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ORIGINAL ARTICLE

Enrichment and characterization of ammonia-oxidizing archaea from the open ocean: phylogeny, physiology and stable isotope fractionation

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Archaeal genes for ammonia oxidation are widespread in the marine environment, but direct physiological evidence for ammonia oxidation by marine archaea is limited. We report the enrichment and characterization of three strains of pelagic ammonia-oxidizing archaea (AOA) from the North Pacific Ocean that have been maintained in laboratory culture for over 3 years. Phylogenetic analyses indicate the three strains belong to a previously identified clade of water column-associated AOA and possess 16S ribosomal RNA genes and ammonia monooxygenase subunit a (*amoA*) genes highly similar (98–99% identity) to those recovered in DNA and complementary DNA clone libraries from the open ocean. The strains grow in natural seawater-based liquid medium while stoichiometrically converting ammonia (NH_3) to nitrite (NO_2^-). Ammonia oxidation by the enrichments is only partially inhibited by allylthiourea at concentrations known to completely inhibit cultivated ammonia-oxidizing bacteria. The three strains were used to determine the nitrogen stable isotope effect ($^{15}\varepsilon_{NH3}$) during archaeal ammonia oxidation, an important parameter for interpreting stable isotope ratios in the environment. Archaeal $^{15}\varepsilon_{NH3}$ ranged from 13‰ to 41‰, within the range of that previously reported for ammonia-oxidizing bacteria. Despite low amino acid identity between the archaeal and bacterial Amo proteins, their functional diversity as captured by $^{15}\varepsilon_{NH3}$ is similar.
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Introduction

Mesophilic archaea are ubiquitous and abundant members of diverse marine environments including coastal waters (Mincer *et al.*, 2007; Beman *et al.*, 2010), marine sediments, estuaries (Mosier and Francis, 2008; Bernhard *et al.*, 2010; Urakawa *et al.*, 2010), stratified basins (Coolen *et al.*, 2007; Lam *et al.*, 2007) and open ocean water columns (Beman *et al.*, 2008; Church *et al.*, 2010; Santoro *et al.*, 2010). The recent cultivation of the first mesophilic marine archaeon, *Nitrosopumilus maritimus*, (Konneke *et al.*, 2005; Martens-Habbena *et al.*, 2009), two thermophilic archaea, *Nitrosocaldus yellowstonii* and *Nitrosophaera gargensis* (Hatzenpichler *et al.*, 2008; de la Torre *et al.*, 2008) and a freshwater archaeon, *Nitrosoarchaeum limnia* (Blainey *et al.*, 2011) established that at least some of these organisms are chemolithoautotrophic ammonia oxidizers. Several studies coupling gene- and cell-based quantification of putative ammonia-oxidizing archaea (AOA) to rate measurements (Wuchter *et al.*, 2006; Beman *et al.*, 2008; Santoro *et al.*, 2010) suggest that nitrification by AOA in the ocean is significant. However, direct demonstration of ammonia-oxidizing activity by the AOA genotypes encountered in the open ocean has not yet been demonstrated.

Ammonia oxidation is the first step of nitrification, a key remineralization reaction in the sea. Work by Olson (1981) and Ward *et al.* (1982) using ^{15}N tracers first suggested that nitrification in the upper ocean could be a significant source of regenerated nutrients for primary production. The quantitative role, however, of nitrification in the euphotic zone is still uncertain. Recent syntheses of nitrification rates made using isotope tracers (Yool *et al.*, 2007; Clark *et al.*, 2008) suggest that globally, nitrification may be a significant source of regenerated N for primary production, supplying as much

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as half of the necessary nitrate. This has important implications for estimates of carbon export based on new production (Eppley and Peterson, 1979); an underestimation of nutrient regeneration would lead to an overestimation of carbon export from the surface ocean (Ward, 2002).

Although instantaneous rate measurements with ^{15}N-labeled compounds provide important insights into the environmental controls on nitrification, they capture only a 'snapshot' view of this undoubtedly time-varying process. An alternative way of quantifying the importance of nitrification is using natural abundance stable isotope ratios to infer the relative importance of different nitrogen cycling processes. In particular, nitrogen and oxygen stable isotope ratios (δ^{15}N and δ^{18}O) in marine nitrate (NO_3^-) can integrate information about the relative sources (nitrification, upward diffusion, atmospheric deposition) and sinks (phytoplankton uptake) of NO_3^- in the euphotic zone (Sigman *et al.*, 2005; Wankel *et al.*, 2007; Casciotti *et al.*, 2008; DiFiore *et al.*, 2009) and provide an independent constraint on the importance of nitrification to new production. In order to interpret these measurements, however, the isotope effects for the various sources and sinks of NO_3^- must be known. The isotope effects for NO_3^- uptake by phytoplankton (Needoba *et al.*, 2003; Granger *et al.*, 2004) and NO_2^- production by nitrifying bacteria (Mariotti *et al.*, 1981; Casciotti *et al.*, 2003, 2010) are relatively well studied, but there are no data on the isotope effects for ammonia oxidation by AOA.

In this study, we describe the enrichment and characterization of three strains of AOA from the water column of the North Eastern Pacific Ocean that oxidize NH_3 to NO_2^-. We used the three strains to estimate the nitrogen kinetic isotope effect ($^{15}\varepsilon_{NH3}$) during ammonia oxidation by AOA. Our results extend the ability for chemoautotrophic ammonia oxidation within the *Archaea* and expand the number of marine archaea for which we have both phylogeny and confirmed metabolic function. We provide important constraints for the interpretation of natural abundance stable isotope ratios for compounds affected by these ubiquitous organisms in the marine nitrogen cycle.

Materials and methods

Enrichment and cultivation

Enrichment cultures were initiated with water collected from the North Eastern Pacific approximately 300 km from shore (California Cooperative Oceanic Fisheries Investigations station 67–90; 35.46°N, 124.91°W) aboard the R/V *Western Flyer* during cruise CN107 in July 2007 (Table 1). Seawater was collected from 25, 75, 150 and 500 m depths using a standard 10 l Niskin rosette sampler equipped with a conductivity-temperature-depth sensor package. Seawater was stored at 22 °C (25 and 75 m waters) and 4 °C (150 and 500 m waters) in 500 ml acid-cleaned polycarbonate bottles. After 20 months of incubation, filter sterile ammonium chloride (NH_4Cl) was added to a final concentration of 10 μmol l^{-1} and the enrichments were monitored for production of NO_2^- using standard colorimetric methods (Strickland and Parsons, 1968). At that time, the 150 m enrichment was moved to 13 °C and maintained at that temperature. Hereafter, the successful enrichments are referred to as CN25, CN75 and CN150 referring to the cruise name and depth of the enrichment inoculums.

Table 1 *In situ* conditions for the starting material for North Pacific Ocean seawater enrichments used for archaeal kinetic isotope fractionation ($^{15}\varepsilon_{NH3}$) determination

Enrichment	*Depth (m)*	*Temp (°C)*	*[NH_4^+] (nmol l^{-1})*	*[NO_2^-] (μmol l^{-1})*
CN25	25	14	40	0.5
CN75	75	12	<15	2.2
CN150	150	10	<15	15.1

Cultures were maintained with transfers of 10–20% (v/v) late exponential phase culture into oligotrophic North Pacific (ONP) medium consisting of: 0.2 μm-filtered North Pacific surface seawater amended with 10–100 μmol l^{-1} NH_4Cl, 1 ml l^{-1} chelated trace elements solution (Balch *et al.*, 1979), 15 μmol l^{-1} KH_2PO_4 and 100 μg ml^{-1} each streptomycin and ampicillin. Maintenance cultures were grown in 200 ml volumes in 250 ml acid-cleaned polycarbonate bottles. Surface seawater for ONP medium was obtained either from the initial collection site or from the Pacific hydrographic station SAFe (Johnson *et al.*, 2007).

Abundance of archaeal and bacterial cells in the enrichments was periodically monitored in 1 ml formaldehyde-fixed culture volumes filtered onto 25 mm diameter, 0.2 μm pore size polycarbonate filters (Millipore GTTP, Billerica, MA, USA) with catalyzed auto reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) and epifluorescence microscopy using probes Cren537–554 and EUB338 I-III (Teira *et al.*, 2004).

Phylogenetic analysis of 16S rRNA and amoA *genes*

DNA was extracted and purified from 25 ml of culture after vacuum filtration onto 25 mm diameter, 0.2 μm pore size Supor membrane filters (Pall, Port Washington, NY, USA) using DNeasy columns (Qiagen, Valencia, CA, USA) as previously described (Santoro *et al.*, 2008). Genes encoding for the 16S ribosomal RNA (rRNA) and the α subunit of ammonia monooxygenase (*amoA*), believed to contain the catalytic site for ammonia oxidation (Hyman and Wood, 1985), were amplified using PCR. Target genes were amplified in 25 μl PCR reactions using the primers: 21F/1492R (archaeal 16S rRNA genes, DeLong, 1992), ArchamoAF/ArchamoAR

(archaeal *amoA*, Francis *et al.*, 2005), 27F/1492R (bacterial 16S rRNA) and amoAF*/amoAR (beta-proteobacterial *amoA*, Stephen *et al.*, 1999) using reaction and thermocycling conditions described in the original papers or as modified in Santoro *et al.*, 2010. Gammaproteobacterial *amoA* genes were amplified using the amoA3F/amoB4R primer set (Purkhold *et al.*, 2000) using the following thermocycling profile: 95 °C for 3 min followed by 35 cycles of: 95 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min. Genomic DNA from *Nitrosococcus oceani* was used as a positive control. PCR products were pooled, purified (MinElute PCR Purification Kit, Qiagen), and cloned using the TOPO-TA cloning kit with pCR4 vector and MACH1 competent cells (Invitrogen, Carlsbad, CA, USA). Plasmids were purified (Mini Prep Spin Kit, Qiagen) and sequenced on an ABI 3730xl sequencer. Twelve archaeal 16S rRNA and *amoA* clones and 24 bacterial 16S rRNA clones were sequenced per enrichment.

16S rRNA gene sequences were imported into the ARB software program (Ludwig *et al.*, 2004) and aligned to a subset of the SILVA SSU reference database, February 2009 release (Pruesse *et al.*, 2007). Archaeal *amoA* sequences were manually aligned and imported into an ARB database maintained by our laboratory containing approximately 2000 environmental *amoA* sequences. Phylogenetic trees were constructed using 1268 nucleotide positions (16S rRNA) or 489 nucleotide positions (*amoA*) using maximum likelihood (RAxML) analyses implemented using the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal v3.0 (www.phylo.org) and visualized using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Archaeal 16S rRNA gene and *amoA* sequences from this study were deposited in GenBank under the accession numbers HQ338108–HQ338109 and JF521547–JF521549.

Growth curves

Growth curves were determined for CN25 and CN75 at 22 °C in replicate 200 ml volumes of ONP medium containing 50 μmol l^{-1} NH_4^+. At each time point, 15 ml of culture was removed. Ten milliliters of culture were frozen for later [NO_2^-] and [NH_4^+] determination. The remaining 5 ml of culture were immediately fixed with formaldehyde (2% final concentration) for four hours and replicate volumes (0.5–2 ml) of fixed culture were vacuum filtered onto 25 mm, 0.2 μm pore size polycarbonate membrane filters (Millipore) and frozen for later CARD-FISH analysis as described above. A minimum of 10 fields of view were counted from each filter using the 100× objective on a Zeiss Axio Scope (Carl Zeiss, Jena, Germany) epifluorescence microscope. [NH_4^+] and [NO_2^-] determinations were made in duplicate using phthaldialdehyde fluorescence (Holmes *et al.*, 1999) and azo dye colorimetry (Strickland and Parsons, 1968), respectively.

Allylthiourea inhibition experiments

To assess the effect of the metal chelator allylthiourea (ATU) on archaeal ammonia oxidation, strain CN25 was grown in triplicate 50 ml volumes in 60 ml polycarbonate screw cap bottles for each of three treatments: no ATU addition (control), 10 mg l^{-1} ATU (86 μmol l^{-1}), and 100 mg l^{-1} (860 μmol l^{-1}). ATU additions were made from a 10 mg ml^{-1} stock solution of ATU (Sigma) dissolved in water. Ammonia oxidation was monitored by the production of NO_2^-, determined using azo dye colorimetry in 1 ml volumes (Strickland and Parsons, 1968). ATU was added to the treatment bottles after initial NO_2^- production was detected in each flask (day 4). Inhibition for the ATU treatments was calculated as the percent decrease in slope of a linear regression fit to the ln[NO_2^-] over time compared with the control bottles.

Nitrogen isotope effect estimates

For each experiment, 25 ml of enrichment culture was inoculated into 175 ml of ONP medium containing 10–75 μmol l^{-1} NH_4^+ in a 250 ml acid-washed, screw-cap polycarbonate bottle. Growth was not observed when experiments were initiated with washed cell suspensions collected by filtration (data not shown), thus all experiments started with an initial [NO_2^-] between 2 and 10 μmol l^{-1} as well as residual NH_4^+ from maintenance cultures, in some cases. Temperature was maintained at 22 °C for CN25 and CN75 and 13 °C for CN150. NO_2^- production was monitored and 15 ml of enrichment culture was removed at each time point and frozen at −20 °C.

Initial and time-course measurements of [NH_4^+] and [NO_2^-] were made in duplicate using phthaldialdehyde fluorescence (Holmes *et al.*, 1999) and azo dye colorimetry (Strickland and Parsons, 1968), respectively, on 2 ml sample volumes. Error in replicate [NH_4^+] analyses ranged from 2% to 7%; error in replicate [NO_2^-] analyses was 0% to 8%. Isotopic measurements of NO_2^- were made by converting NO_2^- to nitrous oxide (N_2O) using the 'azide method' (McIlvin and Altabet, 2005) with 10–20 nmol N per sample. The resulting N_2O was captured using a custom purge and cryogenic trapping system (Casciotti *et al.*, 2002; McIlvin and Casciotti, 2010, 2011) and analyzed using a Finnigan Delta^{PLUS} XP isotope ratio mass spectrometer. Each sample was analyzed in duplicate against RSIL nitrite reference materials N23, N7373 and N10219 run in parallel (Casciotti *et al.*, 2007). Results are reported using delta notation: $\delta^{15}N_{NO2}$ (‰ vs AIR) = $[(^{15}R_{NO2}/^{15}R_{AIR})-1] \times 1000$, where $^{15}R = {}^{15}N{:}{}^{14}N$ and AIR is standard atmospheric N_2. Error in replicate measurements of $\delta^{15}N_{NO2}$ was 0.3‰ or better for all experiments.

Calculation of $^{15}\varepsilon_{NH3}$ requires knowledge of the isotopic composition of the NO_2^- produced over time since an arbitrary starting point. If the initial [NO_2^-] is zero, then the measured $\delta^{15}N_{NO2}$ ($\delta^{15}N_{NO2total}$) is

equal to that produced ($\delta^{15}N_{NO2produced}$). If there is NO_2^- initially present, as in our experiments, the $\delta^{15}N_{NO2produced}$ at any time since the initial time point (i) must be calculated from $\delta^{15}N_{NO2total}$ using the following equations:

$$\begin{aligned}&(^{15}N:^{14}N)_{total}\times[NO_2^-]_{total}\\&=(^{15}N:^{14}N)_{produced}\times[NO_2^-]_{produced}\\&\quad+(^{15}N:^{14}N)_i\times[NO_2^-]_i\end{aligned}\qquad(1a)$$

where $(^{15}N{:}^{14}N)_{total}=(^{15}N{:}^{14}N)_{AIR}\times(\delta^{15}N_{NO2total}/1000+1)$
Rearranging:

$$\begin{aligned}&(^{15}N:^{14}N)_{produced}\\&=\{(^{15}N:^{14}N)_{total}\times[NO_2^-]_{total}\\&\quad-(^{15}N:^{14}N)_i\times[NO_2^-]_i\}/[NO_2^-]_{produced}\end{aligned}\qquad(1b)$$

$$\delta^{15}N_{NO_2}produced=[(^{15}N:^{14}N)_{produced}/(^{15}N:^{14}N)_{AIR}-1]\times1000\qquad(2)$$

The isotope effect ($^{15}\varepsilon_{NH3}$) was then calculated from the $\delta^{15}N_{NO2produced}$ data using the Rayleigh accumulated product equation (after Mariotti *et al.*, 1981):

$$\delta^{15}N_{NO_2produced}=\delta^{15}N_{NH_4initial}+{}^{15}\varepsilon_{NH3}\left(\frac{fln(f)}{1-f}\right)\qquad(3)$$

where $f=[NH_4^+]/[NH_4^+]_{initial}$. By Eqn (3), the slope of a best fit line on a plot of $\delta^{15}N_{NO2}$ vs f *ln(f)/(1-f) yields $^{15}\varepsilon_{NH3}$ and the y intercept corresponds to the starting $\delta^{15}N_{NH4}$. As each experiment was initiated with a mixture of NH_4^+ carried over from the enrichment transfer and 'new' NH_4^+ from freshly prepared medium ($\delta^{15}N\sim-3‰$), minor variations in the $\delta^{15}N_{NH4}$ (and the y intercept) are expected between experiments and therefore each experiment was fitted separately. Fitting and 95% confidence interval calculations were done using IGOR Pro software (v5, WaveMetrics, Inc., Lake Oswego, OR, USA).

Use of the Rayleigh model presumes that ammonia oxidation proceeds as a pseudo one-step reaction with no accumulation of an intermediate product between NH_3 and NO_2^- and no back-reaction of the products (Casciotti *et al.*, 2003). When estimated in this way, several isotope effects are incorporated into $^{15}\varepsilon_{NH3}$ including a $\sim$20–35‰ equilibrium isotope effect between NH_4^+ and NH_3 (Casciotti *et al.*, 2011), as well as any isotope effects for NH_3 or NH_4^+ diffusion. Errors in $^{15}\varepsilon_{NH3}$ resulting from these assumptions, as well as errors resulting from uncertainties in the archaeal ammonia oxidation pathway, are discussed below.

Results

Phylogeny and physiology

Following 20 months of incubation in the dark with no amendments, archaeal *amoA* genes were detected by PCR in all four seawater 'enrichment' bottles (25, 75, 150 and 500 m). On addition of 10 μmol l^{-1} NH_4^+, NO_2^- production was observed in the enrichments from 25, 75 and 150 m within 1 month (data not shown). No NO_2^- production was observed in the 500 m bottle. The NO_2^- producing enrichments are referred to as CN25, CN75 and CN150. All three CN enrichments contained a high proportion of cells hybridizing with the CARD-FISH probe suite CREN537–554 and PCR amplifiable archaeal *amoA* genes. No *amoA* genes from either gamma or betaproteobacteria were detected.

After approximately 1 year of routine transfers into ONP medium (nearly 3 years after initial collection), the enrichments were highly enriched in archaeal cells (Figure 1) and the basic phylogeny and physiology of the enrichments were characterized. A consensus archaeal 16S rRNA gene sequence was obtained from 12 clones from each enrichment. One sequence from CN25 and two sequences from CN75 contained single nucleotide changes (all at different positions) from this consensus sequence.

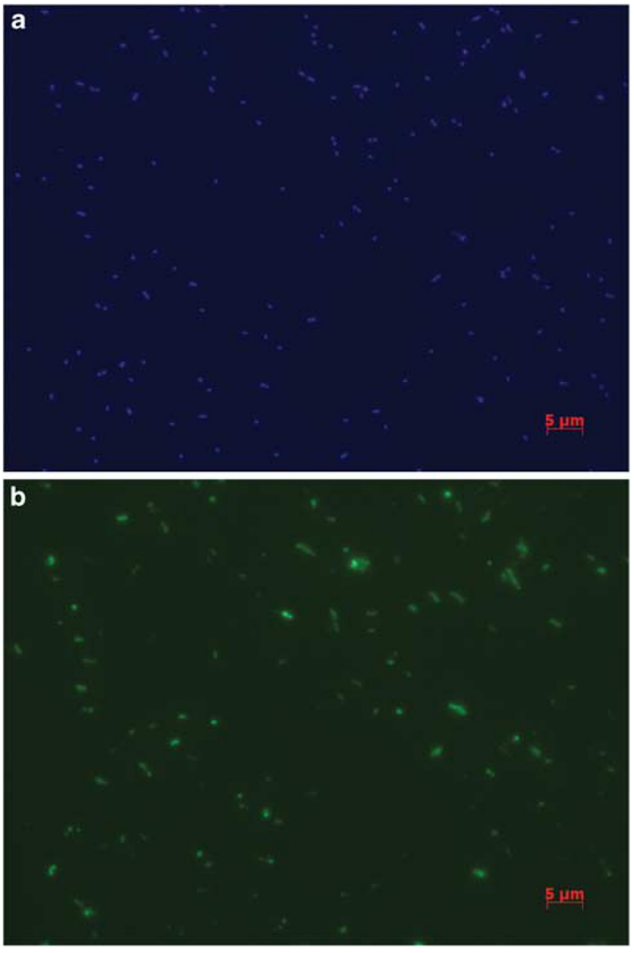

Figure 1 Fluorescence microscopy images of enrichment CN25 stained with (**a**) 4,6-diamidino-2-phenylindole (DAPI) and (**b**) CARD-FISH using the Cren537–554 probe suite. Both images are from the same field of view; the scale bar represents 5 μm.

Phylogenetic analyses place all three sequences within the Marine Group I archaea, now proposed as a new archaeal kingdom—the Thaumarchaeota (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010; Walker *et al.*, 2010) (Figure 2a). The 16S rRNA gene sequences from CN25 and CN75 are 100% identical to each other, and 92% identical to *N. maritimus*. Closest GenBank BLAST matches to the CN25 16S rRNA gene sequence are 98% identical and include sequences from environmental clone libraries from 200 m depth off the Oregon coast (fosmid 4B7, U40238, (Stein *et al.*, 1996)) and an unpublished deep sea hydrothermal vent clone from Suiyo Seamount (AB194001). The CN150 16S rRNA gene is 98% identical to *N. maritimus* and 99% identical to several sequences from the Sargasso Sea metagenome (AACY020033564, (Venter *et al.*, 2004)).

The *amoA* sequences from CN25 and CN75 fall within a cluster of environmental sequences previously termed water column cluster 'A'. They are also 100% identical at the amino acid level to several sequences from the Sargasso Sea metagenome (Venter *et al.*, 2004) (Figure 2b, Supplementary Figure 1) and 84% and 95% identical at the nucleotide and amino acid levels, respectively, to the *amoA* sequence from *N. maritimus*. The CN150 *amoA* gene is also 84% identical to *N. maritimus* at the nucleic acid level. As CN25 and CN75 are identical at the 16S rRNA and *amoA* level, full genome sequencing may be necessary to resolve any strain-level differences between the two enrichments.

Bacterial 16S rRNA gene clone libraries from the CN enrichments did not contain 16S rRNA genes from any genera of known bacterial nitrifiers and contained sequences associated with the genera *Erythrobacter* and *Gracilimonas*. Bacterial *amoA* genes could not be detected using PCR amplification.

Stoichiometric conversion of NH_4^+ to NO_2^- was observed in all three enrichments coincident with an exponential increase in the number of archaeal cells, evidence that the CN strains couple ammonia oxidation to growth (Figure 3). All three CN strains were able to utilize NH_4^+ from the medium below our detection limit of $\sim$200 nM. Bacterial cells, as quantified by CARD-FISH, were not detectible until the archaeal population entered late exponential-early stationary phase. CN25 and CN75, growing at 22 °C, had lag phases of 4–10 days on transferring 10–25% of the original culture volume into new medium, even when cultures were transferred during exponential phase growth. Transfer volumes of $<$10% failed to grow (data not shown). CN150, growing at 13 °C, had lag phases of up to 25 days.

CN25 and CN75 were chosen for more detailed growth studies because of faster NH_4^+ oxidation rates, shorter lag phases, and more consistent growth. The CN25 growth rate at 22 °C was 0.15 day^{-1}; the CN75 growth rate was 0.17 day^{-1}, corresponding to doubling times of about 4–4.6 days. Stationary phase cell densities in CN25 and CN75 were 2.7–3.2 $\times 10^6$ cells ml^{-1} with 86–97% of cells hybridizing with archaeal CARD-FISH probes depending on the growth stage of the enrichment.

ATU is frequently used to inhibit nitrifying activity in environmental studies. We tested the effect of ATU on the ammonia oxidation rate for CN25. An ATU concentration of 10 mg l^{-1} (86 μmol l^{-1}) inhibited the ammonia oxidation rate by 58%; an ATU concentration of 100 mg l^{-1} (860 μmol l^{-1}) completely inhibited ammonia oxidation (Figure 4).

Nitrogen isotopic fractionation

To further investigate the physiology of the enriched strains, we determined their N kinetic isotope effect during ammonia oxidation ($^{15}\varepsilon_{NH3}$). As in growth curve experiments, we observed near stoichiometric conversion of NH_4^+ to NO_2^- over the course of the isotope fractionation experiments, with conservation in the dissolved inorganic nitrogen pool ($NH_4^+ + NO_2^-$) ranging from 74% to 107% with a mean of 97% (Table 2).

Results from replicate experiments are reported individually, as well as an unweighted average of experiments for a given enrichment (Table 2). $^{15}\varepsilon_{NH3}$ for CN25 ranged from 14 to 30‰ with an average of 22 ± 5‰ ($n = 11$), $^{15}\varepsilon_{NH3}$ for CN75 ranged from 10 to 37‰ with an average of 21 ± 10‰ ($n = 6$), and $^{15}\varepsilon_{NH3}$ for CN150 ranged from 16 to 28‰ with an average of 22 ± 5‰ ($n = 7$). Non-linearity was observed in most experiments with CN25 and CN75, with larger $^{15}\varepsilon_{NH3}$ (that is, greater slopes) at the beginning of the growth curve when a large fraction (f) of the initial NH_4^+ remained (Figures 5a and b). This leads to large uncertainties (expressed as 95% confidence intervals) in the slopes for many of the experiments with CN25 and CN75 (Table 2). We did not observe the same non-linearity of $^{15}\varepsilon_{NH3}$ in experiments with CN150 (Figure 5c).

Discussion

Phylogeny

We established three enrichments of marine archaea that stoichiometrically oxidize NH_4^+ to NO_2^-. Multiple lines of evidence suggest that the archaea are the active ammonia oxidizers in the enrichments. Archaeal cells increase exponentially concomitant with an exponential increase in NO_2^-, and *amoA* genes from the enrichments have a high identity to known archaeal ammonia oxidizers. The enrichments lack 16S rRNA and *amoA* genes associated with known γ- or β-proteobacterial ammonia oxidizers. Though no NO_2^- or NO_3^- production was observed in the enrichment from 500 m, this cannot be used to infer a lack of NH_3-oxidizing ability in field populations at this depth, as active NH_3 oxidation was measured *in situ* (Santoro *et al.*, 2010).

Both 16S rRNA and *amoA* gene sequences from the CN strains suggest they are representative of organisms present and active in the open ocean.

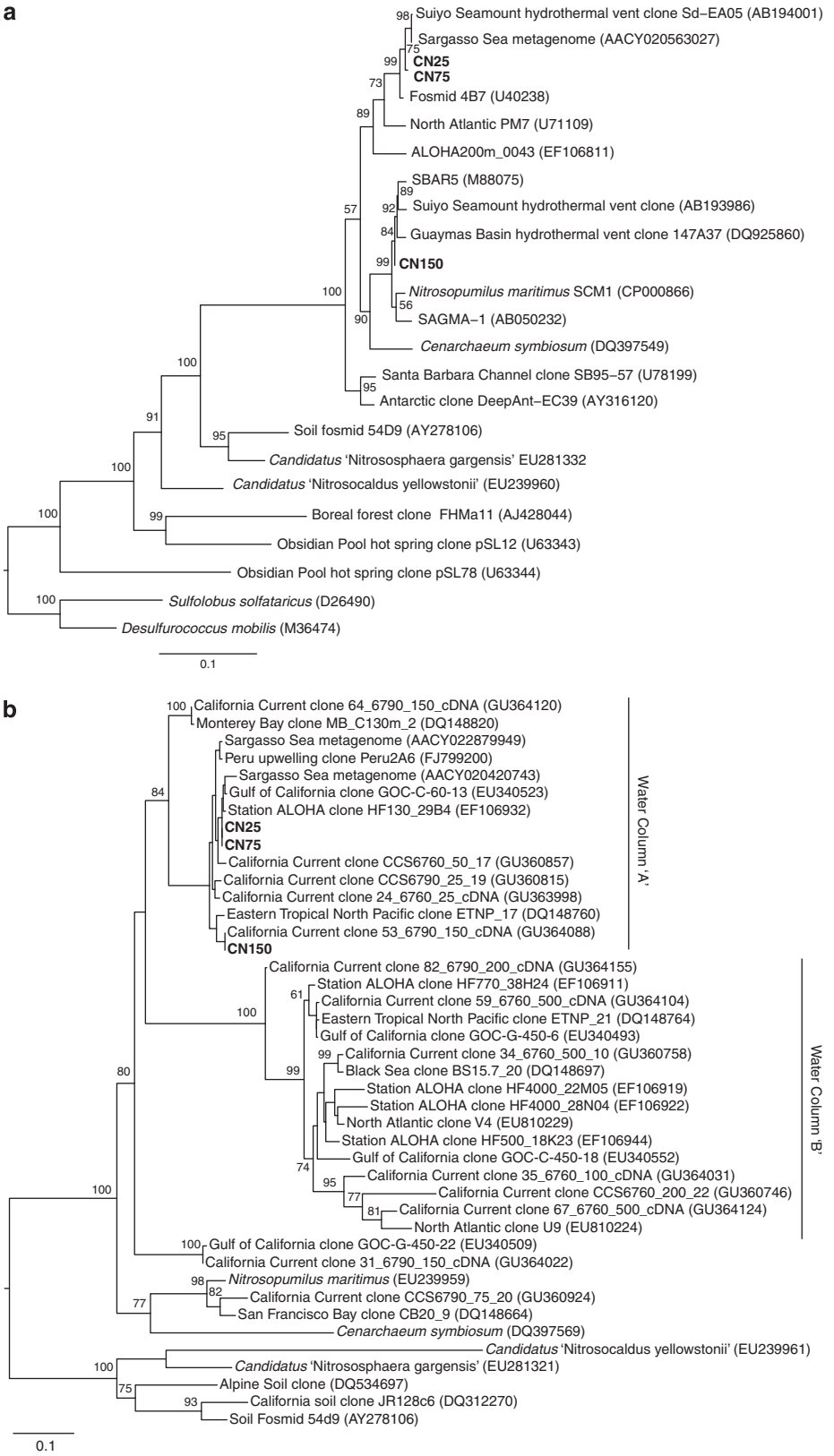

Figure 2 Phylogenetic placement of the CN enrichments based on (**a**) 16S rRNA gene sequences (1268 nucleotide positions) and (**b**) *amoA* gene sequences (489 nucleic acid positions). The CN enrichment sequences are shown in bold. Trees were constructed using maximum likelihood methods (RAxML) using *Sulfolobus solfataricus* as the outgroup for the 16S rRNA tree and the soil and hot spring sequences as the outgroup for the *amoA* tree. Bootstrap support values (>70%) are shown at nodes; some values have been removed from minor nodes for clarity. An expanded *amoA* tree with additional sequences from the study environment is included as Supplementary Figure 1.

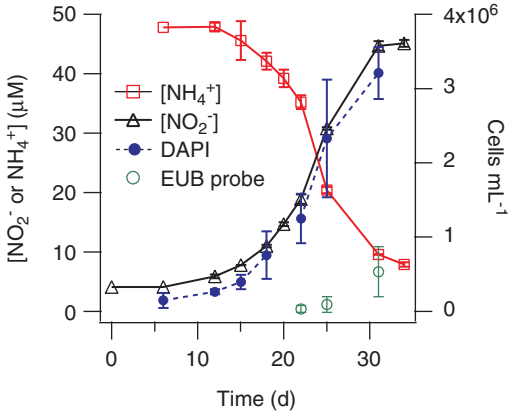

Figure 3 Growth of enrichment CN75 in ONP medium. Error bars denote 1 s.d. and in some cases are smaller than the point. EUB is the CARD-FISH probe suite EUB338 targeting all bacteria.

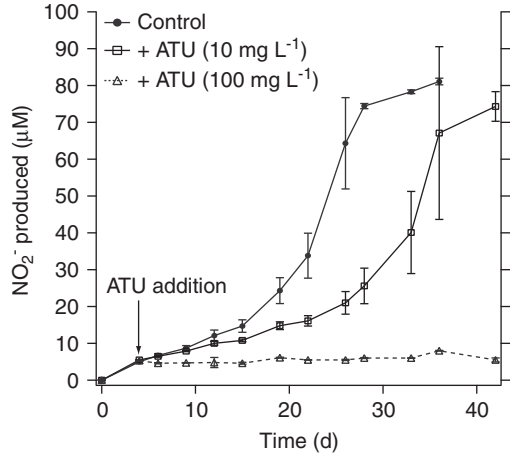

Figure 4 Ammonia oxidation by CN25 in the presence of 10 and 100 mg l^{-1} ATU. Each point represents the mean of three replicate bottles; error bars denote 1 s.d. from the mean.

CN25 and CN75 are 97% identical to *amoA* sequences obtained in DNA-based clone libraries from the environment from which they were enriched (GU360825, (Santoro *et al.*, 2010)) and CN150 is 100% identical to an *amoA* actively expressed in the environment (GU364088). The CN strains are also highly similar on the 16S and *amoA* level to abundant groups of open ocean archaea from a range of oceanic provinces including the Sargasso Sea, Fernandina Island and the coast of Africa as indicated by BLAST searches of the Global Ocean Survey dataset (Rusch *et al.*, 2007). Previous *amoA*-based phylogenies of AOA have shown that water column-derived sequences fall into two clusters termed 'A' and 'B' (Francis *et al.*, 2005) thought to represent a depth-dependent partitioning of AOA (Hallam *et al.*, 2006; Mincer *et al.*, 2007; Beman *et al.*, 2008). The exact physiological basis for the partitioning of archaeal *amoA* genotypes is unknown, but could include adaptations to temperature, light or substrate availability. Based on *amoA* sequences, all three CN strains belong to the shallow water column clade 'A'. Our data now associate a 16S rRNA genotype with at least some members of the clade 'A' archaea that are distinct from *N. maritimus* and are likely to represent a new genus within the Thaumarchaea.

Physiology

The growth rate of CN25 and CN75 (0.17 day^{-1}) in ONP medium is slower than growth rates reported for the cultivated AOA *N. maritimus* (0.65 day^{-1} at 30 °C, (Martens-Habbena *et al.*, 2009) and *N. yellowstonii* (0.8 day^{-1} at 72 °C, (de la Torre *et al.*, 2008) and cultivated marine ammonia-oxidizing bacteria (AOB) (0.34–0.77 day^{-1}, (Prosser, 1990). These differences can be partially explained by the lower cultivation temperature of the CN enrichments (22 °C) relative to *N. maritimus* and *N. yellowstonii*. Further optimization of the culture medium may lead to increases in the growth rates reported here. The per-cell ammonia oxidation rate for the CN strains, ~2 fmol NO_2^- $cell^{-1}$ day^{-1} is lower than *N. maritimus* (13 fmol $cell^{-1}$ day^{-1}, (Martens-Habbena *et al.*, 2009)), but similar to a mesocosm enrichment of archaea from North Atlantic (2–4 fmol $cell^{-1}$ day^{-1})(Wuchter *et al.*, 2006), and estimates of *in situ* per-cell activity rates in the California Current (0.2–15 fmol $cell^{-1}$ day^{-1}) (Santoro *et al.*, 2010).

Multiple isolation strategies, including the addition of antibiotics and size fractionation through 0.45 μm filters, did not yield pure cultures of any of the CN strains. Dilution-to-extinction approaches were also not successful, as high dilutions of the CN strains did not grow (data not shown). *N. yellowstonii* and *N. gargensis*, the two cultivated thermophilic strains of AOA, also could not be established in pure culture (Hatzenpichler *et al.*, 2008; de la Torre *et al.*, 2008). This may be because there is a cooperative relationship between the bacteria and the archaea in these enrichment cultures, as has been reported in cultures of the marine chlorophyte *Prochlorococcus* (Morris *et al.*, 2008), and low cell density dilutions that do not contain the associated heterotrophic bacteria are unable to grow. Interactions between the heterotrophic bacteria and autotrophic archaea in the enrichment, and in the ocean, will be an exciting area of future research.

We observed partial inhibition of archaeal ammonia-oxidizing activity by ATU at 86 μmol l^{-1}, a concentration known to completely inhibit cultivated AOB (Hooper and Terry, 1973) and bacteria-rich environmental samples (Ginestet *et al.*, 1998). Near complete inhibition of ammonia oxidation in CN25 was observed at 860 μmol l^{-1} ATU. The mechanism of ATU inhibition in AOB is thought to be chelation of the Cu active site in the Amo protein (Bedard and Knowles, 1989). Our results are consistent with observations of *N. gargensis* (Hatzenpichler *et al.*, 2008) and support the idea that ATU-inhibited nitrification rates may represent conservative estimates of AOA-only activity in the field.

Table 2 Experimental results for kinetic isotope effect ($^{15}\varepsilon_{NH3}$) determinations during of archaeal ammonia oxidation

Enrichment	*Experiment #*	$^{15}\varepsilon_{NH3}$ (‰)	*CI 95%* (‰)	*Initial* $[NH_4^+]$ ($\mu mol\,l^{-1}$)	$[N+N]_{final}$: $[N+N]_{initial}$	NH_4^+ *oxidation rate* ($\mu mol\,l^{-1}\,day^{-1}$)[a]
CN25	1	25	1	15	1.05	3.0
		28	28	15	1.05	2.5
		30	83	15	1.05	2.1
	2	25	52	9	0.99	0.4
	3	26	21	16	0.74	0.8
		14	4	16	0.98	0.9
		15	3	14	1.07	0.4
	4	20	7	76	1.03	4.5
		21	6	77	1.02	4.5
		20	4	77	1.02	4.5
		20	4	76	0.97	4.4
	Mean ± s.d.	22 ± 5‰				
CN75	1	37	58	10	0.81	0.4
		20	5	10	1.05	0.4
		14	NaN[b]	9	1.07	0.5
	2	23	20	16	0.90	0.8
		19	11	17	0.90	0.9
		10	8	14	1.06	0.2
	Mean ± s.d.	21 ± 10‰				
CN150	1	16	3	25	1.04	0.4
		20	1	25	1.03	0.6
	2	18	4	46	0.94	3.4
		28	17	48	0.88	1.7
	3	23	2	49	0.90	4.1
		28	4	50	0.88	4.5
		22	4	52	0.85	3.7
	Mean ± s.d.	22 ± 5‰				

Abbreviations: CI 95%, 95% confidence interval; [N+N], sum of the measured $[NH_4^+]$ and $[NO_2^-]$.
[a]Approximate NH_4^+ oxidation rates were calculated from the change in $[NO_2^-]$ and the time elapsed between the first and final timepoints, and may underestimate the actual rate in experiments where the final time point was after all NH_4^+ had been consumed.
[b]Calculating a confidence interval was not possible for this experiment because only two points were available to calculate the slope.

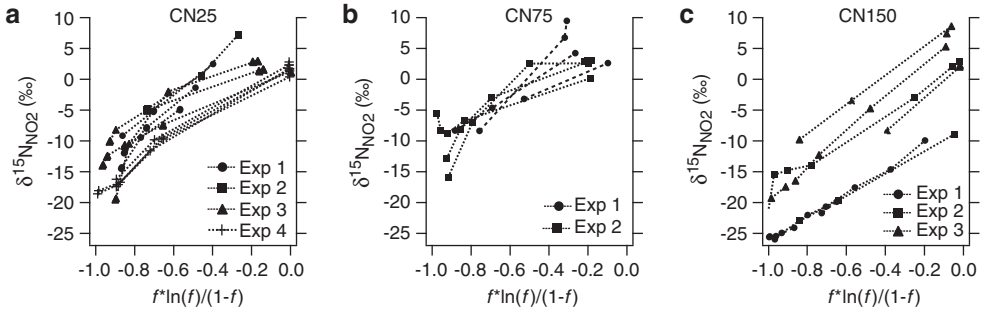

Figure 5 $\delta^{15}N_{NO2}$ during ammonia oxidation in batch culture for the archaeal enrichments (**a**) CN25, (**b**) CN75 and (**c**) CN150. Kinetic isotope effects for ammonia oxidation ($^{15}\varepsilon_{NH3}$) were calculated using linear regression of $\delta^{15}N_{NO2}$ vs $f * \ln(f)/(1-f)$ as described in the text and are reported in Table 2. f is the fraction of the initial $[NH_4^+]$ remaining in the culture.

Further, these results suggest that ATU should not be relied on for complete inhibition of nitrifying activity in environmental samples. For example, nitrification rate measurements in the California Current showed variable (0–90%) but on average low levels of inhibition by ATU (mean 35%, $n = 11$) consistent with other lines of evidence supporting AOA activity in that study (Santoro *et al.*, 2010). On the other hand, Lam *et al.*, 2009 reported complete inhibition of ammonium oxidation in the presence of 86 $\mu mol\,l^{-1}$ ATU in the Eastern Tropical South Pacific oxygen minimum zone. The physiology behind the differential responses of AOA and AOB and different environmental communities to this inhibitor is unknown, but could be the result of different metal active sites for enzymes in the ammonia oxidation pathway between the two groups of organisms or differences in metal active sites or affinities of enzymes, or differences in trace metal availability in different field locations.

Nitrogen isotopic fractionation

Multiple experiments with the three archaeal enrichments suggest a $^{15}\varepsilon_{NH3}$ for archaeal ammonia oxidation of approximately 22%. This value falls

within the reported $^{15}\varepsilon_{NH3}$ for bacterial ammonia oxidation of 14% to 42% (Mariotti *et al.*, 1981; Casciotti *et al.*, 2003, 2010). Thus, despite genome-inferred differences in the ammonia oxidation pathways of AOA and AOB (Walker *et al.*, 2010), a difference in the magnitude of $^{15}\varepsilon_{NH3}$ between these two groups was not apparent.

The physiological reasons for the large range in $^{15}\varepsilon_{NH3}$ for both AOA and AOB are unknown, but could result from changes in growth state of the organisms or violations of assumptions associated with using the Rayleigh accumulated product equation to estimate $^{15}\varepsilon_{NH3}$ from $\delta^{15}N_{NO2}$ measurements. Factors to consider include variations in substrate concentration, ammonia oxidation rate and loss of N to unmeasured pools. Furthermore, unbalanced growth could lead to accumulation of intermediate products in the multi-step oxidation pathway, leading to larger errors involved with characterization of ammonia oxidation as a single-step unidirectional reaction. At high starting [NH_4^+], expressed $^{15}\varepsilon_{NH3}$ values were consistently around 20–22‰, whereas at low starting [NH_4^+], expressed $^{15}\varepsilon_{NH3}$ values were more variable (both higher and lower) and greater uncertainty was involved in their estimation (Table 2). Therefore, no direct relationship can be drawn between [NH_4^+] and $^{15}\varepsilon_{NH3}$. Similarly, there is no direct relationship between ammonia oxidation rate (or oxidation rate/[NH_4^+]) and $^{15}\varepsilon_{NH3}$ (Table 2).

Although dissolved inorganic nitrogen conservation ([$NH_4^+ + NO_2^-$]) was not 100% in all experiments, the majority of experiments had NO_2^- recoveries over 90% (Table 2), which is within the range reported for pure cultures of AOB (Mariotti *et al.*, 1981; Casciotti *et al.*, 2002). There is likely to be some loss of NH_4^+ because of uptake for anabolic metabolism by AOA and the bacteria in the enrichments, but this sink should be small and consistent across experiments. This process would alter observed $^{15}\varepsilon_{NH3}$ values in proportion to the amount of NH_4^+ assimilated and the isotope effect for NH_4^+ assimilation (4–27‰; (Hoch *et al.*, 1992)). However, we emphasize that this effect is likely to be small and note that relatively high estimates of $^{15}\varepsilon_{NH3}$, not low ones, were observed where recovery was low (Table 2). We did not make measurements of [NO_3^-] in the experiments described here, but we have not observed NO_2^- loss over time and have no evidence that NO_2^- is further oxidized to NO_3^- in any of the enrichments.

In addition to variation between experiments, we found evidence for variable $^{15}\varepsilon_{NH3}$ during archaeal ammonia oxidation within some experiments, with the greatest $^{15}\varepsilon_{NH3}$ expressed at the early stages of growth. This was particularly apparent for CN25 and CN75 enrichments grown at low [NH_4^+] ($<20\,\mu M$), which exhibited a considerable lag phase before the commencement of ammonia oxidation. Variable ε values have been observed in many other organisms, including denitrifiers (Granger *et al.*, 2008), methane oxidizers (Templeton *et al.*, 2006), sulfate reducers (Habicht *et al.*, 2005) and nitrite oxidizers (Casciotti, 2009; Buchwald and Casciotti, 2010), and during assimilation of NH_4^+ by heterotrophic bacteria (Hoch *et al.*, 1992) but has not previously been reported in ammonia-oxidizing organisms. We explore two hypotheses that could explain a large apparent isotope effect at the early stages of growth (Figure 6).

The first hypothesis is that, in the early stages of growth in batch culture, diffusion of NH_3 and/or NH_4^+ between the growth medium and the periplasm is rapid relative to the oxidation rate ($R_{diff,NH3}$ or $R_{diff,NH4} > R_{AMO}$) and enzymatic ammonia oxidation is the rate-limiting step. In this scenario, the enzyme-level isotope effect ($^{15}\varepsilon_{AMO}$), plus the equilibrium isotope effect between NH_3 and NH_4^+ ($^{15}\varepsilon_{eq} = 19‰$; (Hermes *et al.*, 1985), should dominate the observed $^{15}\varepsilon_{NH3}$ early in the experiment. In the later stages of growth, the reaction may become diffusion limited ($R_{diff} < R_{AMO}$) and the isotope effect for diffusion ($^{15}\varepsilon_{diff}$) would dominate. A similar mechanism has been examined for variations in expressed isotopic fractionation during NH_4^+ assimilation by bacteria (Hoch *et al.*, 1992) and marine algae (Pennock *et al.*, 1996), as well as CO_2 uptake by algae (Laws *et al.*, 1997) and plants (O'Leary, 1981). Diffusion of NH_3 in aqueous solution is estimated to have an isotope effect of 20‰ (Hoch *et al.*, 1992), which is similar to the late-stage $^{15}\varepsilon_{NH3}$ values we observed. However, this scenario would predict a direct relationship between [NH_4^+] and $^{15}\varepsilon_{NH3}$ in experiments starting at different initial NH_4^+ concentrations, which is not observed in our data (Table 2). If anything, we observe larger $^{15}\varepsilon_{NH3}$ values at low initial [NH_4^+]. Therefore, we reject the hypothesis of diffusion limitation in controlling $^{15}\varepsilon_{NH3}$ in these experiments.

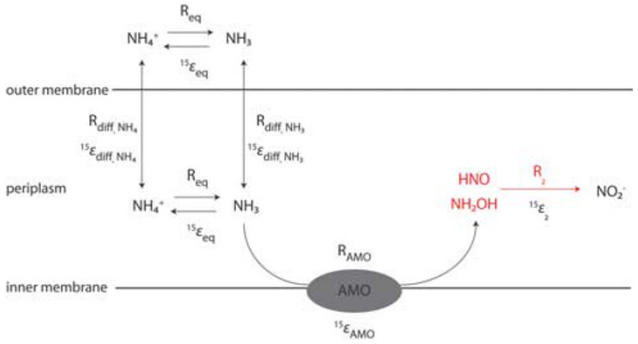

Figure 6 Potential pathways of NH_3 acquisition and oxidation by AOA that could lead to a variable kinetic isotope effect ($^{15}\varepsilon_{NH3}$) (Walker *et al.*, 2010). R_{eq} and $^{15}\varepsilon_{eq}$ are the rate and equilibrium isotope effect for NH_3/NH_4^+ equilibration, $R_{diff,NH4}$ and $^{15}\varepsilon_{diff,NH4}$ are the rate and kinetic isotope effect for NH_4^+ diffusion, $R_{diff,NH3}$ and $^{15}\varepsilon_{diff,NH3}$ are the rate and kinetic isotope effect for NH_3 diffusion, R_{AMO} and $^{15}\varepsilon_{AMO}$ are the rate and kinetic isotope effect for ammonia oxidation by ammonia monooxygenase (Amo), and R_2 and $^{15}\varepsilon_2$ are the rate and kinetic isotope effect for the second step of the ammonia oxidation pathway. Major uncertainties in the archaeal oxidation pathway are shown in red.

It has been assumed here that the Amo-catalyzed reaction occurs in the periplasm, as it does in AOB, and that active transport of NH_3 or NH_4^+ into the periplasm from the medium does not occur. Genes for putative Amt transporters have been identified in the genomes of both AOB (Arp *et al.*, 2007) and AOA (Hallam *et al.*, 2006; Walker *et al.*, 2010; Blainey *et al.*, 2011) and expression of these genes in environmental samples appears proportional to expression of *amo* genes (Stewart *et al.*, 2011). At this time it is uncertain whether Amt supplies N for anabolic metabolism serves a regulatory function (Arp *et al.*, 2007), or supplies substrate to the Amo enzyme. However, if Amt is localized for transport across the cytoplasmic membrane (Andrade *et al.*, 2005), it would be difficult to envision a role for Amt in the $^{15}\varepsilon_{NH3}$ variations observed here.

A second hypothesis to explain the within-experiment variations in $^{15}\varepsilon_{NH3}$ is that during early exponential phase growth, the first step of ammonia oxidation proceeds more quickly than the second step ($R_{AMO} > R_2$) and an as-yet unknown intermediate (NH_2OH or HNO) accumulates. This intermediate pool would be isotopically depleted relative to NH_4^+ outside the cell, and a second isotope effect would be expressed ($^{15}\varepsilon_2$; Figure 6) resulting in the production of NO_2^- with a large apparent isotope effect. If the intermediate pool is eventually used up and ammonia oxidation proceeds as a pseudo one-step reaction, the apparent isotope effect should decrease to $^{15}\varepsilon_{AMO}$ (plus $^{15}\varepsilon_{eq}$) late in the experiment. This hypothesis is consistent with the fact that curvature in the Rayleigh plots was most prominent where long lag phases preceded ammonia oxidation, which may be associated with imbalanced growth early in the experiment. Interestingly, if this hypothesis is correct, the similarity between average $^{15}\varepsilon_{NH3}$ (22‰) and $^{15}\varepsilon_{eq}$ (19‰); (Hermes *et al.*, 1985) may indicate a small kinetic isotope effect for Amo. Use of the Rayleigh distillation equation with measurements of $\delta^{15}N_{NH4}$ would get around the potential problem from accumulation of intermediates, although it would still be affected by NH_3/NH_4^+ equilibration and NH_4^+ transport effects discussed above. At this time there are many additional uncertainties in the archaeal ammonia oxidation pathway (Klotz and Stein, 2008; Walker *et al.*, 2010) including the exact chemical intermediates, the localization of the respiratory enzymes within the cell, and the role of active NH_3/NH_4^+ transport that must be resolved before the mechanisms causing variable $^{15}\varepsilon_{NH3}$ can be fully explained.

The ultimate goal of determining species-level isotope effects is to better interpret natural abundance stable isotope ratios ($\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$) in the environment. The average $\delta^{15}N$ of marine NO_3^- is set by the balance of N_2 fixation and N removal by denitrification and anammox (Brandes and Devol, 2002), although nitrification can affect the partitioning of ^{15}N between dissolved and particulate N (Wankel *et al.*, 2007). The potential for variable $^{15}\varepsilon_{NH3}$ adds an extra level of complexity to the interpretations of $\delta^{15}N_{NO3}$ in the environment.

Nitrification is also the main determinant in setting the average $\delta^{18}O$ of marine NO_3^- (Casciotti *et al.*, 2002; Sigman *et al.*, 2009) and N_2O (Ostrom *et al.*, 2000; Popp *et al.*, 2002). Determining the oxygen isotope effects for archaeal ammonia oxidation will therefore be an important next step in advancing the use of the dual isotope signatures of NO_3^- and N_2O to understand the marine nitrogen cycle.

Conflict of interest

The authors declare no conflict of interest.

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