abundance (sample)} = \left(\text{ng} \times {}^{\text{abundance}}/_{\text{mol}}\right) \div \left(\text{bp} \times {}^{\text{ng}}/_{\text{g}} \times {}^{\text{g}}/_{\text{mol of bp}}\right)$$

and then converted to abundance per mL seawater filtered, assuming 100% extraction efficiency. Gene abundances are reported with errors averaged from the triplicate qPCR reactions.

3. Results

3.1. Nutrient Profiles

[16] Depth profiles of nitrate have been published previously and NH_4^+ concentrations were usually undetectable (Figure 2) [*Bulow et al.*, 2010]. The maximum NO_2^- concentration of the PNM was higher at Station 2 (0.85 μM at 30 m) than at Station 1 (0.50 μM at 50m) and Station 3 (0.41 μM at 70m). The thickness and intensity of the OMZ at Stations 1 and 2 were similar: oxygen concentrations declined to ≤ 1 μM between 100 and 150 m and increased to > 10 μM again by ~1100 m depth (Figure 2). The OMZ at Station 3 was less thick, with the oxygen concentration declining to ≤ 1 μM by 110 m and increasing to ~10 μM by 300 m.

3.2. Archaeal and Bacterial *amoA* Gene Abundance

[17] Both AOA and AOB *amoA* genes were detected at every depth sampled at each station in the Arabian Sea (Figure 3). Archaeal *amoA* genes were 35 to 216 times more abundant than bacterial *amoA* genes, above and below the OMZ (Figure 4). Gene abundances of AOA *amoA* genes were the greatest within the oxycline ($2.90 \pm 0.29 \times 10^5$ copies/mL at Station 2, 150 m) and were least abundant at the deepest depths. The lowest number of copies was detected at 916 m at Station 3, with $1.57 \pm 0.32 \times 10^4$ copies/mL. Overall, gene abundance varied among depths by a factor of five. AOA *amoA* genes were also detected within the OMZ at Station 2 at a depth with undetectable oxygen concentrations where gene abundance was measured but ammonia oxidation rates were not.

[18] Gene abundances of β-AOB *amoA* genes were the greatest above and within the oxycline, up to $4.04 \pm 0.42 \times 10^3$ copies/mL at Station 1, 102 m, and the least abundant at the deepest depth, with only 180.4 ± 16.2 copies/mL detected at Station 3, 916 m (Figure 3). AOB *amoA* genes were also detected within the OMZ at depths with undetectable oxygen concentrations. Gene abundance varied among depths by more than a factor of 20.

3.3. Ammonia Oxidation Rates

[19] Ammonia oxidation rates were measured at similar depths to those at which gene abundance was measured, but from different casts. Separate incubations received additions of $^{15}NH_4^+$ + ampicillin to determine the contribution of AOB. There were no significant differences (paired t tests, all $p > 0.18$) between incubations with and without the ampicillin treatments. The rates from both experiments are shown in Figure 5. Because they were not significantly different, however, the treatments with and without ampicillin were treated as replicates. All rates are reported in the text as the average between the duplicate experiments with and without the ampicillin treatment, and error reported is the propagated error from the standard deviation from each experiment. Ammonia oxidation rates ranged from undetectable to 21.1 ± 0.1 nmol l^{-1} d^{-1} (Figure 5). Rates were undetectable at Station 2, 1041 m in the $^{15}NH_4^+$ treatment and at Station 3, 900 m in the ampicillin treatment. The highest

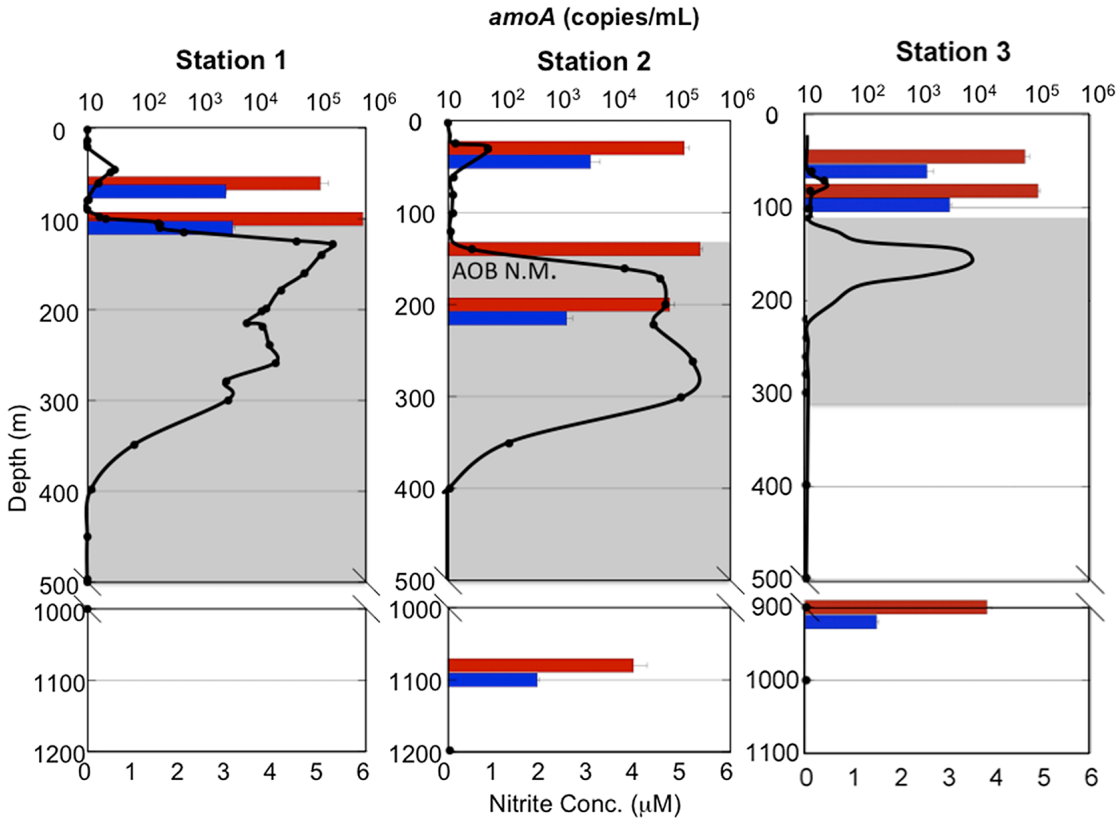

Figure 3. Archaeal and bacterial *amoA* gene abundance as measured by qPCR. AOA *amoA* gene abundance is shown in red, and AOB *amoA* gene abundance is shown in blue. AOA and AOB were quantified at the same depth. Error bars are the standard deviation of triplicate qPCR runs. Closed circles are nitrite concentration (μM), and the depth interval with an oxygen concentration below 10 μM is shaded in gray. N.M. indicates "not measured."

rates at each station were measured at the depth of the PNM. Ammonia oxidation rates were between 1.5 $\pm$ 0.2 and 4.6 $\pm$ 0.4 nmol l^{-1} d^{-1} within the oxycline at the top of the Secondary Nitrite Maximum. There were very low but detectable rates of ammonia oxidation (average 0.07 $\pm$ 0.05 nmol l^{-1} d^{-1}) below the OMZ.

4. Discussion

[20] AOA appear to be the dominant ammonia oxidizers in the Arabian Sea during the Southwest Monsoon/Fall Intermonsoon season. Both AOA and AOB *amoA* genes were detected at all locations sampled in the Arabian Sea, but AOA *amoA* genes were at least an order of magnitude (Figure 4) more abundant than AOB *amoA* genes. AOA have been shown to be more abundant than AOB in a number of marine environments [*Wuchter et al.*, 2006; *Mincer et al.*, 2007; *Beman et al.*, 2008, 2010; *Santoro et al.*, 2010]. If marine AOA have the same very high specific affinity for ammonium as calculated for the cultured *Candidatus Nitrosopumilus maritimus* strain SCM1 [*Martens-Habbena et al.*, 2009], this may provide the physiological basis for allowing them to successfully outcompete AOB in the open ocean, where ammonium concentrations are usually well below 1 μM.

[21] There were no significant differences between the rates measured from experiments with and without ampicillin,

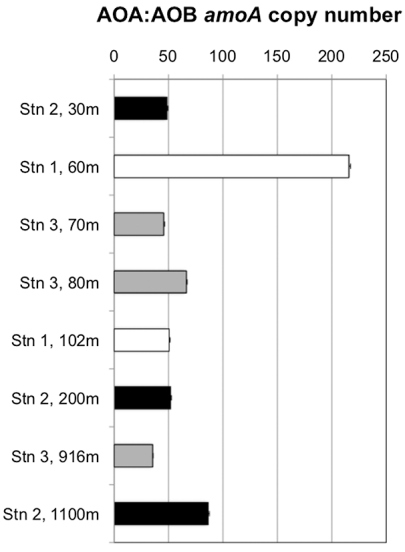

Figure 4. The relative abundance of *amoA* gene abundance as a ratio of AOA to AOB as quantified by qPCR. AOB *amoA* genes were not quantified at Station 2, 150 m. Error bars are the propagated error (standard deviation) from triplicate qPCR runs.

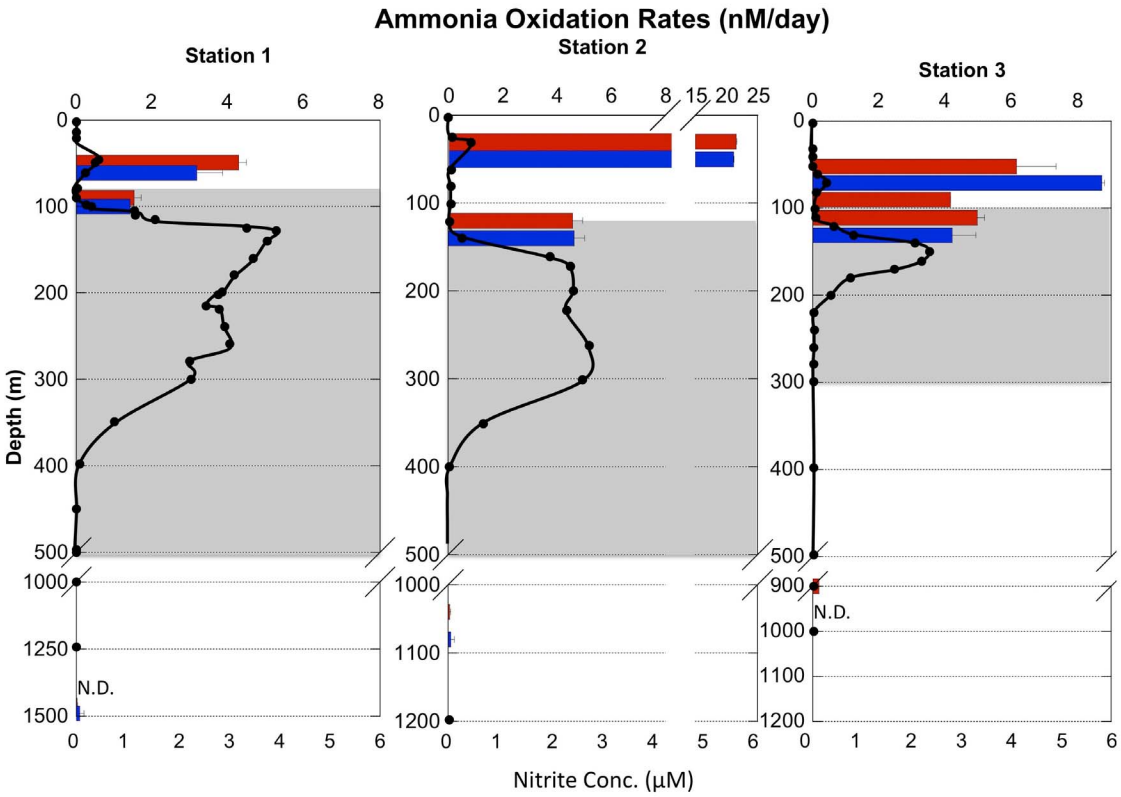

Figure 5. Ammonia oxidation rates in nmol l^{-1} d^{-1}. Treatments with only the $^{15}NH_4$ tracer addition are represented by blue bars, while treatments with the $^{15}NH_4$ + ampicillin are represented by red bars. The two treatments were performed at the same depth. No ampicillin experiment was performed at Station 3, 80 m. Error bars show the range from duplicate experiments. Nitrite concentration (μM) is shown in closed circles, and the depth interval with an oxygen concentration below 10 μM is shaded in gray. N.D. indicates that, in one treatment, no rate was detected. Note low but significant rates below OMZ.

suggesting that the AOB were not sensitive to the ampicillin during the timeframe of the experiment, and the rates were not influenced by the treatments. Ampicillin is most effective in growing bacterial cells, and the AOB were probably not growing fast enough to be affected in the 24 h timeframe. Without further study, however, it is not possible to determine whether the AOB were affected by the ampicillin. If the ampicillin treatment did inhibit the AOB, the rates of bacterial ammonia oxidation either must have been negligible compared to the archaeal rates, or the archaeal production increased with the decreased competition.

[22] There is some debate as to whether the rates of transformation measured by tracer additions are, by definition, artifactual and therefore should be considered potential rates. Previous experiments in Saanich Inlet by *Ward and Kilpatrick* [1990] showed that ammonia oxidation rates were independent of ammonium concentrations above 0.2 μM. These results suggest an even lower half-saturation constant in situ than in culture, given that the cultivated marine AOA strain *Nitrosopumilus maritimus* has a half saturation constant of 0.132 μM NH_3+NH_4^+ for ammonia oxidation [*Martens-Habbena et al.*, 2009]. If the natural assemblage of ammonia oxidizers was functioning at the maximum rate at ambient substrate concentrations, then our 0.5 μM $^{15}NH_4^+$ tracer addition would not affect the ammonia oxidation rates, and the measured rates are reflective of real, in situ rates, and not potential rates. Without knowing the in situ half saturation constant, it is uncertain whether the measured rates are real or potential. The affinity of natural assemblages of ammonia oxidizers should be investigated with more sensitive tracer and concentration measurements.

[23] The ammonia oxidation rates measured in this study are in the same range as those previously measured by similar tracer methods in the Pacific off Baja California (5–20 nmol l^{-1} d^{-1} [*Ward and Zafiriou,* 1988]), in the Central California Current (9–210 nmol l^{-1} d^{-1} [*Santoro et al.*, 2010]), in the Guaymas Basin (up to 90 nmol l^{-1} d^{-1} [*Beman et al.*, 2008]), in the Carmen Basin (up to 40 nmol l^{-1} d^{-1} [*Beman et al.*, 2008]), in Monterey Bay (27.8–73.8 nmol l^{-1} d^{-1} [*Ward,* 2005]), in the Atlantic (up to 10 nmol l^{-1} d^{-1} [*Clark et al.*, 2008]), and on the surface of the Arabian Sea (5–100 nmol l^{-1} d^{-1} [*McCarthy et al.*, 1999]).

[24] Ambient NO_2^- concentrations correlated positively with ammonia oxidation rates (Figure 6) with $R^2 = 0.71$ and $p < 0.001$ for a linear fit. Still, as biological processes are complex, the linear regression is not necessarily an appropriate a priori assumption, and using a Spearman rank correlation, the NO_2^- concentrations remain significantly correlated ($\rho = 0.83$, $p < 0.0001$) with the ammonia oxidation rates. Furthermore, NO_2^- concentration maxima have been associated with NH_4^+ concentration peaks in the Arabian Sea [*Nicholls et al.*, 2007].

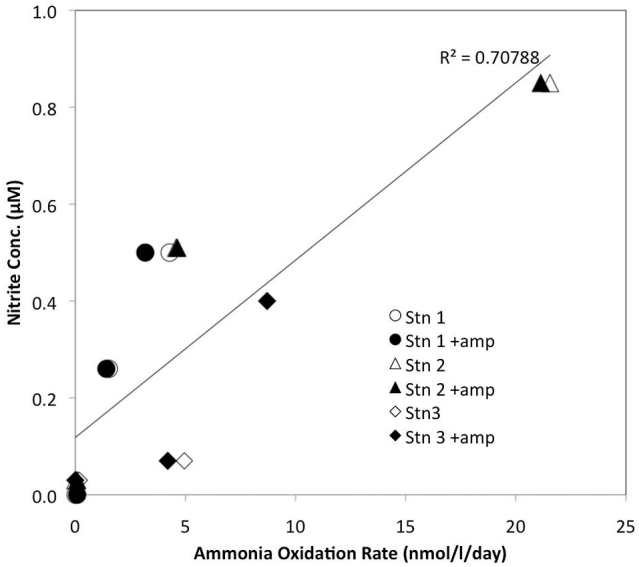

Figure 6. The relationship between ammonia oxidation rate and corresponding nitrite concentration at a given depth (n = 17). Station 1 is represented by circles, Station 2 is represented by triangles, and Station 3 is represented by diamonds. Open symbols, no ampicillin; closed symbols, plus ampicillin.

[25] There was no significant correlation between AOA *amoA* gene abundance and oxidation rate (p = 0.80), in agreement with the findings of *Bernhard et al.* [2010] in the Plum Island Sound estuary and *Santoro et al.* [2010] in the Central California Current. There was also no correlation between AOA:AOB *amoA* abundances and NO_2^- concentrations (p = 0.46), in contrast to the findings of *Beman et al.* [2010], indicating that further work is required to understand the relationship between *amoA* gene abundance and ammonia oxidation rates.

4.1. Ammonia Oxidation in the Surface Layer

[26] The highest rates of ammonia oxidation all occurred in the depth zone of the PNM, suggesting that AOO are significant producers of NO_2^- at the PNM. An earlier study in the Arabian Sea [*McCarthy et al.*, 1999] measured rates of both NO_2^- and NH_4^+ regeneration comparable to the rates of uptake of those nutrients, which implies that maintenance of the PNM does not require diffusive or advective fluxes from other sources. *McCarthy et al.* [1999] also found that, during the first half of the solar day, integrated rates of NH_4^+ oxidation to NO_2^- were twice as great as the rates of photoautotrophic NO_2^- uptake, suggesting an important role for nitrification in sustaining the PNM. Using the maximum rate measured in this study and the range of PNM turnover time scales reported by *McCarthy et al.* [1999] of a few days to a month, ammonia oxidation could be responsible for 10%–100% of the supply rate of NO_2^- required to maintain the PNM. While far from definitive, the potential importance of nitrification suggests that further research is warranted in the Arabian Sea to explore the temporal dynamics between nitrification and phytoplankton production and uptake of NO_2^- that maintain the PNM.

4.2. Ammonia Oxidation in the Oxycline

[27] New evidence suggests that AOA are capable of producing N_2O [*Santoro et al.*, 2011]. This supports the conventional view that nitrification is the dominant mechanism of marine N_2O production, based on the robust correlation between ΔN_2O and apparent oxygen utilization and because $\Delta N_2O/NO_3^-$ ratios tend to increase with decreasing O_2 in a manner consistent with laboratory studies of AOB [*Nevison et al.*, 2003]. With the discovery that AOA are capable of producing N_2O, the implication from these data that the AOA are actively nitrifying within the oxycline supports the theory that nitrification is a significant source of N_2O production in the oxycline [*Nevison et al.*, 2003].

[28] It has been suggested that ammonia oxidation in the oxycline supports anaerobic ammonia oxidation (anammox) [*Lam et al.*, 2009]. Both AOO and anammox bacteria require NH_4^+ as a substrate, but anammox also requires NO_2^-, which could be provided by aerobic ammonia oxidation. Anammox rates from the same cruise have been reported previously [*Bulow et al.*, 2010; *Ward et al.*, 2009]. Where anammox was detected, the rates were low, but the same order of magnitude as those measured for aerobic ammonia oxidation in the oxycline in this study. However, anammox was not detected at depths with ambient $O_2 > 1$ μM [*Bulow et al.*, 2010; *Ward et al.*, 2009], and therefore not at the depths where we report ammonia oxidation within the oxycline. A recent study by *Pitcher et al.* [2011] similarly reported that AOA and anammox lipids were not detected at the same depths within the Arabian Sea oxycline. Therefore, it is unlikely that ammonia oxidation produces the NO_2^- substrate for anammox bacteria, but rather that denitrification is the source of the NO_2^-.

4.3. Ammonia Oxidation in the Mesopelagic Zone

[29] The role of *Crenarchaeota* in the deep ocean has yet to be resolved. A study by *Agogué et al.* [2008] showed a marked decrease in total *amoA* gene abundance and crenarchaeal *amoA*:16S gene abundance from 150 m to 1000 m, suggesting that most mesopelagic Crenarchaeota are not autotrophic ammonia oxidizers but rather are living heterotrophically. In contrast, this study and others [*Beman et al.*, 2010; *Church et al.*, 2010; *Mincer et al.*, 2007] show that AOA *amoA* gene abundances are high ($\sim 10^4$–10^6 copies/mL) within this depth interval, suggesting that most—if not all—*Crenarchaeota* have the *amoA* gene. Possibly this discrepancy is due to methodological differences, as *Konstantinidis et al.* [2009] have shown that the primers used by *Agogué et al.* [2008] have some significant mismatches with a deep crenarchaeal metagenome sequence.

[30] Crenarchaeal 16S gene abundance was not quantified directly here, but AOA *amoA* gene abundance was on the order of 10^5 copies/mL at $\sim$1000 m depth, indicative of a large crenarchaeal population. The abundance of AOA raises the question of whether they are living as autotrophs, mixotrophs or heterotrophs. *Ingalls et al.* [2006] reported that 83% of archaeal lipids (and therefore biomass) at 670 m at the HOT Station in Hawaii contained carbon incorporated through an autotrophic pathway. *Hansman et al.* [2009] used the $\Delta^{14}C$ signature from DNA at HOT to calculate that 95% of the microbial community at 670 m and 36% at 1000 m were autotrophic. The two complete genomes of AOA

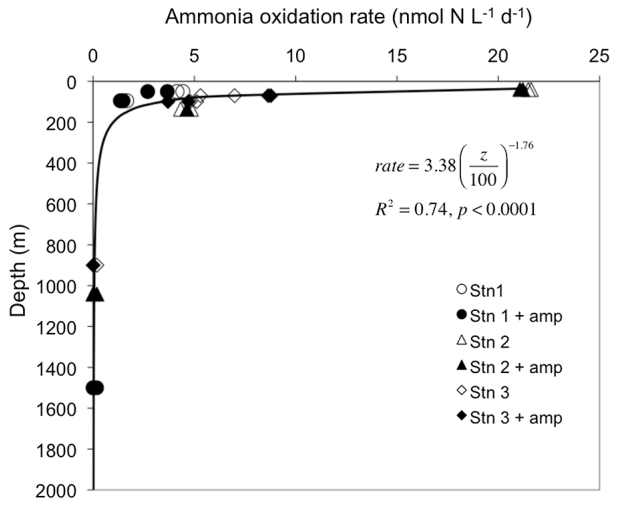

Figure 7. Analytical curve fit to the measured ammonia oxidation rates. A power function [*Martin et al.*, 1987] was fit to ammonia oxidation rates from every sample depth combined into one profile and was used to calculate the integrated nitrification rate.

[*Hallam et al.*, 2006; *Walker et al.*, 2010] suggest that AOA have the capability of living mixotrophically. Thus, the results of *Ingalls et al.* [2006] imply that 83% of the *Crenarchaeota* are autotrophic ammonia oxidizers and 17% are heterotrophic, or they are all AOA that utilize a mixotrophic lifestyle or any combination in between.

[31] The abundance of AOA at ~1000 m has further implications for the mesopelagic N and C cycles, because nitrate, rather than nitrite, accumulates in the deep ocean. Thus, the community of AOA must be supporting a population of nitrite-oxidizing bacteria (NOB) capable of further oxidizing the nitrite resulting from ammonium oxidation [*Ward,* 2011]. Since nitrite oxidation is even less energy yielding (~one third of the energy yield from ammonia oxidation [*Gottschalk,* 1986]), the biomass of NOB would be lower than that of AOB plus AOA, but still a substantial contribution to the total microbial biomass. *Hansell and Ducklow* [2003] reported total microbial biomass on the order of 50,000 cells/mL below the Arabian Sea OMZ at 1000 m, and the average *amoA* copies mL^{-1} at 1000 m in this study was 17,450 copies mL^{-1}. Assuming there is one copy of *amoA* per AOA and NOB abundance is one third that of the AOA, that suggests that ~50% of the microbial biomass is autotrophic.

[32] *Ward* [2011] argued that this prevalence of autotrophy in the mesopelagic ocean requires a rethinking of controls on biogeochemical fluxes and remineralization. If the majority of the microbial biomass at depth is supported by autotrophy, then much of the microbial metabolism is controlled by the flux of the ammonium supplied by remineralization of raining organic matter. N regeneration is coupled to the rate of respiration of organic matter, and the rate can be estimated from the flux of organic carbon captured in sediment traps [*Suess,* 1980; *Martin et al.*, 1987]. The annually averaged C flux over the 100–1510 m depth interval at Joint Global Ocean Flux Study (JGOFS) Station 4 (15°20′ – 15°59′N, 61°30′E) is 4.542 mmol C m^{-2} d^{-1} 5 (10°N, 65°E), and over the 100 m – 2363 m depth interval at JGOFS Station 5 is 2.404 mmol C m^{-2} d^{-1} [*Lee et al.*, 1998], which are the closest JGOFS stations to our three stations. Using the C:N (6.67) of captured organic matter from the same data set [*Millero et al.*, 1998], the annual average N remineralization rate is 360 μmol N m^{-2} d^{-1} at JGOFS Station 5 and 681 μmol N m^{-2} d^{-1} at JGOFS Station 4. A Martin-style power curve (Figure 7; $R^2 = 0.74$, $p < 0.0001$) fit to the ammonia oxidation rates (consolidated into a single depth profile due to the small number of samples at each station) can be used to estimate the integrated nitrification rate with depth [*Martin et al.*, 1987; *Ward and Zafiriou,* 1988]. The flux curve calculated from the integration of the rate distribution would have an exponential *b* coefficient of 0.76, which is very similar to those published by *Berelson* [2001] in the surrounding JGOFS stations (range of 0.72 to 0.75). From the regressed fit, the integrated nitrification rate is 405 μmol N m^{-2} d^{-1} over the 100–2363 m depth interval. Compared to the N flux calculated from sediment trap carbon fluxes, this integrated rate accounts for ~60%–110% of the ammonium from remineralized organic matter over this depth interval, implying that nitrification is directly coupled to organic remineralization. This implies that the autotrophic and nitrifying microbial biomass at ~1000 m is responsible for consumption of all the ammonium from the remineralization flux in the deep ocean. The organic matter flux therefore constrains both the biomass of the nitrifiers supported and the rate at which they are able to nitrify.

[33] If a large percentage of the microbial biomass at depth is supported by nitrification, the AOO are nitrifying very slowly. The rates of ammonia oxidation at the deepest depths observed in this study were very low. Using the measured nitrification rate at 900m (0.2 nmol l^{-1} d^{-1}) and assuming one copy of *amoA* per AOA cell, the per cell ammonia oxidation rate would be 1.3×10^{-17} mol N $cell^{-1}$ d^{-1}. This value is similar to the per cell ammonia oxidation rate for the HOT station between 150 and 1000 m calculated from remineralization rates based on vertical flux [*Ward,* 2011] of 10^{-17} mol N $cell^{-1}$ d^{-1}. Both of these rates at ~1000 m are 1 to 3 orders of magnitude lower than the values calculated by *Santoro et al.* [2010] for the California Current at 67 m of 0.2 to 15×10^{-15} mol N $cell^{-1}$ d^{-1}, which are still very slow. To achieve the nitrification rates measured with the population of AOA detected, not only would the per cell rate need to be very slow, but the biomass turnover time would be about 1500 days, assuming 1 *amoA* gene per AOA cell, 25 N oxidized per C fixed [*Könneke et al.*, 2005], and 9 fg C/cell [*Herndl et al.*, 2005].

[34] Calculated per cell rates and generation times that are orders of magnitude below those measured in culture [*Könneke et al.*, 2005] suggest that further investigation is required to understand the role of AOA in the mesopelagic zone. The assumption of one *amoA* gene per AOA cell may not be valid. *Church et al.* [2010] reported that the ratio of AOA *amoA* transcript number to *amoA* gene abundance declined drastically with decreasing depth (although the current 16S primers may not target all of the marine AOA, possibly underestimating total crenarchaeal numbers). The carbon fixation pathway in the dominant marine AOA may also be more efficient than the 3-hydroxypropionate/

4-hydroxybutryrate pathway in *Candidatus Nitrosopumulis maritimus* [*Walker et al.*, 2010]. The comparison of the measured nitrification rates with the sediment trap fluxes shows that nitrification plays an important role converting the remineralized ammonium to nitrate in the mesopelagic zone, but that nitrification is likewise constrained by the organic matter flux. The calculated per cell rates and turnover times are difficult to reconcile with supporting such a large population of nitrifying autotrophs, suggesting that some or all of the AOA may be mixotrophic or even heterotrophic.

[35] This and other recent studies emphasize the importance of reconsidering the role of nitrification in the open ocean. At the surface in productive areas of the ocean, nitrification in the photic zone provides recycled nitrate that is used in primary production and contributes to the maintenance of the PNM. In OMZs, especially within the oxycline, nitrification remains a potential source of N_2O, which will need to be reconsidered as the pathways for production of N_2O by AOA are investigated. The role of AOA within the OMZ is still a mystery, and further work is required to determine the pathways AOA might use for sustaining growth under hypoxic to anoxic conditions. The abundance of autotrophic organisms below the euphotic zone may require a shift in thinking from the traditional view that the flux of organic carbon sustains a microbial population dominated by heterotrophs to sustaining a small population of heterotrophs, which supply the ammonium that supports a larger autotrophic nitrifier community. This autotrophic assemblage then performs primary production in situ at depth, but this is not new production since it is dependent upon ammonium and oxygen originally produced by photosynthesis in the surface ocean. There has been little work focused on microbial abundance and metabolism below the euphotic zone, and any more definitive conclusions will require further investigation.

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A. R. Babbin, A. Jayakumar, and B. B. Ward, Department of Geosciences, Princeton University, Guyot Hall, Washington Road, Princeton, NJ 08544, USA.

S. E. Newell, Department of Ecology and Evolutionary Biology, Princeton University, Guyot Hall, Washington Road, Princeton, NJ 08544, USA. (sebulow@princeton.edu)