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Molecular and biogeochemical evidence for ammonia oxidation by marine Crenarchaeota in the Gulf of California

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Nitrification plays an important role in marine biogeochemistry, yet efforts to link this process to the microorganisms that mediate it are surprisingly limited. In particular, ammonia oxidation is the first and rate-limiting step of nitrification, yet ammonia oxidation rates and the abundance of ammonia-oxidizing bacteria (AOB) have rarely been measured in tandem. Ammonia oxidation rates have not been directly quantified in conjunction with ammonia-oxidizing archaea (AOA), although mounting evidence indicates that marine Crenarchaeota are capable of ammonia oxidation, and they are among the most abundant microbial groups in the ocean. Here, we have directly quantified ammonia oxidation rates by ¹⁵N labeling, and AOA and AOB abundances by quantitative PCR analysis of ammonia monooxygenase subunit A (*amoA*) genes, in the Gulf of California. Based on markedly different archaeal *amoA* sequence types in the upper water column (60 m) and oxygen minimum zone (OMZ; 450 m), novel *amoA* PCR primers were designed to specifically target and quantify 'shallow' (group A) and 'deep' (group B) clades. These primers recovered extensive variability with depth. Within the OMZ, AOA were most abundant where nitrification may be coupled to denitrification. In the upper water column, group A tracked variations in nitrogen biogeochemistry with depth and between basins, whereas AOB were present in relatively low numbers or undetectable. Overall, ¹⁵NH₄⁺ oxidation rates were remarkably well correlated with AOA group A *amoA* gene copies ($r^2 = 0.90$, $P < 0.001$), and with 16S rRNA gene copies from marine Crenarchaeota ($r^2 = 0.85$, $P < 0.005$). These findings represent compelling evidence for an archaeal role in oceanic nitrification.

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Introduction

The element nitrogen (N) acts as an essential limiting nutrient over broad expanses of the ocean and plays a central role in marine ecology and biogeochemistry. Recent evidence indicates that N regenerated by nitrification in the upper water column directly fuels 10–25% of marine primary production (Yool *et al.*, 2007), yet the first and rate-limiting step of nitrification, ammonia oxidation, is thought to be carried out by a limited group of microorganisms, both in terms of diversity—confined to a few groups of betaproteobacterial and gammaproteobacterial ammonia-oxidizing bacteria

(β -AOB and γ -AOB) (Purkhold *et al.*, 2000)—and in terms of abundance—typically less than 0.1% of microbial assemblages (Ward, 2000). In fact, a shortfall between the apparent abundance of AOB and inferred nitrification rates has been recognized for some time (Watson, 1965).

Recovery of ammonia monooxygenase subunit A (*amoA*) genes associated with microorganisms from the domain Archaea (Venter *et al.*, 2004; Schleper *et al.*, 2005) raised the possibility that an entirely unexpected and exceptionally abundant group of microbes may be involved in nitrification (Francis *et al.*, 2007). In particular, although marine Crenarchaeota have been shown to numerically dominate microbial communities throughout the water column (DeLong *et al.*, 1994; Karner *et al.*, 2001), there has been limited insight into the biogeochemical and ecological roles of these organisms following their initial identification in the ocean (DeLong, 1992; Fuhrman *et al.*, 1992; Francis *et al.*, 2007). Consistent with a role in nitrification—the stepwise oxidation of ammonia

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(NH₃) to nitrite (NO₂⁻) to nitrate (NO₃⁻)—several studies have indicated that a large proportion of the Crenarchaeota are autotrophic (Kuypers *et al.*, 2001; Pearson *et al.*, 2001; Wuchter *et al.*, 2003; Ingalls *et al.*, 2006; Kirchman *et al.*, 2007). Most convincingly, cultivation of the autotrophic ammonia-oxidizing archaeon (AOA) *Nitrosopumilus maritimus* confirmed that at least some mesophilic Crenarchaeota are capable of ammonia oxidation (Könneke *et al.*, 2005), and archaeal *amoA* genes appear to be widespread in water columns and sediments where nitrification is expected to be important (Francis *et al.*, 2005).

Direct measurements of nitrification rates in the ocean remain surprisingly limited, however, and amount to about a dozen studies in total (Ward, 1987; Ward *et al.*, 1989; Yool *et al.*, 2007). Of these, only two have directly quantified nitrification rates and the abundance of AOB in tandem, finding limited correspondence between maximum rates and maximum abundances (Ward, 1987; Ward *et al.*, 1989). None have quantified AOA—although archaeal *amoA* was expressed in the nitrification zone of the Black Sea (Lam *et al.*, 2007), and archaeal *amoA* gene copies vastly outnumber bacterial *amoA* copies on land and in the sea (Leininger *et al.*, 2006; Wuchter *et al.*, 2006). This study is the first to directly measure nitrification rates (using ¹⁵N-labeled NH₄⁺) in the ocean in conjunction with quantification of both AOB and AOA. By sampling at 5–15 m depth intervals in the upper water column of the Gulf of California (GOC), we were able to resolve both organisms and processes, and our results may be broadly representative of ammonia oxidation in the upper water column. In addition, we demonstrate the ability to quantitatively separate the crenarchaeal community into two distinct clades based on the archaeal *amoA* gene—potentially further refining the distribution of ammonia oxidation activity within the marine Crenarchaeota and throughout the ocean.

Materials and methods

Sampling

Two stations (27°01'N, 111°25'W in the Guaymas Basin and 26°04'N, 110°07'W in the Carmen Basin) were sampled in the GOC between 23 July and 12 August 2005 aboard the R/V *New Horizon*. Temperature and chlorophyll concentrations were measured using a Seabird SBE 9 CTD equipped with a Seapoint fluorometer; oxygen concentrations were measured using an SBE oxygen sensor and corrected based on Winkler titrations ($r^2 = 0.998$, $n = 112$ for the cruise). Water samples were collected using a rosette of 10-l PVC sample bottles, with two bottles collected at each depth. From each sample bottle, aliquots were taken for analysis of nutrient concentrations (Strickland and Parsons, 1972; Holmes *et al.*, 1999) and, with the exception of the

samples from 0 to 100 m in the Carmen Basin, the remaining volume was filtered through 142 mm diameter 0.2 µm Durapore filters (Millipore, Billerica, MA, USA). For the samples from 0 to 100 m in the Carmen Basin, 1 l of seawater was filtered through 47 mm diameter 0.2 µm Durapore filters. All filters were frozen at -80 °C until DNA extraction.

DNA extraction

DNA was extracted from filters following the protocol of Santoro and Boehm (2007). Filters with a diameter of 47 mm were extracted in their entirety, whereas those with a diameter of 143 mm were cut into quarter sections, with one section used in each extraction. Briefly, filters or filter sections were incubated with 800 µl lysis buffer (20 mM EDTA, 400 mM NaCl, 750 mM sucrose and 50 mM Tris) and 100 µl of 10% SDS at 99 °C for 1 min using a dry heat block, and following the addition of 100 µl proteinase K (10 mg ml⁻¹), incubated at 55 °C for 4 h. Lysates were then purified using Qiagen DNeasy columns following the addition of 500 µl of 100% ethanol according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). DNAs from the upper 100 m were further purified using Montage filter units (Millipore).

DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and assayed in triplicate. $A_{260/280}$ ratios were 1.8 ± 0.2 s.d., and samples from the upper water column in the Carmen Basin spanning a range of DNA concentrations correlated ($r^2 = 0.98$) with DNA concentrations measured using PicoGreen fluorescence (Molecular Probes, Eugene, OR, USA) and standards of 0.1, 0.5, 1.0 and 5.0 ng DNA per microliter. Where complete water column profiles of AOA are displayed, gene copy numbers are normalized per milliliter based on the volume of seawater that was filtered, the proportion of filter extracted and assuming a completely efficient extraction procedure. Where samples from the upper water column are considered independently, gene copies are normalized to per nanogram DNA due to variation in all of the above assumptions for the per milliliter calculation.

PCR and analysis of community composition

Archaeal *amoA* gene fragments were amplified, cloned and sequenced using PCR primers (Arch-amoAF and Arch-amoAR) and conditions as described previously (Francis *et al.*, 2005). Nucleotide sequences were assembled and edited using Sequencher v.4.2 (GeneCodes, Ann Arbor, MI, USA), and nucleotide alignments were generated using MacClade (<http://macclade.org>). Abundance-based Sørensen-type similarity indices (Chao *et al.*, 2005) were calculated using the program SONS (Schloss and Handelsman, 2006) and neighbor-joining

phylogenetic trees (based on Jukes-Cantor-corrected distances) were constructed based on alignments of DNA sequences using ARB (<http://www.arb-home.de>) (Ludwig *et al.*, 2004). GOC sequences from 60 and 450 m were compared with all previously reported sequences at least 540 bp in length from marine water columns (Francis *et al.*, 2005; Hallam *et al.*, 2006b; Wuchter *et al.*, 2006; Coolen *et al.*, 2007; Lam *et al.*, 2007; Mincer *et al.*, 2007), coastal and estuarine sediments (Francis *et al.*, 2005; Beman and Francis, 2006), soils (Francis *et al.*, 2005; Leininger *et al.*, 2006), wastewater treatment plants (Park *et al.*, 2006) and corals (Beman *et al.*, 2007)—as well as reference sequences from soil fosmid 54d9 (Schleper *et al.*, 2005), *Cenarchaeum symbiosum* (Hallam *et al.*, 2006b) and *N. maritimus* (Könneke *et al.*, 2005). Distance- and parsimony-based bootstrap analyses were conducted in PAUP *4.0b10 (Sinauer Associates, Sunderland, MA, USA) and used to estimate the reliability of phylogenetic reconstructions with 200 replicates. These sequence data have been submitted to the GenBank database under accession nos. EU340464–EU340556.

Primer design and quantitative PCR analysis

Based on the depth-related differences identified above, comparison of 20 different sequences each from groups A and B revealed several regions of the archaeal *amoA* gene that differ between the two groups. One of these regions was selected for design of new forward primers that individually target the two groups: Arch-amoAFA (5'-ACACCAGTTTGGYTACCWTC DGC-3') and Arch-amoAFB (5'-CATC CRATGTGGATTCCATCDTG-3'). These primers were used in quantitative PCR (qPCR) reactions with the Arch-amoAR reverse primer of Francis *et al.* (2005) and were tested against sequenced clones from both clusters A and B. In no case did plasmids extracted from clones show cross-amplification; that is, the Arch-amoAFA primer amplified sequences from only cluster A, and Arch-amoAFB amplified sequences from only cluster B (both product sizes were approximately 340 bp). Based on *in silico* analysis, the Arch-amoAFA primer may also amplify some non-group A sequence types in sedimentary environments (Francis *et al.*, 2005) and '*N. maritimus*-like' sequence types in the ocean. However, *N. maritimus*-like sequences were not recovered in any of our clone libraries and have only rarely been recovered in marine metagenomic libraries (Venter *et al.*, 2004; Hallam *et al.*, 2006b; Mincer *et al.*, 2007); as a result, data from this assay are referred to as 'group A' in the main text, although there may be a small contribution from the *N. maritimus*-like group.

Quantitative PCR assays were adapted from or followed established protocols. For the group-specific and general archaeal *amoA* qPCR assays, the following reaction chemistry was used: 12.5 µl

SYBR Premix F (Epicentre Biotechnologies, Madison, WI, USA), an additional 2 mM MgCl₂, 0.4 µM of each primer, 40 ng µl⁻¹ BSA, 1.25 U AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA) and 1–6 ng DNA in a final volume of 25 µl. Group A and general qPCR assays were run in triplicate and the group B assay in duplicate using the following cycling parameters: 95 °C for 4 min, followed by 30 cycles of 95 °C for 30 s, 53/55/56 °C (general/group B/group A assays) for 45 s and 72 °C for 60 s, with a detection step at the end of each cycle. Plasmids containing cloned and sequenced *amoA* gene fragments were used as qPCR standards in 10-fold dilution series from 4.0×10^7 to 10^2 copies; the clone GOC-G-60-9 (GenBank accession no. EU340472) was used for the general and group A assay and GOC-C-450-8 (GenBank accession no. EU340542) for the group B assay. PCR efficiencies and correlation coefficients for standard curves were as follows: for the general assay, 103% and $r^2 = 0.990$; for the group A assay, 87.8–103% and $r^2 = 0.991$ –0.997; for the group B assay, 91.7% and $r^2 = 0.999$.

16S rRNA genes from marine group 1 Crenarchaeota were quantified using the same reaction chemistry with the primers GI_741F and GI_956R (Mincer *et al.*, 2007). Cycling conditions were similar to those of Mincer *et al.* (2007); however, the total number of cycles was reduced from 50 to 30, and the length of the detection step was increased from 1 to 7 s. The cloned sequence arc11april01.150 (JM Beman *et al.*, unpublished) was used as a standard in 10-fold dilutions from 1.0×10^7 to 10^2 copies ($r^2 = 0.976$); PCR efficiency was 104%.

β-AOB and γ-AOB were quantified using a similar approach. β-AOB primers amoAF and amoA2R (Rotthauwe *et al.*, 1997) were used with a reaction chemistry similar to that mentioned above, but with 1.25 U Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA), 5–15 ng DNA and without additional MgCl₂. This assay was run in duplicate due to higher DNA template requirements using the following protocol: 95 °C for 5 min followed by 40 cycles of 94 °C for 45 s, 56 °C for 30 s, 72 °C for 60 s and a detection step at 81 °C for 7 s. The clone HB_A_0206_G01 (GenBank accession EU155190; Santoro *et al.*, in press) was used as a qPCR standard in a dilution series from 2.1×10^7 to 10^1 copies ($r^2 = 0.994$ –0.995). PCR efficiencies were 103–109%. Following Lam *et al.* (2007), the reaction chemistry, cycling conditions and primers amoA-3F and amoB-4R of Purkhold *et al.* (2000) were used to detect γ-AOB; however, no samples were rigorously quantified since only two exhibited amplification.

For all qPCR assays, product specificity was verified by both dissociation curves and agarose gel electrophoresis, and we tested for PCR inhibition by spiking a known quantity of standard with 1 µl of each sample. The only sample for which inhibition was a factor was the 45 m sample from the Carmen

Basin; as a result, this sample was not included in our analyses.

¹⁵N ammonia oxidation rates

NH₄⁺ oxidation rates were measured using ¹⁵N-labelled NH₄⁺. *In situ* NH₄⁺ concentrations were measured onboard and determined to be at most 10–50 nM; under such conditions, it is recommended that 50–100 nM ¹⁵NH₄⁺ be added as a minimal yet measurable amount of isotopic label (Ward *et al.*, 2005). In this study, ¹⁵NH₄⁺ was added up to a concentration of 55 nM, which is comparable to the highest measured concentrations (36–42 nM), but represents a significant enrichment at some depths. However, rates at some of these depths (for example, 30 m in both basins and 35 m at Guaymas) remained extremely low (<0.3 nmol l⁻¹ day⁻¹) despite this potential source of enrichment; in addition, depths with similar initial NH₄⁺ concentrations nonetheless had very different rates. *In situ* 25-h incubations were carried out in acid-washed, DI-rinsed polycarbonate bottles (0.25 l) mounted on a free-floating array; features of this array are described by Prah *et al.* (2005).

Accumulation of ¹⁵N in NO₂⁻ or NO₃⁻ by oxidation of ¹⁵N-NH₄⁺ was measured using the denitrifier method of Sigman *et al.* (2001). This method has the advantage of capturing ¹⁵N accumulation in both pools, with high sensitivity and low sample volumes. Briefly, cell concentrates of *Pseudomonas aureofaciens* (ATCC 13985) were used to convert sample NO₃⁻ and NO₂⁻ to N₂O under O₂-free conditions in 20 ml serum vials. N₂O is subsequently stripped from the vial, cryofocused (Sansone *et al.*, 1997) and introduced into the carrier stream of a Finnigan MAT252 mass spectrometer through an online gas chromatographic front end. N₂O is thereby separated from residual CO₂ and its δ¹⁵N value determined from masses 44, 45 and 46 as described previously (Dore *et al.*, 1998; Popp *et al.*, 2002). Uptake of ¹⁵N into particulate matter was not measured and rates were not corrected for isotope dilution due to NH₄⁺ turnover; that is, oxidation rates were calculated based on the accumulation of ¹⁵N in the NO₃⁻ plus NO₂⁻ pool relative to the initial ¹⁵N-labeled NH₄⁺ pool without further adjustment. Duplicate samples from 50 m depth in the Guaymas Basin had an s.d. of 0.5 nmol l⁻¹ day⁻¹ (mean rate was 93.1 nmol l⁻¹ day⁻¹), as did duplicate samples from 35 m in the Carmen Basin (mean rate was 12.5 nmol l⁻¹ day⁻¹); all other samples were run without replication.

Results and discussion

Nitrogen biogeochemistry in the GOC

The GOC is a biologically productive (Zeitzschel, 1969) and diverse (Roberts *et al.*, 2002) subtropical sea bordered by the Baja California peninsula and

Figure 1 Location of the Gulf of California and sampling stations in the Guaymas and Carmen Basins. Image from MODIS-Aqua covers the west coast of North America from Sinaloa, Mexico to San Francisco, California, USA and depicts average surface chlorophyll concentrations during summer 2005.

mainland Mexico (Figure 1). The element N plays an essential role in the ecology and biogeochemistry of GOC because it strongly limits primary production: for example, N-rich agricultural runoff fuels large phytoplankton blooms in the central GOC from November through April (Beman *et al.*, 2005), and cyanobacterial N₂ fixation occurs in surface waters during highly stratified conditions in summer (White *et al.*, 2007). In fact, chronic N-deficiency (relative to other nutrients) occurs from top to bottom in the GOC water column (Alvarez-Borrego *et al.*, 1978; Beman *et al.*, 2005; White *et al.*, 2007)—during our study, dissolved NO₃⁻ concentrations in surface waters were below the detection limit (<0.03 µM), while dissolved phosphorus concentrations exceeded 0.3 µM (White *et al.*, 2007). Under these conditions, microbially-mediated N cycling—including nitrification—is an important determinant of availability of this limiting nutrient.

This study focuses on two basins in the GOC, the Guaymas Basin and the Carmen Basin (Figure 1), and two depth ranges: 30–60 m, where we expected near-surface nitrification to be active and which was the primary focus; and 300–650 m, where nitrification may take place under suboxic (<10 µM O₂) conditions. The California Undercurrent transports suboxic waters from the eastern tropical North Pacific (ETNP) to the central GOC at depths of approximately 400–800 m (Roden, 1958), and the GOC is characterized by an oxygen minimum zone (OMZ) at these depths. Anaerobic N-loss processes such as anaerobic ammonium oxidation or denitrification are active in the cores of OMZs, and nitrification is expected to occur on the suboxic fringes, where both NH₄⁺ and O₂ are available (Ward, 2000).

In July 2005, the GOC water column was characterized by highly stratified conditions, with surface water temperatures of approximately 30 °C declining sharply to 20–23 °C at 45–50 m depth (Figure 2a). Chlorophyll concentrations were maximal at 40–45 m depth in the Carmen Basin (Figure 2b), whereas O₂ concentrations declined rapidly from subsurface values of approximately 200 $\mu\text{mol kg}^{-1}$ to less than 10 $\mu\text{mol kg}^{-1}$ at 300 m, and below 5 $\mu\text{mol kg}^{-1}$ at 550 m depth (Figure 2c). NO₃⁻ concentrations increased from undetectable concentrations (<0.03 μM) in the 0–40 m depth range to approximately 40 μM at 650 m in the OMZ (Figure 2d). NO₂⁻ concentrations exhibited two maxima: a primary NO₂⁻ maximum below the euphotic zone at 45–50 m, and a secondary NO₂⁻ maximum in the OMZ at 550 m (Figure 2e). A 1–2 μM decrease in NO₃⁻ concentrations corresponded with the secondary NO₂⁻ maximum, which is classically interpreted as evidence of *in situ* denitrification—that is, sequential reduction of NO₃⁻ to NO₂⁻ and eventually to N₂ gas; alternatively, anaerobic ammonium oxidation may be important in the GOC given its significance in virtually all marine OMZs studied to date (Kuypers *et al.*, 2005; Francis *et al.*, 2007; Hamersley *et al.*, 2007). Irrespective of the mechanism by which these features are generated, they are indicative of anaerobic N metabolism at 550 m depth in GOC.

Crenarchaeota and nitrogen biogeochemistry: surface to 700 m

Based on qPCR analysis of archaeal *amoA* gene copy numbers, profiles of AOA abundance exhibited a

double-peak pattern similar to that of NO₂⁻. A subsurface peak (50–60 m) in archaeal *amoA* gene copies occurred just below the primary NO₂⁻ maximum in both basins (Figures 2f, g, 5b, d, g and i), while a secondary peak occurred at 450 m depth (Figures 2f and g). Gene copies at 450 m were comparable to those in the upper portion of the water column, and reached 10⁴ copies per milliliter in the Carmen Basin. Relatively high archaeal *amoA* abundances under suboxic conditions are in line with a number of previous studies: expansions of marine archaea may occur during oceanic anoxic events (Kuypers *et al.*, 2001), archaeal *amoA* genes have been recovered from the OMZ of the ETNP and from the suboxic zone of the Black Sea (Francis *et al.*, 2005), and archaeal *amoA* copy numbers were maximal in the suboxic zone of the Black Sea (Coolen *et al.*, 2007; Lam *et al.*, 2007). In an interesting parallel, the distribution of marine Crenarchaeota based on their membrane lipid, crenarchaeol, showed consistently high abundances at 450 m in the OMZ of the Arabian Sea (Sinninghe Damste *et al.*, 2002).

Since anaerobic N removal processes appear to consume NO₃⁻ and generate the secondary NO₂⁻ maximum at 550 m, increased archaeal *amoA* copy numbers at 450 m could be representative of coupled nitrification–denitrification at 450–500 m. Reduced numbers at 550–650 m relative to 300–450 m are consistent with anaerobic metabolism being more important at these depths. Although ammonia oxidation rates were not measured in the OMZ, nitrous oxide (N₂O) data collected in the Guaymas Basin in 2001 support such a transition: at 505 and 555 m depth, isotopomers of N₂O (the relative

Figure 2 Profiles from 0 to 700 m of temperature (a), chlorophyll *a* (b), dissolved oxygen (c), NO₃⁻ (d) and NO₂⁻ (e) in the Carmen Basin, and *amoA* gene copies from the Carmen (f) and Guaymas Basins (g). Data from 0 to 100 m are from the group A archaeal *amoA* assay and 300–650 m from the general assay. Error bars denote one standard error of the triplicate quantitative PCR (qPCR) measurement and are not visible when less than the width of the data point.

distribution of N isotopes in the N_2O molecule) were indicative of N_2O production by both denitrification and nitrification, while at 405 m, nitrification was the dominant process (no data are available from 450 m) (Yamagishi *et al.*, 2007).

Quantitative distribution of AOA groups

Due to high copy numbers at 450 and 60 m (Figures 2f and g), archaeal *amoA* PCR clone libraries were generated from both these depths in both basins. Remarkably different *amoA* sequence types were recovered from 60 and 450 m despite similar abundances at the two depths. In the Guaymas Basin, the Sorensen's abundance-based similarity (L_{abd}) between 60 and 450 m libraries was 0.47 ± 0.29 , while in the Carmen Basin, L_{abd} was 0.17 ± 0.14 (operational taxonomic units defined as 5% nucleotide sequence difference). These values represent the probability that a sequence type found in either library will be found in both libraries (Chao *et al.*, 2005; Schloss and

Handelsman, 2006), and by way of contrast, $L_{\text{abd}} = 0.92 \pm 0.11$ for the two 450 m libraries and 1.0 ± 0 for the two 60 m libraries—that is, libraries drawn from the same depths in different basins are essentially identical.

These similarities and differences are clear based on phylogenetic analysis, as all 48 archaeal *amoA* sequences from both 60 m libraries clustered with other water column sequences in Water Column Cluster A (Francis *et al.*, 2005), whereas 50% of sequences from 450 m in the Guaymas Basin and 25% from 450 m in the Carmen Basin fell into this cluster (Figure 3). The remaining sequences from the 450 m fell predominantly into Water Column Cluster B with the majority of sequences recovered from the secondary NO_2^- maximum in the ETNP (Francis *et al.*, 2005), sequences from 200 to 4000 m depth at station ALOHA (Hallam *et al.*, 2006b; Mincer *et al.*, 2007) and sequences from 500 m in Monterey Bay (Hallam *et al.*, 2006b; Mincer *et al.*, 2007).

Marine crenarchaeal communities exhibit a similar depth-related separation based on analysis

Figure 3 Phylogenetic relationships among archaeal *amoA* sequences from the GOC and previously-reported sequences. The water column group A cluster is shaded light gray and the group B cluster is shaded dark gray; next to these clusters, bar graphs indicate the number of sequences recovered in clone libraries from the GOC that are contained in each cluster. The four bars in each graph represent the combination of two different depths—60 and 450 m—sampled in two basins—the Guaymas and Carmen Basins (two sequences from 450 m in the Guaymas Basin also form a branch below cluster A). Metagenomic sequences from the Sargasso Sea (accession no. AACY01435967) and German soil (accession no. AJ627422), as well as *amoA* genes from *N. maritimus* (accession no. DQ085098) and *C. symbiosum* (accession no. DQ397569), are shown in boldface next to the clusters containing them. This neighbor-joining tree is based on Jukes-Cantor-corrected DNA distances, and is rooted with the large cluster in the lower portion of the tree containing soil sequences. The distance scale bar is at the lower left and bootstrap values (> 60%) are indicated at branch points, with distance bootstrap values above the line and parsimony values below. GOC, Gulf of California.

of 16S rRNA genes (Massana *et al.*, 2000) and sequencing of the intergenic (or internal transcribed) spacer between the 16S and 23S rRNA genes (Garcia-Martinez and Rodriguez-Valera, 2000). Specifically, group 1 Crenarchaeota partial 16S rRNA gene sequences from eight different sampling locations grouped predominantly into two clusters, with one cluster containing sequences from both the upper water column and deeper waters (cluster I- α), and the other cluster containing sequences drawn exclusively from water depths of 200 m and greater (cluster 1- γ) (Massana *et al.*, 2000). Similarly, group 1 Crenarchaeota partial 16S rRNA gene sequences and ITS sequences from the Antarctic Ocean and Mediterranean Sea grouped into at least three clusters containing sequences from a mix of surface and deep water samples (Crena-S1, S2 and A clusters), while another cluster (Crena-D) contained sequences from only deep waters; these patterns were consistent for both 16S rRNA and ITS sequences (Garcia-Martinez and Rodriguez-Valera, 2000). Based on the archaeal *amoA* gene, distinct water column groups A and B were first identified by Francis *et al.* (2005), and Hallam *et al.* were the first to suggest that group A and group B represent different 'shallow' and 'deep' 'ecotypes' (Hallam *et al.*, 2006b). Additional evidence for this phylogenetic separation based on the archaeal *amoA* gene was reported by Mincer *et al.* (2007), who further suggested that it may be due to photoinhibition-resistance adaptations.

To explore their distribution and possible ecological roles, we designed unique PCR primers to target the 'shallow' group A and 'deep' group B archaeal *amoA* clades. These primers were found to be specific for *amoA* sequence types from these groups, with no evident cross-amplification; however, based on 100% sequence identity with the *N. maritimus amoA* gene, the group A forward primer may also target '*N. maritimus*-like' *amoA* sequence types (Figure 3; see Materials and methods). In line with the results from cloning and sequencing, qPCR data indicated that group A was present throughout the water column, from the near-surface to 650 m depth. At first glance, both groups constituted a relatively constant proportion of total *amoA* copies in the 300–650 m depth range—group B comprised 57% ($\pm 15\%$ s.d.) and group A 43 $\pm 15\%$ of group A and B copy numbers added together (Figure 4). However, departures from these percentages varied with depth, and the greatest discrepancy occurred at 450 m, despite similar peaks in abundance: group A comprised 54% of total copies in the Guaymas Basin and only 25% in the Carmen Basin. These numbers are virtually identical to the proportions recovered in clone libraries (50% and 25%).

For the deep samples overall, the sum of the group A and B assays correlated with the general assay ($r^2 = 0.89$; Figure 4) with a slope slightly greater than 1 (1.15). Given that the general archaeal *amoA* primers were designed based on limited sequence

Figure 4 Distribution of ammonia-oxidizing archaea groups A and B in the oxygen minimum zone based on archaeal *amoA* qPCR analysis. Group A (light gray) and group B (black) *amoA* gene copy numbers are shown in comparison to those from the general *amoA* assay (dark gray). Oxygen profiles are also shown for comparative purposes (note the different scales for the two profiles). qPCR, quantitative PCR.

data available at that time (Francis *et al.*, 2005), it is not surprising that they may underestimate gene copies in some cases. However, these and at least five other archaeal *amoA* primer sets have been used for PCR applications (Francis *et al.*, 2005; Könneke *et al.*, 2005; Hallam *et al.*, 2006b; Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Mincer *et al.*, 2007), and the specificity of all of these primer sets for different AOA groups is largely untested. Based on our results, the dynamics of these and other AOA groups are intriguing and deserve more detailed study.

Crenarchaeota and ammonia oxidation: surface to 100 m

In contrast to deep waters, AOA communities in the upper water column (0–100 m) were composed entirely of group A—in no case was the ‘deep’ archaeal *amoA* qPCR assay significantly different from zero for these samples. However, the distribution of these organisms varied tremendously over this depth range, reflecting fundamental differences in N biogeochemistry both within and between basins. In the Guaymas Basin, chlorophyll concentrations, NO_3^- concentrations and NO_2^- concentrations all exhibited increases or maxima at shallower depths than in the Carmen Basin. The chlorophyll maximum occurred at 30–40 m in the Guaymas Basin, and 40–50 m in the Carmen Basin (White *et al.*, 2007). NO_3^- increased from undetectable concentrations to similar concentrations at 70 m in both the basins, yet NO_3^- increased more rapidly with increasing depth in Guaymas—for example, at 50 m, NO_3^- exceeded $10\ \mu\text{M}$ in the Guaymas Basin and was less than $2\ \mu\text{M}$ in the Carmen Basin (Figures 5a and f). The primary NO_2^- maximum ($0.3\ \mu\text{M}$) occurred at 40 m in the Guaymas Basin and 50 m in the Carmen ($0.4\ \mu\text{M}$)—10 m deeper in the water column (Figures 5b and g). NH_4^+ concentrations were greatest at 40 m in the Guaymas Basin, and maximal NH_4^+ concentrations occurred at 40 and 50 m in the Carmen Basin (Figures 5b and g). NH_4^+ concentrations were higher overall in the Guaymas Basin than in the Carmen Basin.

Ammonia oxidation rates were quantified by tracing the incorporation of $^{15}\text{NH}_4^+$ into the $\text{NO}_2^- + \text{NO}_3^-$ pool, and paralleled differences in N biogeochemistry. $^{15}\text{NH}_4^+$ oxidation rates were quantified at depths from 30 to 60 m in both basins and ranged from 0.01 to $93.1\ \text{nmol N l}^{-1}\text{ day}^{-1}$, with both mean and median rates between 34 and $35\ \text{nmol N l}^{-1}\text{ day}^{-1}$ (Figures 5c and h). Adding to the relatively limited number of nitrification rate measurements available globally (Yool *et al.*, 2007), the measurements reported here are the first from the GOC and among only a handful from the Pacific Ocean. However, $^{15}\text{NH}_4^+$ oxidation rates measured in the Guaymas and Carmen Basins are in line with previously reported rates from the ETNP and elsewhere in the Pacific: Sutka *et al.* (2004) found

maximum rates of $24.7\ \text{nmol N l}^{-1}\text{ day}^{-1}$ at 65 m depth in the ETNP, whereas Ward and Zafiriou (1988) measured rates of approximately $20\ \text{nmol N l}^{-1}\text{ day}^{-1}$ in the ETNP near the mouth of the GOC. $^{15}\text{NH}_4^+$ oxidation rates ranged from 20 to $80\ \text{nmol N l}^{-1}\text{ day}^{-1}$ in the Monterey Bay and were not related to AOB diversity (Ward, 2005), whereas rates ranged from 1.0 to $137.4\ \text{nmol N l}^{-1}\text{ day}^{-1}$ at station ALOHA (Dore and Karl, 1996). In the GOC, maximum $^{15}\text{NH}_4^+$ oxidation rates were greater in the Guaymas Basin (93.1 versus $39.4\ \text{nmol N l}^{-1}\text{ day}^{-1}$), and the depth profiles from these basins were distinct: $^{15}\text{NH}_4^+$ oxidation rates increased gradually and plateaued in the Carmen Basin, whereas rates peaked sharply and declined in the Guaymas Basin (Figures 5c and h).

Despite such pronounced variability, there were remarkable similarities between $^{15}\text{NH}_4^+$ oxidation rates and the distribution of AOA group A. In both basins, maximum $^{15}\text{NH}_4^+$ oxidation rates occurred at the same depths as maximum group A archaeal *amoA* gene copies, and just below the NO_2^- maximum: 50 m in Guaymas (14 100 copies per nanogram DNA) and 60 m in the Carmen Basin (10 600 copies per nanogram DNA). Both maximum rates and maximum *amoA* copy numbers in Guaymas were greater than those in the Carmen Basin (Figures 5c, d, h and i). Within the limits of our sampling regime in the Carmen Basin, $^{15}\text{NH}_4^+$ oxidation rates increased from 0.01 to $39.4\ \text{nmol l}^{-1}\text{ day}^{-1}$ from 30 to 60 m, while *amoA* copies increased by several orders of magnitude, eventually declining between 60 and 90 m (Figures 5h and i). Most striking were the depth-resolved similarities between $^{15}\text{NH}_4^+$ oxidation rates, *amoA* copy numbers and crenarchaeal 16S rRNA gene copy numbers in the Guaymas Basin (Figures 5c, d and e): all three profiles increased in unison from 35 to 50 m, and decreased in unison from 50 to 60 m.

As all of these data indicate, $^{15}\text{NH}_4^+$ oxidation rates are strongly and significantly correlated with group A *amoA* copy numbers ($r^2 = 0.90$, $P < 0.001$, $n = 8$; Figure 6). This relationship is robust despite variation in both parameters that ranges over several orders of magnitude, and holds across multiple depths and between two different basins—In addition, there is a similar correlation between marine group 1 Crenarchaeota 16S rRNA gene copies (per nanogram DNA) and $^{15}\text{NH}_4^+$ oxidation rates ($r^2 = 0.85$, $P < 0.005$, $n = 8$; Figure 6). As these relationships imply, marine group 1 Crenarchaeota 16S rRNA gene copies and group A archaeal *amoA* gene copies are also significantly correlated ($r^2 = 0.82$, $P < 0.005$, $n = 8$). The slope of this relationship is 2.5, which is virtually identical to the findings of Wuchter *et al.* (2006)—these authors reported a slope of 2.5 between archaeal *amoA* and crenarchaeal cell counts by catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH), and 2.8 for archaeal *amoA* gene

Figure 5 Vertical distributions from 0 to 100 m in the Gulf of California of: NO_3^- (a, f); NO_2^- (orange symbols) and $10 \times \text{NH}_4^+$ concentrations (dark red symbols) (b, g); $^{15}\text{NH}_4^+$ oxidation rates (c, h); group A archaeal *amoA* gene copies (d, i); and marine *Crenarchaeota* 16S rRNA gene copies (e, j). Top panels (a–e) are from the Guaymas Basin and bottom panels (f–j) from the Carmen Basin. Error bars denote one standard error of the triplicate quantitative PCR (qPCR) measurement and are not visible when less than the width of the data point. Gray-shaded regions denote areas of active nitrification in the two basins where all parameters shift rapidly with depth.

copies and 16S rRNA gene copies from *Crenarchaeota*. These findings suggest that similar to various β -AOB, which are known to have 2–3 copies of the *amo* operon (Norton *et al.*, 2002), marine *Crenarchaeota* may possess more than one *amoA* copy per genome. However, only one *amoA* copy was reported in the genome of the sponge symbiont *C. symbiosum* (Hallam *et al.*, 2006a), and it seems likely that definitive information will only emerge following further genome sequencing. Last, although gene copies on a per milliliter basis are more variable (due to differences in extraction efficiency among samples, differences in the volume of water filtered and variation in the proportion of filter extracted), marine group 1 *Crenarchaeota* 16

rRNA gene copy numbers per milliliter correlated with $^{15}\text{NH}_4^+$ oxidation rates ($r^2 = 0.71$, $P < 0.01$, $n = 8$).

Altogether, it seems plausible that AOA group A is actively involved in ammonia oxidation in the upper water column of the GOC. Whether or not this extends to group B—or to other depths or regions of the ocean—is, at present, unknown. However, *amoA* genes corresponding with group A were shown to be expressed in the modeled nitrification zone of the Black Sea (Lam *et al.*, 2007), which clearly lends additional support for their involvement in ammonia oxidation. Expression of archaeal *amoA* genes in mRNA was not directly assayed in this study due to methodological and logistical

constraints; yet *amoA* gene copies are probably an accurate reflection of community structure in the upper water column of the GOC. Decoupling between gene copy numbers and *amoA* expression was documented only within the suboxic zone of the Black Sea (Lam *et al.*, 2007), and highly stratified conditions during summertime in the GOC result in a stable physical configuration, such that AOA populations are likely to be at near steady-state with biogeochemical conditions. For example, the profiles observed here follow the expected progression from ammonification to ammonia oxidation to nitrite oxidation with increasing depth in the water column (Codispoti and Christensen, 1985; Ward, 2000)—as evidenced by peak NH_4^+ concentrations at 35–40 m, peak NO_2^- concentrations at 40–50 m, maximum ammonia oxidation rates and archaeal *amoA* gene copies at 50–60 m and an eventual increase in NO_3^- concentrations at 50 m and below

Figure 6 Correlation between $^{15}\text{NH}_4^+$ oxidation rates and marine Crenarchaeota 16S rRNA gene copies (gray), and group A archaeal *amoA* gene copies (black) across both basins. Regression equations with standard errors of slopes and intercepts are as follows: $y = 0.045 \pm 0.007 * (16\text{S rRNA gene copies}) + 0.35 \pm 7.43$; $y = 0.017 \pm 0.002 * (\text{amoA gene copies}) - 1.97 \pm 6.32$.

(Figure 5). Because NO_2^- and NH_4^+ are produced and consumed by multiple processes, including ammonia oxidation, concentrations of NO_2^- and NH_4^+ are not necessarily expected to correlate with *amoA* gene copies throughout the water column—however, in some cases they might be related over certain depth ranges, or, more likely, through time (for example, Murray *et al.*, 1999; Wuchter *et al.*, 2006).

These data may instead capture coincident variation in biogeochemical properties and microbial abundances along the transition from surface layers to the deep ocean; however, this seems like an oversimplification given variation over several orders of magnitude for both *amoA* copies and $^{15}\text{NH}_4^+$ oxidation rates, as well as distinct biogeochemical differences between basins. For example, NH_4^+ is higher, NO_2^- lower and both rates and *amoA* copies higher in the Guaymas Basin than in the Carmen Basin (Figure 5)—which may indicate a more dynamic or developing stage of nitrification in the Guaymas Basin—yet where rates are similar between the basins (for example, 60 m), *amoA* copy numbers are similar. Clearly, N biogeochemistry, $^{15}\text{NH}_4^+$ oxidation rates and AOA group A co-vary strongly both with depth and between basins, and it is unlikely that such well-defined synchrony is entirely coincidental.

Bacteria and ammonia oxidation: surface to 100 m

Ammonia-oxidizing members of the bacterial domain exhibited a substantially different pattern of variation. γ -AOB are widely distributed geographically (Ward and O'Mullan, 2002) and ammonia oxidation by γ -AOB may be important in the Black Sea suboxic zone (Lam *et al.*, 2007). However, in 20 samples from the North Atlantic, γ -AOB were not detected by qPCR, while both AOA and β -AOB were present and quantifiable (Wuchter *et al.*, 2006); also γ -AOB were not amplifiable in a range of samples collected in the polar oceans (Hollibaugh *et al.*, 2002). In the GOC, γ -AOB *amoA* sequences were

Table 1 AOB *amoA* amplification and abundance

Guaymas Basin			Carmen Basin		
Depth (m)	β -AOB (<i>amoA</i> copies per nanogram DNA)	γ -AOB	Depth (m)	β -AOB (<i>amoA</i> copies per nanogram DNA)	γ -AOB
30	—	—	0	—	—
35	—	—	10	—	—
40	—	—	30	—	+
45	—	—	45	—	+
50	65 ± 4	—	60	243 ± 19	—
60	214 ± 67	—	90	24 ± 6	—

Abbreviations: AOB, ammonia-oxidizing bacteria; *amoA*, ammonia monooxygenase subunit A.

β -AOB abundance based on *amoA* copies (per nanogram DNA) is shown ($\pm$ s.e.); quantitative amplification of γ -AOB is indicated by a '+' sign. For either AOB group, a '—' sign indicates lack of amplification by qPCR.

amplifiable by qPCR only at 30 and 45 m in the Carmen Basin, and as a result were not directly quantified (Table 1). β -AOB were below the detection limit of 21 copies per nanogram DNA in all samples from 0 to 45 m, and were significantly less abundant than AOA at 50 m in the Guaymas Basin (65 versus 14 100 copies per nanogram DNA). β -AOB were present in similar proportions in both basins at 60 m (Table 1), and decreased by an order of magnitude from 60 to 90 m in the Carmen Basin, from 243 ± 19 to 24 ± 6 copies per nanogram DNA. Overall, AOA outnumbered β -AOB by a factor of 37–217, with peak β -AOB abundances found at 60 m.

This distribution is generally consistent with studies that have quantified AOB in conjunction with measuring ammonia oxidation rates. Previous authors used immunofluorescence to enumerate AOB and measured $^{15}\text{NH}_4^+$ oxidation rates in the Southern California Bight, and found limited correspondence between AOB abundance and oxidation rates: although maximum rates and abundances occurred at the same depths, these parameters were decoupled in the rest of the water column (Ward, 1987). Similarly, Ward *et al.* (1989) noted some correspondence between maximum $^{15}\text{NH}_4^+$ oxidation rates and maximum AOB abundances off the coast of Peru; however, no significant correlations were found. Although nitrification is known to actively occur on sinking particles (Karl *et al.*, 1984) and may be important in the GOC water column, the filtering procedure used here should capture any particle-associated microorganisms in addition to planktonic AOB. A disconnect between AOB and $^{15}\text{NH}_4^+$ oxidation rates does not preclude bacterial ammonia oxidation, and at depths where AOB are present, they may actively contribute to ammonia oxidation. However, β -AOB were not quantifiable and γ -AOB were not amplified at depths where ammonia oxidation rates exceed $15 \text{ nmol NI}^{-1} \text{ day}^{-1}$, and where AOA are present in substantial amounts based on their gene copy numbers (for example, 40 and 45 m in the Guaymas Basin).

Ultimately, the most logical explanation for a strong and significant correlation between $^{15}\text{NH}_4^+$ oxidation rates and both archaeal *amoA* and 16S rRNA gene copy numbers is that marine Crenarchaeota are actively oxidizing ammonia in the GOC. More specifically, the AOA group A *amoA* primers developed in this study target an archaeal *amoA* sequence type that is known to be expressed in the marine environment, and group A gene copy numbers are correlated with $^{15}\text{NH}_4^+$ oxidation rates in the upper water column of the GOC. These findings may be broadly representative of how and where this AOA group is biogeochemically active in the ocean, but they may not apply to group B or to deeper waters. The combination of stable isotope biogeochemistry and molecular microbial ecology employed in this study will determine whether or not this is the case.

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