

Enrichment and characterization of ammonia-oxidizing archaea from the open ocean:

2 Phylogeny, physiology, and stable isotope fractionation

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Running head: Isotope fractionation by ammonia-oxidizing archaea

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Abstract

2 Archaeal genes for ammonia oxidation are widespread in the marine environment, but
direct physiological evidence for ammonia oxidation by marine archaea is limited. We
4 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
6 culture for over three years. Phylogenetic analyses indicate the three strains belong to a
previously identified clade of water column-associated AOA and possess 16S rRNA
8 genes and ammonia monooxygenase subunit a (*amoA*) genes highly similar (98-99%
identity) to those recovered in DNA and cDNA clone libraries from the open ocean. The
10 strains grow in natural seawater-based liquid medium while stoichiometrically converting
ammonium (NH_4^+) to nitrite (NO_2^-). Ammonia oxidation by the enrichments is only
12 partially inhibited by allylthiourea at concentrations known to inhibit cultivated
ammonia-oxidizing bacteria. The three strains were used to determine the nitrogen stable
14 isotope effect ($^{15}\epsilon_{\text{NH}_3}$) during archaeal ammonia oxidation, an important parameter for
interpreting stable isotope ratios in the environment. Archaeal $^{15}\epsilon_{\text{NH}_3}$ ranged from 13-
16 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
18 functional diversity as captured by $^{15}\epsilon_{\text{NH}_3}$ is similar.

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Introduction

2 Mesophilic archaea are ubiquitous and abundant members of diverse marine
environments including coastal waters (Beman *et al.*, 2010; Mincer *et al.*, 2007, and
4 references therein), marine sediments, estuaries (Bernhard *et al.*, 2010; Mosier and
Francis, 2008; Urakawa *et al.*, 2010), stratified basins (Coolen *et al.*, 2007; Lam *et al.*,
6 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*
8 *maritimus*, (Konneke *et al.*, 2005; Martens-Habbena *et al.*, 2009), two thermophilic
archaea, *Nitrosocaldus yellowstonii* and *Nitrososphaera gargensis* (de la Torre *et al.*,
10 2008; Hatzenpichler *et al.*, 2008), and a freshwater archaeon, *Nitrosoarchaeum limnia*
(Blainey *et al.*, 2011) established that at least some of these organisms are
12 chemolithoautotrophic ammonia oxidizers. Several studies coupling gene- and cell-based
quantification of putative ammonia-oxidizing archaea (AOA) to rate measurements
14 (Beman *et al.*, 2008; Santoro *et al.*, 2010; Wuchter *et al.*, 2006) suggest that nitrification
by AOA in the ocean is significant. However, direct demonstration of ammonia-
16 oxidizing activity by the AOA genotypes encountered in the open ocean has not yet been
demonstrated.

18 Ammonia oxidation is the first step of nitrification, a key remineralization
reaction in the sea. Work by Olsen (1981) and Ward *et al.* (1982) using ¹⁵N isotope
20 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
22 nitrification in the euphotic zone is still uncertain. Recent syntheses of nitrification rates
made using isotope tracers (Clark *et al.*, 2008; Yool *et al.*, 2007) suggest that globally,

nitrification may be a significant source of regenerated N for primary production,
2 supplying as much as half of the necessary nitrate. This has important implications for
estimates of carbon export based on new production (Eppley and Peterson, 1979); an
4 underestimation of nutrient regeneration would lead to an overestimation of export from
the surface ocean (Ward, 2002).

6 Though instantaneous rate measurements with ^{15}N -labeled compounds provide
important insights into the environmental controls on nitrification, they capture only a
8 ‘snapshot’ view of this undoubtedly time-varying process. An alternative way of
quantifying the importance of nitrification is using natural abundance stable isotope ratios
10 to infer the relative importance of different nitrogen cycling processes. In particular,
nitrogen and oxygen stable isotope ratios ($\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) in marine nitrate (NO_3^-) can
12 integrate information about the relative sources (nitrification, upward diffusion,
atmospheric deposition) and sinks (phytoplankton uptake) of NO_3^- in the euphotic zone
14 (Casciotti *et al.*, 2008; DiFiore *et al.*, 2009; Sigman *et al.*, 2005; Wankel *et al.*, 2007) and
provide an independent constraint on the importance of nitrification to new production.
16 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
18 phytoplankton (Granger *et al.*, 2004; Needoba *et al.*, 2003) and NO_2^- production by
nitrifying bacteria (Casciotti *et al.*, 2010; Casciotti *et al.*, 2003; Mariotti *et al.*, 1981) are
20 relatively well studied, but there are no data on the isotope effects for ammonia oxidation
by AOA.

22 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

NO₂⁻. We used the three strains to estimate the nitrogen kinetic isotope effect (¹⁵ε_{NH3})
2 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
4 which we have both phylogeny and confirmed metabolic function. We provide important
constraints for the interpretation of natural abundance stable isotope ratios for these
6 ubiquitous organisms in the marine nitrogen cycle.

8 **Materials and Methods**

Enrichment and cultivation. Enrichment cultures were initiated with water from the North
10 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*
12 during cruise CN107 in July 2007 (Table 1). Seawater was collected from 25 m, 75 m,
150 m, and 500 m depths using a standard 10 L Niskin rosette sampler equipped with a
14 conductivity-temperature-depth sensor package. Seawater was stored at 22°C (25 m and
75 m waters) and 4°C (150 m and 500 m waters) in 500 mL acid-cleaned polycarbonate
16 bottles. After 20 months of incubation, filter sterile ammonium chloride (NH₄Cl) was
added to a final concentration of 10 μmol L⁻¹ and the enrichments were monitored for
18 production of NO₂⁻ using standard colorimetric methods (Strickland and Parsons, 1968).
At that time, the 150 m enrichment was moved to 13°C and maintained at that
20 temperature. Hereafter, the successful enrichments are referred to as CN25, CN75, and
CN150 referring to the cruise name and depth of the enrichment inoculums.

22 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 $\mu\text{mol L}^{-1}$ NH_4Cl , 1 mL L^{-1} chelated
2 trace elements solution (Balch *et al.*, 1979), 15 $\mu\text{mol L}^{-1}$ KH_2PO_4 and 100 $\mu\text{g mL}^{-1}$ each
streptomycin and ampicillin. Maintenance cultures were grown in 200 mL volumes in
4 250 mL acid-cleaned polycarbonate bottles. Surface seawater for ONP medium was
obtained either from the initial collection site or from the Pacific hydrographic station
6 SAFe (Johnson *et al.*, 2007).

Abundance of archaeal and bacterial cells in the enrichments was periodically
8 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
10 catalyzed auto reporter deposition-fluorescent in situ hybridization (CARD-FISH) and
epifluorescence microscopy using probes Cren537-554 and EUB338 I-III (Teira *et al.*,
12 2004).

Phylogenetic analysis of 16S rRNA and amoA genes. DNA was extracted and purified
14 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
16 (Qiagen, Valencia, CA, USA) as previously described (Santoro *et al.*, 2008). Genes
encoding for the 16S rRNA and the α subunit of ammonia monooxygenase (*amoA*),
18 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
20 the primers: 21F/1492R (archaeal 16S rRNA genes, DeLong (1992)),
ArchamoAF/ArchamoAR (archaeal *amoA*, Francis *et al.* 2005), 27F/1492R (bacterial 16S
22 rRNA), and amoAF*/amoAR (betaproteobacterial *amoA*, Stephen *et al.* (1999)) using
reaction and thermocycling conditions described in the original manuscripts or as

modified in Santoro *et al.* (2010). Gammaproteobacterial *amoA* genes were amplified
2 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 sec, 50°C
4 for 45 sec, 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,
6 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
8 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
10 enrichment.

16S rRNA gene sequences were imported into the ARB software program
12 (Ludwig *et al.*, 2004) and aligned to a subset of the SILVA SSU reference database, Feb
2009 release (Pruesse *et al.*, 2007). Archaeal *amoA* sequences were manually aligned and
14 imported into an ARB database maintained by our laboratory containing approximately
2000 environmental *amoA* sequences. Phylogenetic trees were constructed using 1,268
16 nucleotide positions (16S rRNA) or 489 nucleotide positions (*amoA*) using maximum
likelihood (RAxML) analyses implemented using the Cyberinfrastructure for
18 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
20 *amoA* sequences from this study were deposited in GenBank under the accession
numbers HQ338108-HQ338109 and JF521547-JF521549.

22 *Growth curves.* Growth curves were determined for CN25 and CN75 at 22°C in replicate
200 mL volumes of ONP medium containing 50 $\mu\text{mol L}^{-1}$ NH_4^+ . At each time point, 15

mL of culture was removed. Ten milliliters of culture were frozen for later $[\text{NO}_2^-]$ and
2 $[\text{NH}_4^+]$ 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)
4 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
6 above. A minimum of ten fields of view were counted from each filter using the 100X
objective on a Zeiss Axio Scope epifluorescence microscope. $[\text{NH}_4^+]$ and $[\text{NO}_2^-]$
8 determinations were made in duplicate using phthaldialdehyde fluorescence (Holmes *et*
al., 1999) and azo dye colorimetry (Strickland and Parsons, 1968), respectively.

10 *Allylthiourea inhibition experiments.* To assess the effect of the metal chelator
allylthiourea (ATU) on archaeal ammonia oxidation, strain CN25 was grown in triplicate
12 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 $\mu\text{mol L}^{-1}$), and 100 mg L^{-1} (860 $\mu\text{mol L}^{-1}$).
14 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^- ,
16 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
18 flask (day 4). Inhibition for the ATU treatments was calculated as the percent decrease in
slope of a linear regression fit to the $\ln[\text{NO}_2^-]$ over time compared with the control
20 bottles.

Nitrogen isotope effect estimates. For each experiment, 25 mL of enrichment culture was
22 inoculated into 175 mL of ONP medium containing 10-25 $\mu\text{mol L}^{-1}$ 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
2 shown), thus all experiments started with an initial $[\text{NO}_2^-]$ between 2 and $10 \mu\text{mol L}^{-1}$ as
well as residual NH_4^+ from maintenance cultures, in some cases. Temperature was
4 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
6 -20°C .

Initial and time-course measurements of $[\text{NH}_4^+]$ and $[\text{NO}_2^-]$ were made in
8 duplicate using phthaldialdehyde fluorescence (Holmes *et al.*, 1999) and azo dye
colorimetry (Strickland and Parsons, 1968), respectively, on 2 mL sample volumes.
10 Error in replicate $[\text{NH}_4^+]$ analyses ranged from 2-7%; error in replicate $[\text{NO}_2^-]$ analyses
was 0-8%. Isotopic measurements of NO_2^- were made by converting NO_2^- to N_2O using
12 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
14 (Casciotti *et al.*, 2002; McIlvin and Casciotti, 2010; McIlvin and Casciotti, 2011) and
analyzed using a Finnigan Delta^{PLUS} XP isotope ratio mass spectrometer. Each sample
16 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:
18 $\delta^{15}\text{N}_{\text{NO}_2}$ (‰ vs. AIR) = $[(^{15}\text{R}_{\text{NO}_2} / ^{15}\text{R}_{\text{AIR}}) - 1] \times 1000$, where $^{15}\text{R} = ^{15}\text{N}:^{14}\text{N}$ and AIR is
standard atmospheric N_2 . Error in replicate measurements of $\delta^{15}\text{N}_{\text{NO}_2}$ was 0.3‰ or better
20 for all experiments.

Calculation of $^{15}\epsilon_{\text{NH}_3}$ requires knowledge of the isotopic composition of the NO_2^-
22 produced over time since an arbitrary starting point. If the initial $[\text{NO}_2^-]$ is zero, then the
measured $\delta^{15}\text{N}_{\text{NO}_2}$ ($\delta^{15}\text{N}_{\text{NO}_2\text{total}}$) is equal to that produced ($\delta^{15}\text{N}_{\text{NO}_2\text{produced}}$). If there is

NO₂⁻ initially present, as in our experiments, the $\delta^{15}\text{N}_{\text{NO}_2\text{produced}}$ at any time since the

2 initial time point (*i*) must be calculated from $\delta^{15}\text{N}_{\text{NO}_2\text{total}}$ using the following equations:

$$(^{15}\text{N}:^{14}\text{N})_{\text{total}} * [\text{NO}_2^-]_{\text{total}} = (^{15}\text{N}:^{14}\text{N})_{\text{produced}} * [\text{NO}_2^-]_{\text{produced}} + (^{15}\text{N}:^{14}\text{N})_i * [\text{NO}_2^-]_i \quad \text{Eq (1a)}$$

4 where $(^{15}\text{N}:^{14}\text{N})_{\text{total}} = (^{15}\text{N}:^{14}\text{N})_{\text{AIR}} * (\delta^{15}\text{N}_{\text{NO}_2\text{total}}/1000 + 1)$

Rearranging:

$$6 \quad (^{15}\text{N}:^{14}\text{N})_{\text{produced}} = \{ (^{15}\text{N}:^{14}\text{N})_{\text{total}} * [\text{NO}_2^-]_{\text{total}} - (^{15}\text{N}:^{14}\text{N})_i * [\text{NO}_2^-]_i \} / [\text{NO}_2^-]_{\text{produced}} \quad \text{Eq}$$

(1b)

$$8 \quad \delta^{15}\text{N}_{\text{NO}_2\text{produced}} = [(^{15}\text{N}:^{14}\text{N})_{\text{produced}} / (^{15}\text{N}:^{14}\text{N})_{\text{AIR}} - 1] * 1000 \quad \text{Eq (2)}$$

The isotope effect ($^{15}\epsilon_{\text{NH}_3}$) was then calculated from the $\delta^{15}\text{N}_{\text{NO}_2\text{produced}}$ data using the

10 Rayleigh accumulated product equation (after Mariotti *et al.*, 1981):

$$\delta^{15}\text{N}_{\text{NO}_2\text{produced}} = \delta^{15}\text{N}_{\text{NH}_4\text{initial}} + ^{15}\epsilon_{\text{NH}_3} \left(\frac{f \ln(f)}{1-f} \right) \quad \text{Eq (3)}$$

12 where $f = [\text{NH}_4^+] / [\text{NH}_4^+]_{\text{initial}}$. By Eq (3), the slope of a best fit line on a plot of $\delta^{15}\text{N}_{\text{NO}_2}$
vs. $f * \ln(f) / (1-f)$ yields $^{15}\epsilon_{\text{NH}_3}$ and the y-intercept corresponds to the starting $\delta^{15}\text{N}_{\text{NH}_4}$.

14 Because each experiment was initiated with a mixture of NH₄⁺ carried over from the
enrichment transfer and ‘new’ NH₄⁺ from freshly prepared medium ($\delta^{15}\text{N} \sim -3\%$), minor
16 variations in the $\delta^{15}\text{N}_{\text{NH}_4}$ (and the y-intercept) are expected between experiments and
therefore each experiment was fitted separately. Fitting and 95% confidence interval
18 calculations were done using IGOR Pro software (v5, WaveMetrics, Inc., Lake Oswego,
OR, USA).

20 Use of the Rayleigh model presumes that ammonia oxidation proceeds as a
pseudo one-step reaction with no accumulation of an intermediate product between NH₃
22 and NO₂⁻ and no back-reaction of the products (Casciotti *et al.*, 2003). When estimated

in this way, several isotope effects are incorporated into $^{15}\epsilon_{\text{NH}_3}$ including a ~20-35%
2 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 $^{15}\epsilon_{\text{NH}_3}$ resulting from these
4 assumptions, as well as errors resulting from uncertainties in the archaeal ammonia
oxidation pathway, are discussed below.

6

Results

8 *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
10 ‘enrichment’ bottles (25 m, 75 m, 150 m, and 500 m). On addition of $10 \mu\text{mol L}^{-1} \text{NH}_4^+$,
 NO_2^- production was observed in the enrichments from 25 m, 75 m, and 150 m within
12 one 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
14 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
16 either gamma or betaproteobacteria were detected.

After approximately one year of routine transfers into ONP medium (nearly three
18 years after initial collection), the enrichments were highly enriched in archaeal cells (Fig.
1) and the basic phylogeny and physiology of the enrichments were characterized. A
20 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
22 nucleotide changes (all at different positions) from this consensus sequence.

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.*,
2 2008; Spang *et al.*, 2010; Walker *et al.*, 2010)(Fig. 2a). The 16S rRNA gene sequences
from CN25 and CN75 are 100% identical to each other, and 92% identical to *N.*
4 *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
6 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
8 rRNA gene is 98% identical to *N. maritimus* and 99% identical to several sequences from
the Sargasso Sea metagenome (AACY020033564, (Venter *et al.*, 2004)).

10 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
12 the amino acid level to several sequences from the Sargasso Sea metagenome (Venter *et*
al., 2004)(Fig. 2b) and 84% and 95% identical at the nucleotide and amino acid levels,
14 respectively, to the *amoA* sequence from *N. maritimus*. The CN150 *amoA* gene is also
84% identical to *N. maritimus* at the nucleic acid level. Because CN25 and CN75 are
16 identical at the 16S rRNA and *amoA* level, full genome sequencing may be necessary to
resolve any strain-level differences between the two enrichments.

18 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
20 associated with the genera *Erythrobacter* and *Gracilimonas*. Bacterial *amoA* genes could
not be detected using PCR amplification.

22 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 (Fig. 3). All three CN strains were able
2 to utilize NH_4^+ from the medium below our detection limit of ~200 nM. Bacterial cells,
as quantified by CARD-FISH, were not detectable until the archaeal population entered
4 late exponential-early stationary phase. CN25 and CN75, growing at 22°C, had lag phases
of 4-10 days upon transferring 10 – 25% of the original culture volume into new medium,
6 even when cultures were transferred during exponential phase growth. Transfer volumes
of less than 10% failed to grow (data not shown). CN150, growing at 13°C, had lag
8 phases of up to 25 days.

CN25 and CN75 were chosen for more detailed growth studies due to faster NH_4^+
10 oxidation rates, shorter lag phases, and more consistent growth. The CN25 growth rate at
22°C was 0.15 d^{-1} ; the CN75 growth rate was 0.17 d^{-1} , corresponding to doubling times of
12 about 4-4.6 d. 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
14 on the growth stage of the enrichment.

Allythiourea (ATU) is frequently used to inhibit nitrifying activity in
16 environmental studies. We tested the effect of ATU on the ammonia oxidation rate for
CN25. An ATU concentration of 10 mg L^{-1} ($86 \mu\text{mol L}^{-1}$) inhibited the ammonia
18 oxidation rate by 58%; an ATU concentration of 100 mg L^{-1} ($860 \mu\text{mol L}^{-1}$) completely
inhibited ammonia oxidation (Fig. 4).

20

Nitrogen isotopic fractionation. To further investigate the physiology of the enriched
22 strains, we determined their N kinetic isotope effect during ammonia oxidation ($^{15}\epsilon_{\text{NH}_3}$).
As in growth curve experiments, we observed near stoichiometric conversion of NH_4^+ to

NO₂⁻ over the course of the isotope fractionation experiments, with conservation in the
2 DIN pool (NH₄⁺ + NO₂⁻) ranging from 74% to 107% with a mean of 97% (Table 2).

Results from replicate experiments are reported individually, as well as an
4 unweighted average of experiments for a given enrichment (Table 2). ¹⁵ε_{NH3} for CN25
ranged from 14-30‰ with an average of 22 ± 5‰ (n = 11), ¹⁵ε_{NH3} for CN75 ranged from
6 10-37‰ with an average of 21 ± 10‰ (n = 6), and ¹⁵ε_{NH3} for CN150 ranged from 16-
28‰ with an average of 22 ± 5‰ (n = 7). Non-linearity was observed in most
8 experiments with CN25 and CN75, with larger ¹⁵ε_{NH3} (i.e. greater slopes) at the
beginning of the growth curve when a large fraction (f) of the initial NH₄⁺ remained (Fig.
10 5a,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
12 the same non-linearity of ¹⁵ε_{NH3} in experiments with CN150 (Fig. 5c).

14 **Discussion**

Phylogeny. We established three enrichments of marine archaea that stoichiometrically
16 oxidize NH₄⁺ to NO₂⁻. Multiple lines of evidence suggest that the archaea are the active
ammonia oxidizers in the enrichments. Archaeal cells increase exponentially
18 concomitant with an exponential increase in NO₂⁻ and *amoA* genes from the enrichments
have a high identity to known archaeal ammonia oxidizers. The enrichments lack 16S
20 rRNA and *amoA* genes associated with known γ- or β-proteobacterial ammonia-
oxidizers. Though no NO₂⁻ or NO₃⁻ production was observed in the enrichment from 500
22 m, this cannot be used to infer a lack of NH₃-oxidizing ability in field populations at this
depth as active NH₃ oxidation was measured in situ (Santoro *et al.*, 2010).

Both 16S rRNA and *amoA* gene sequences from the CN strains suggest they are
2 representative of organisms present and active in the open ocean. CN25 and CN75 are
97% identical to *amoA* sequences obtained in DNA-based clone libraries from the
4 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).
6 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,
8 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
10 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
12 (Beman *et al.*, 2008; Hallam *et al.*, 2006; Mincer *et al.*, 2007). The exact physiological
basis for the partitioning of archaeal *amoA* genotypes is unknown but could include
14 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
16 16S rRNA genotype with at least some members of the clade ‘A’ archaea distinct from *N.*
maritimus that are likely to represent a new genus within the Thaumarchaea.

18

Physiology. The growth rate of CN25 and CN75 (0.17 d^{-1}) in ONP medium is slower
20 than growth rates reported for the cultivated AOA *N. maritimus* (0.65 d^{-1} at 30°C ,
(Martens-Habbena *et al.*, 2009) and *N. yellowstonii* (0.8 d^{-1} at 72°C , (de la Torre *et al.*,
22 2008) and cultivated marine AOB ($0.34 - 0.77 \text{ d}^{-1}$, (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
2 may lead to increases in the growth rates reported here. The per-cell activity rate for the
CN strains, $\sim 2 \text{ fmol NO}_2^- \text{ cell}^{-1} \text{ d}^{-1}$, is lower than *N. maritimus* ($13 \text{ fmol cell}^{-1} \text{ d}^{-1}$,
4 (Martens-Habbena *et al.*, 2009)), but similar to a mesocosm enrichment of archaea from
North Atlantic ($2\text{-}4 \text{ fmol cell}^{-1} \text{ d}^{-1}$)(Wuchter *et al.*, 2006), and estimates of *in situ* per-cell
6 activity rates in the California Current ($0.2\text{-}15 \text{ fmol cell}^{-1} \text{ d}^{-1}$) (Santoro *et al.*, 2010).

Multiple isolation strategies, including the addition of antibiotics and size
8 fractionation through $0.45 \mu\text{m}$ 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
10 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 (de
12 la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008). This may be because there is a
cooperative relationship between the bacteria and the archaea in these enrichment
14 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
16 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
18 future research.

We observed partial inhibition of archaeal ammonia-oxidizing activity by ATU at
20 $86 \mu\text{mol L}^{-1}$, a concentration known to completely inhibit cultivated AOB (Hooper and
Terry, 1973) and bacterial-rich environmental samples (Ginestet *et al.*, 1998), and near
22 complete inhibition of ammonia oxidation in CN25 was observed at $860 \mu\text{mol L}^{-1}$ 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
2 observations of *N. gargensis* (Hatzenpichler *et al.*, 2008) and support the idea that ATU-
inhibited rates may represent conservative estimates of AOA-only activity in the field.
4 Further, these results suggest that ATU should not be relied on for complete inhibition of
nitrifying activity in environmental samples. For example, nitrification rate
6 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
8 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
10 $\mu\text{mol L}^{-1}$ ATU in the Eastern Tropical South Pacific oxygen minimum zone. The
physiology behind the differential response of AOA and AOB and different
12 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
14 two groups of organisms or differences in trace metal availability or trace metal affinity
in different field locations.

16

Nitrogen isotopic fractionation. Multiple experiments with the three archaeal enrichments
18 suggest a $^{15}\epsilon_{\text{NH}_3}$ for archaeal ammonia oxidation of approximately 22‰. This value falls
within the reported $^{15}\epsilon_{\text{NH}_3}$ for bacterial ammonia oxidation of 14 to 42‰ (Casciotti *et al.*,
20 2010; Casciotti *et al.*, 2003; Mariotti *et al.*, 1981). Thus, despite genome-inferred
differences in the ammonia oxidation pathways of AOA and AOB (Walker *et al.*, 2010),
22 a difference in the magnitude of $^{15}\epsilon_{\text{NH}_3}$ between these two groups was not apparent.

The physiological reasons for the large range in $^{15}\epsilon_{\text{NH}_3}$ for both AOA and AOB
2 are unknown, but could result from changes in growth state of the organisms or violations
of assumptions associated with using the accumulated product equation to estimate $^{15}\epsilon_{\text{NH}_3}$
4 from $\delta^{15}\text{N}_{\text{NO}_2}$ measurements. Factors to consider include variations in substrate
concentration, ammonia oxidation rate, and loss of N to unmeasured pools. Furthermore,
6 unbalanced growth could lead to accumulation of intermediate products in the multi-step
oxidation pathway, leading to larger errors involved with characterization of ammonia
8 oxidation as a single-step unidirectional reaction. At high starting $[\text{NH}_4^+]$, expressed
 $^{15}\epsilon_{\text{NH}_3}$ values were consistently around 20-22‰, whereas at low starting $[\text{NH}_4^+]$,
10 expressed $^{15}\epsilon_{\text{NH}_3}$ values were more variable (both higher and lower) and greater
uncertainty was involved in their estimation (Table 2). Therefore, no direct relationship
12 can be drawn between $[\text{NH}_4^+]$ and $^{15}\epsilon_{\text{NH}_3}$. Likewise, there is no direct relationship
between ammonia oxidation rate (or oxidation rate/ $[\text{NH}_4^+]$) and $^{15}\epsilon_{\text{NH}_3}$ (Table 2).

14 Though DIN conservation ($[\text{NH}_4^+ + \text{NO}_2^-]$) was not 100% in all experiments, the
majority of experiments had NO_2^- recoveries over 90% (Table 2), which is within the
16 range reported for pure cultures of AOB (Casciotti *et al.*, 2002; Mariotti *et al.*, 1981).
There is likely to be some loss of NH_4^+ due to uptake for anabolic metabolism by AOA
18 and the bacteria in the enrichments, but this sink should be small and consistent across
experiments. This process could alter observed $^{15}\epsilon_{\text{NH}_3}$ values in proportion to the amount
20 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
22 relatively high estimates of $^{15}\epsilon_{\text{NH}_3}$, not low ones, were observed where recovery was low
(Table 2). We did not make measurements of $[\text{NO}_3^-]$ in the experiments described here,

but we have not observed NO_2^- loss over time and have no evidence that NO_2^- is further
2 oxidized to NO_3^- in any of the enrichments.

In addition to variation between experiments, we found evidence for variable
4 $^{15}\epsilon_{\text{NH}_3}$ during archaeal ammonia oxidation within some experiments, with the greatest
 $^{15}\epsilon_{\text{NH}_3}$ expressed at the early stages of growth. This was particularly apparent for CN25
6 and CN75 grown at low $[\text{NH}_4^+]$ ($<20 \mu\text{M}$), which exhibited a considerable lag phase prior
to the commencement of ammonia oxidation. Variable ϵ values have been observed in
8 many other organisms, including denitrifiers (Granger *et al.*, 2008), methane oxidizers
(Templeton *et al.*, 2006), sulfate reducers (Habicht *et al.*, 2005) and nitrite oxidizers
10 (Buchwald and Casciotti, 2010; Casciotti, 2009), and during assimilation of NH_4^+ by
heterotrophic bacteria (Hoch *et al.*, 1992) but has not previously been reported in
12 ammonia-oxidizing organisms. We explore two hypotheses that could explain a large
apparent isotope effect at the early stages of growth (Fig. 6).

14 The first hypothesis is that, in the early stages of growth in batch culture,
diffusion of NH_3 and/or NH_4^+ between the periplasm and the growth medium is rapid
16 relative to the oxidation rate ($R_{\text{diff},\text{NH}_3}$ or $R_{\text{diff},\text{NH}_4} > R_{\text{AMO}}$) and enzymatic ammonia
oxidation is the rate-limiting step. In this scenario, the enzyme-level isotope effect
18 ($^{15}\epsilon_{\text{AMO}}$), plus the equilibrium isotope effect between NH_3 and NH_4^+ ($^{15}\epsilon_{\text{eq}} = 19\text{‰}$;
(Hermes *et al.*, 1985), should dominate the observed $^{15}\epsilon_{\text{NH}_3}$ early in the experiment. In the
20 later stages of growth, the reaction may become diffusion limited ($R_{\text{diff}} < R_{\text{AMO}}$) and the
isotope effect for diffusion ($^{15}\epsilon_{\text{diff}}$) would dominate. A similar mechanism has been
22 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 NH₃ in
2 aqueous solution is estimated to have an isotope effect of 20‰ (Hoch *et al.*, 1992), which
is similar to the late-stage ¹⁵ε<sub>NH₃ values we observed. However, this scenario would
4 predict a direct relationship between [NH₄⁺] and ¹⁵ε<sub>NH₃ in experiments starting at
different initial NH₄⁺ concentrations, which is not observed in our data (Table 2). If
6 anything, we observe larger ¹⁵ε<sub>NH₃ values at low initial [NH₄⁺]. Therefore, we reject the
hypothesis of diffusion limitation in controlling ¹⁵ε_{NH₃ in these experiments.}</sub></sub></sub>

8 It has been assumed here that the Amo-catalyzed reaction occurs in the periplasm,
as it does in AOB, and that active transport of NH₃ or NH₄⁺ into the periplasm from the
10 medium does not occur. Genes for putative Amt transporters have been identified in the
genomes of both AOB (Arp *et al.*, 2007) and AOA (Blainey *et al.*, 2011; Hallam *et al.*,
12 2006; Walker *et al.*, 2010) and expression of these genes in environmental samples
appears proportional to expression of *amo* genes (Stewart *et al.*, 2011). At this time it is
14 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
16 localized for transport across the cytoplasmic membrane (Andrade *et al.*, 2005), it would
be difficult to envision a role for Amt in the ¹⁵ε_{NH₃ variations observed here.}

18 A second hypothesis to explain the within-experiment variations in ¹⁵ε<sub>NH₃ is that
during early exponential phase growth, the first step of ammonia oxidation proceeds
20 more quickly than the second step (R_{AMO} > R₂) and an as-yet unknown intermediate
(NH₂OH or HNO) accumulates. This intermediate pool would be isotopically depleted
22 relative to NH₄⁺ outside the cell, and a second isotope effect would be expressed (¹⁵ε₂;
Figure 6) resulting in the production of NO₂⁻ with a large apparent isotope effect. If the</sub>

intermediate pool is eventually used up and ammonia oxidation proceeds as a pseudo
2 one-step reaction, the apparent isotope effect should decrease to $^{15}\epsilon_{\text{AMO}}$ (plus $^{15}\epsilon_{\text{eq}}$) late in
the experiment. This hypothesis is consistent with the fact that curvature in the Rayleigh
4 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
6 hypothesis is correct, the similarity between average $^{15}\epsilon_{\text{NH}_3}$ (22‰) and $^{15}\epsilon_{\text{eq}}$ (19‰);
(Hermes *et al.*, 1985)) may indicate a small kinetic isotope effect for Amo. Use of the
8 Rayleigh distillation equation with measurements of $\delta^{15}\text{N}_{\text{NH}_4}$ would get around the
potential problem from accumulation of intermediates, although it would still be affected
10 by $\text{NH}_3/\text{NH}_4^+$ equilibration and NH_4^+ transport effects discussed above. At this time there
are many additional uncertainties in the archaeal ammonia oxidation pathway (Klotz and
12 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 $\text{NH}_3/\text{NH}_4^+$
14 transport that must be resolved before the mechanisms causing variable $^{15}\epsilon_{\text{NH}_3}$ can be
fully explained.

16 The ultimate goal of determining species-level isotope effects is to better interpret
natural abundance stable isotope ratios ($\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{NO}_3}$) in the environment. The
18 average $\delta^{15}\text{N}$ 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
20 the partitioning of ^{15}N between dissolved and particulate N (Wankel *et al.*, 2007). The
potential for variable $^{15}\epsilon_{\text{NH}_3}$ adds an extra level of complexity to the interpretations of
22 $\delta^{15}\text{N}_{\text{NO}_3}$ in the environment.

Nitrification is also the main determinant in setting the average $\delta^{18}\text{O}$ of marine
2 NO_3^- (Casciotti *et al.*, 2002; Sigman *et al.*, 2009) and nitrous oxide (N_2O) (Ostrom *et al.*,
2000; Popp *et al.*, 2002). Determining the oxygen isotope effects for archaeal ammonia
4 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.

6

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18

Figure Legends

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

Figure 2. Phylogenetic placement of the CN enrichments based on (a) 16S rRNA gene
6 sequences (1,268 nucleotide positions) and (b) *amoA* gene sequences (489 nucleic acid
positions). The CN enrichment sequences are shown in bold. Trees were constructed
8 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
10 the *amoA* tree. Bootstrap support values (>70%) are shown at nodes; some values have
been removed from minor nodes for clarity.

12 Figure 3. Growth of enrichment CN75 in ONP medium. Error bars denote one standard
deviation and in some cases are smaller than the point.

14 Figure 4. Ammonia oxidation by CN25 in the presence of 10 and 100 $\mu\text{g mL}^{-1}$
allylthiourea (ATU). Each point represents the mean of three replicate bottles; error bars
16 denote one standard deviation from the mean.

Figure 5. $\delta^{15}\text{N}_{\text{NO}_2}$ during ammonia oxidation in batch culture for the archaeal enrichments
18 (a) CN25, (b) CN75, and (c) CN150. Kinetic isotope effects for ammonia oxidation
($^{15}\epsilon_{\text{NH}_3}$) were calculated using linear regression of $\delta^{15}\text{N}_{\text{NO}_2}$ vs. $f * \ln(f)/(1-f)$ as described
20 in the text and are reported in Table 2. f is the fraction of the initial $[\text{NH}_4^+]$ remaining in
the culture.

22 Figure 6. Potential pathways of NH_3 acquisition and oxidation by ammonia-oxidizing
archaea that could lead to a variable kinetic isotope effect ($^{15}\epsilon_{\text{NH}_3}$) (Walker *et al.*, 2010).

R_{eq} and $^{15}\epsilon_{\text{eq}}$ are the rate and equilibrium isotope effect for $\text{NH}_3/\text{NH}_4^+$ equilibration,
2 $R_{\text{diff,NH}_4}$ and $^{15}\epsilon_{\text{diff,NH}_4}$ are the rate and kinetic isotope effect for NH_4^+ diffusion, $R_{\text{diff,NH}_3}$
and $^{15}\epsilon_{\text{diff,NH}_3}$ are the rate and kinetic isotope effect for NH_3 diffusion, R_{AMO} and $^{15}\epsilon_{\text{AMO}}$
4 are the rate and kinetic isotope effect for ammonia oxidation by ammonia
monooxygenase (Amo), and R_2 and $^{15}\epsilon_2$ are the rate and kinetic isotope effect for the
6 second step of the ammonia oxidation pathway. Major uncertainties in the archaeal
oxidation pathway are shown in red.

8

Supplemental Figure 1. Phylogenetic tree of 87 archaeal *amoA* sequences chosen to
2 illustrate the relationship between the CN enrichments (shown in red), cultivated
ammonia-oxidizing archaea, and environmental sequences from the enrichment site and
4 other Pacific locations. This Jukes-Cantor distance based tree is based on a 489 base pair
alignment of the *amoA* gene.
6

Table 1. *In situ* conditions for the starting material for North Pacific Ocean seawater

2 enrichments used for archaeal kinetic isotope fractionation ($^{15}\epsilon_{\text{NH}_3}$) determination.

Enrichment	Depth (m)	Temp (°C)	[NH ₄ ⁺] (nmol L ⁻¹)	[NO ₃ ⁻] (μmol L ⁻¹)
CN25	25	14	40	0.5
CN75	75	12	<15	2.2
CN150	150	10	<15	15.1

4

Table 2. Experimental results for kinetic isotope effect ($^{15}\epsilon_{\text{NH}_3}$) determinations during of
 2 archaeal ammonia oxidation. CI 95% is the 95% confidence interval. [N+N] is the sum
 of the measured $[\text{NH}_4^+]$ and $[\text{NO}_2^-]$. SD is standard deviation.

Enrichment	Experimen t #	$^{15}\epsilon_{\text{NH}_3}$ (‰)	CI 95% (‰)	Initial [NH_4^+] ($\mu\text{mol L}^{-1}$)	$[\text{N+N}]_{\text{final}}: [\text{N+}$ $\text{N}]_{\text{initial}}$	NH_4^+ oxidation rate ^a ($\mu\text{mol L}^{-1}$ d^{-1})	
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
	4	14	4	4	16	0.98	0.9
			15	3	14	1.07	0.4
			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			
	22 ± mean ± SD		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	
21 ± mean ± SD		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	
22 ± mean ± SD		5‰					

4 ^aApproximate NH_4^+ oxidation rates were calculated from the change in $[\text{NO}_2^-]$ and the time elapsed
 between the first and final timepoints, and may underestimate the actual rate in experiments where the final
 6 time point was after all NH_4^+ had been consumed.

^bCalculating a confidence interval was not possible for this experiment because only two points were
2 available to calculate the slope.

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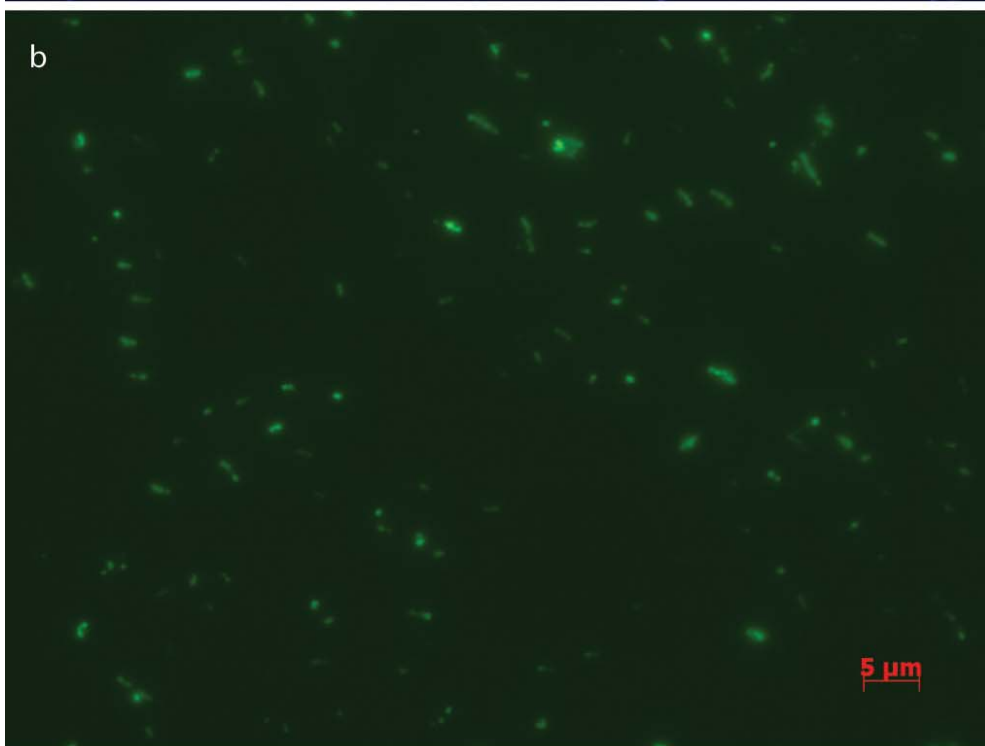


Fig. 1

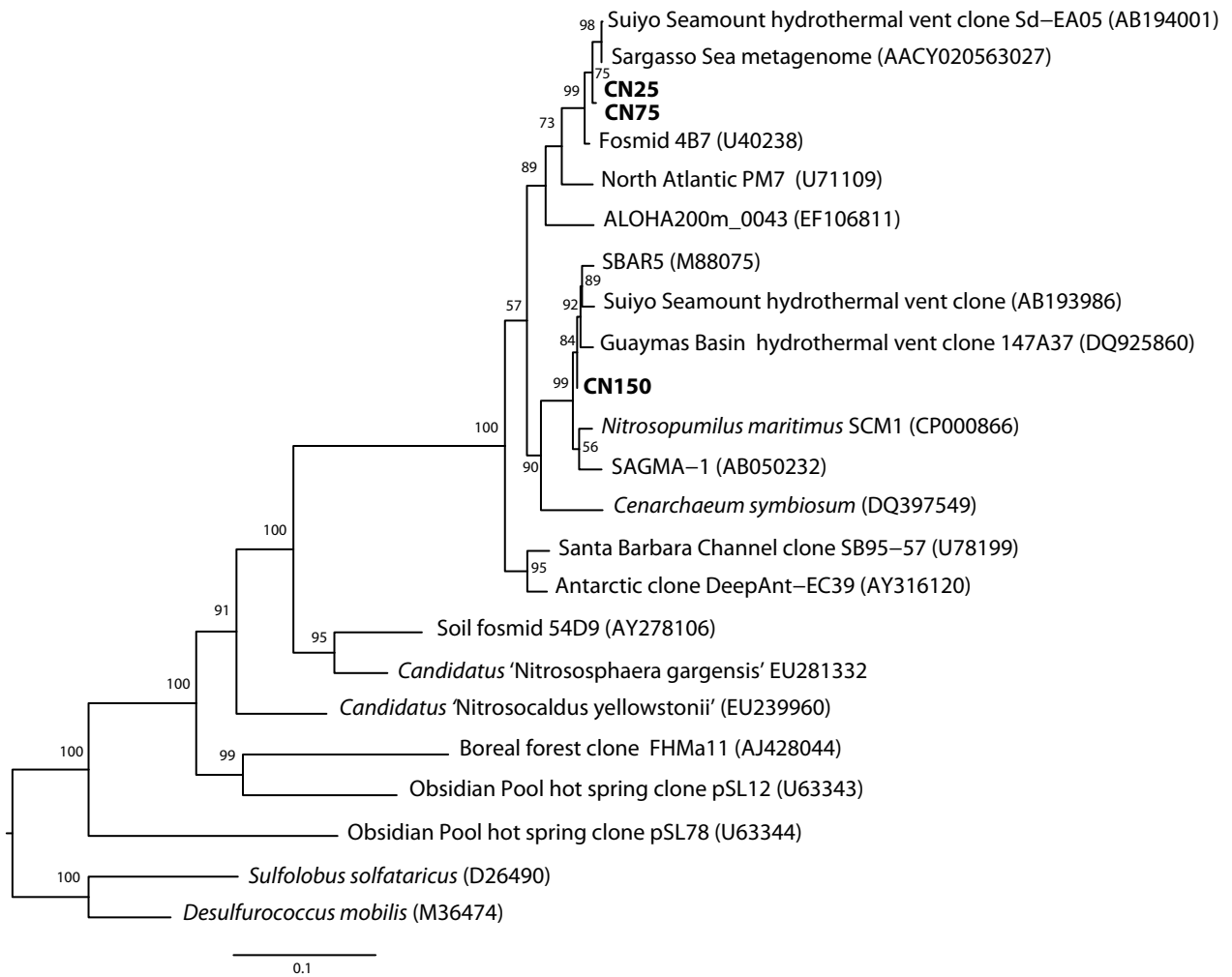


Fig. 2a

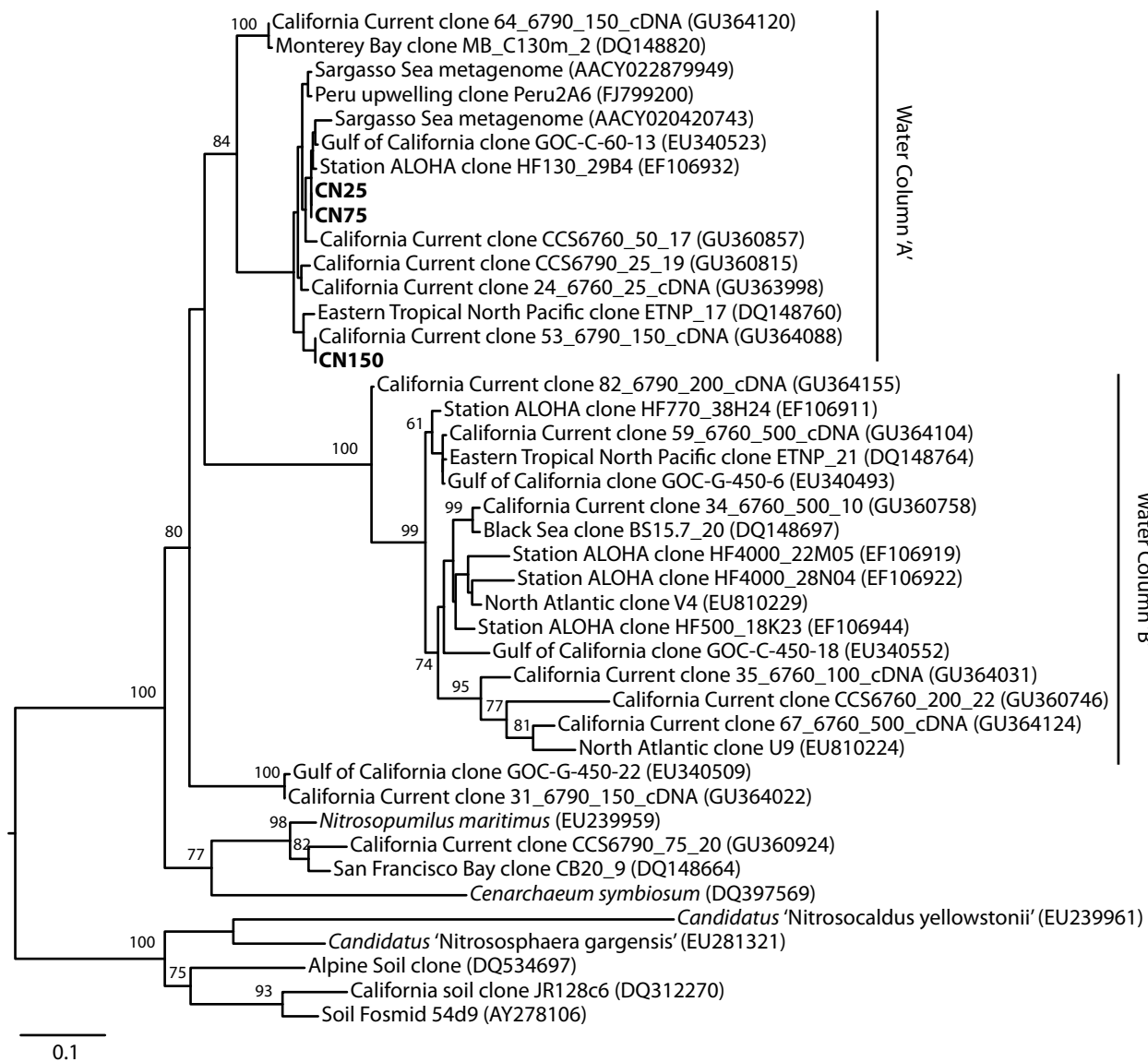


Fig. 2b

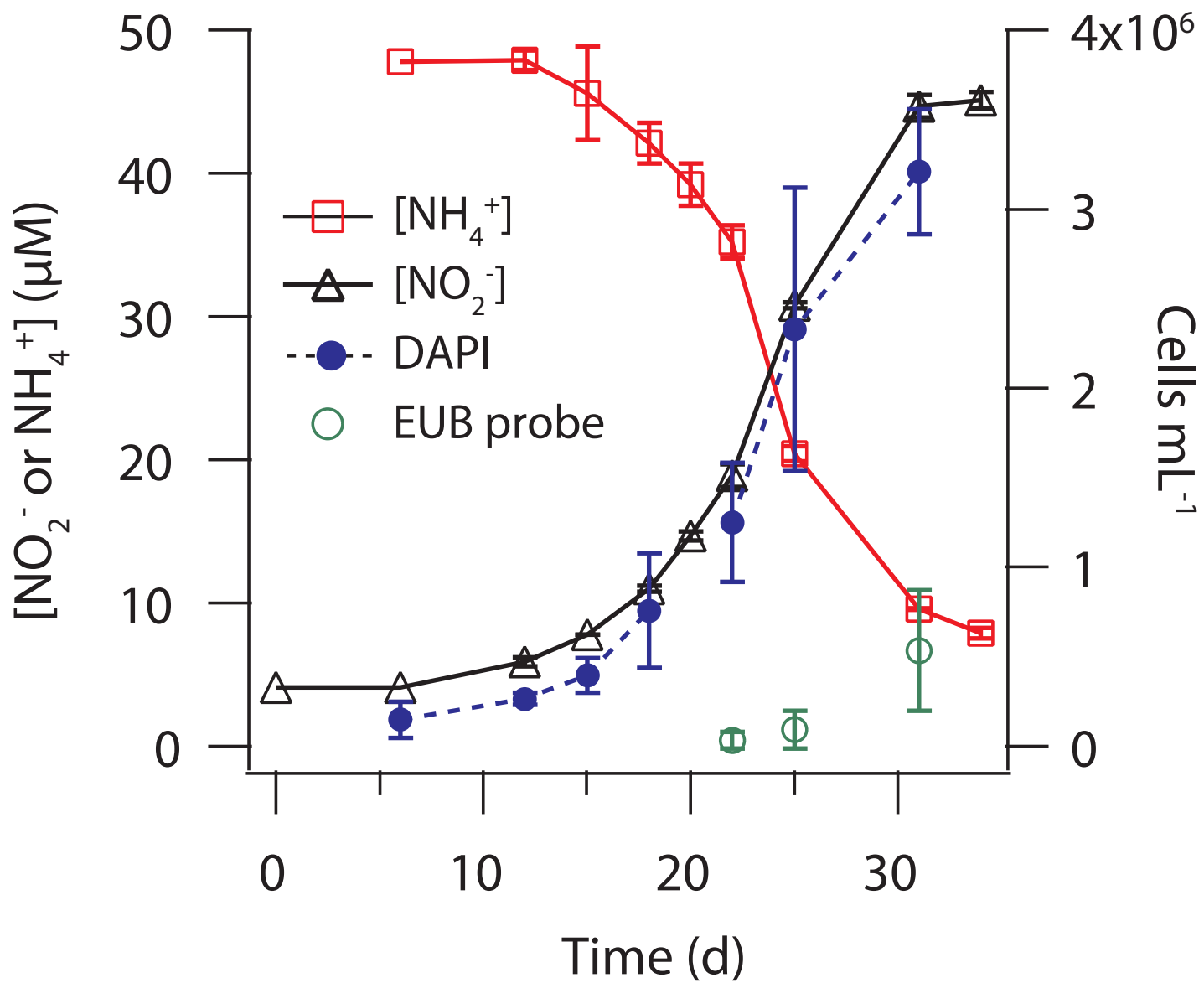


Fig. 3

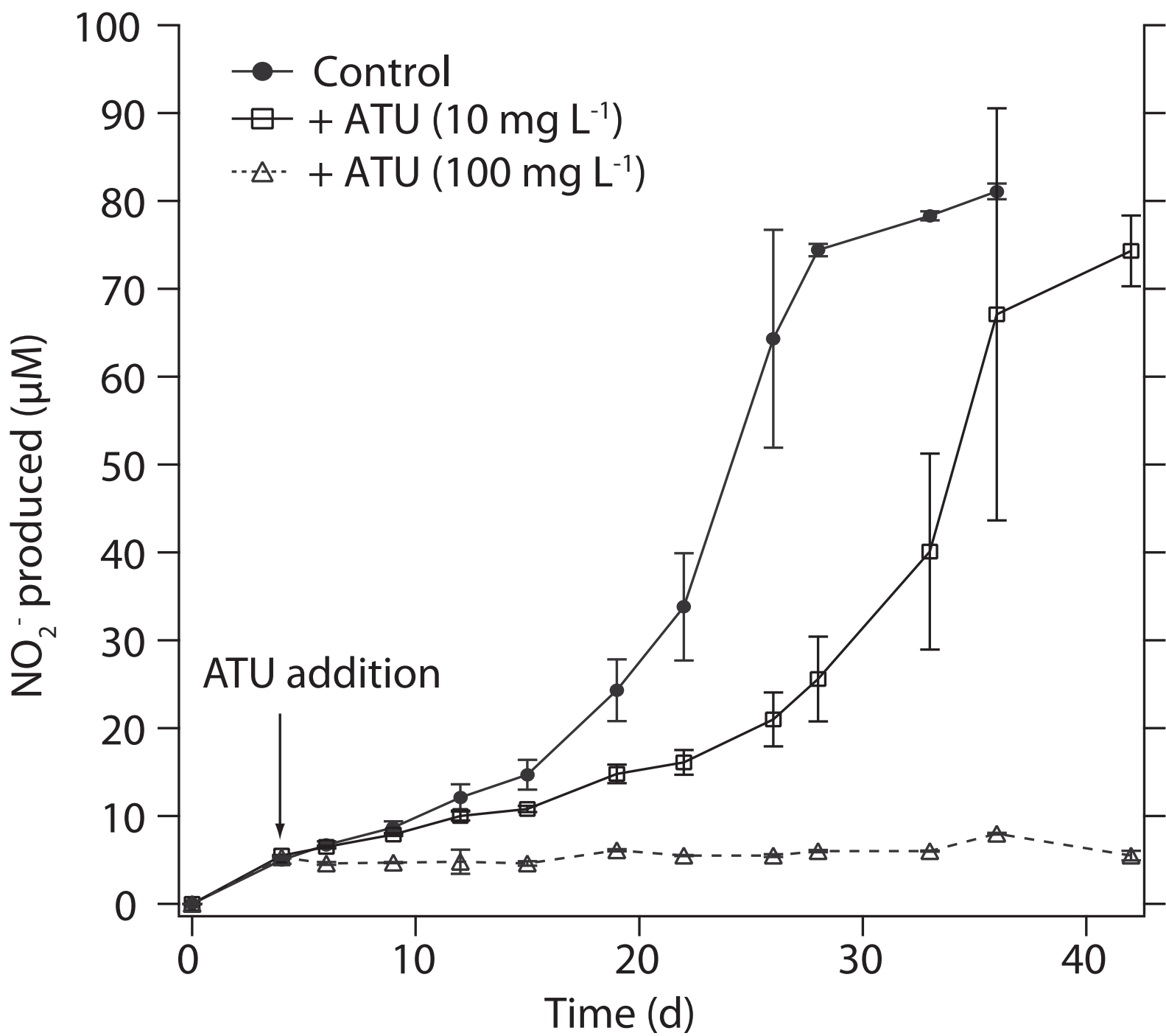


Fig. 4

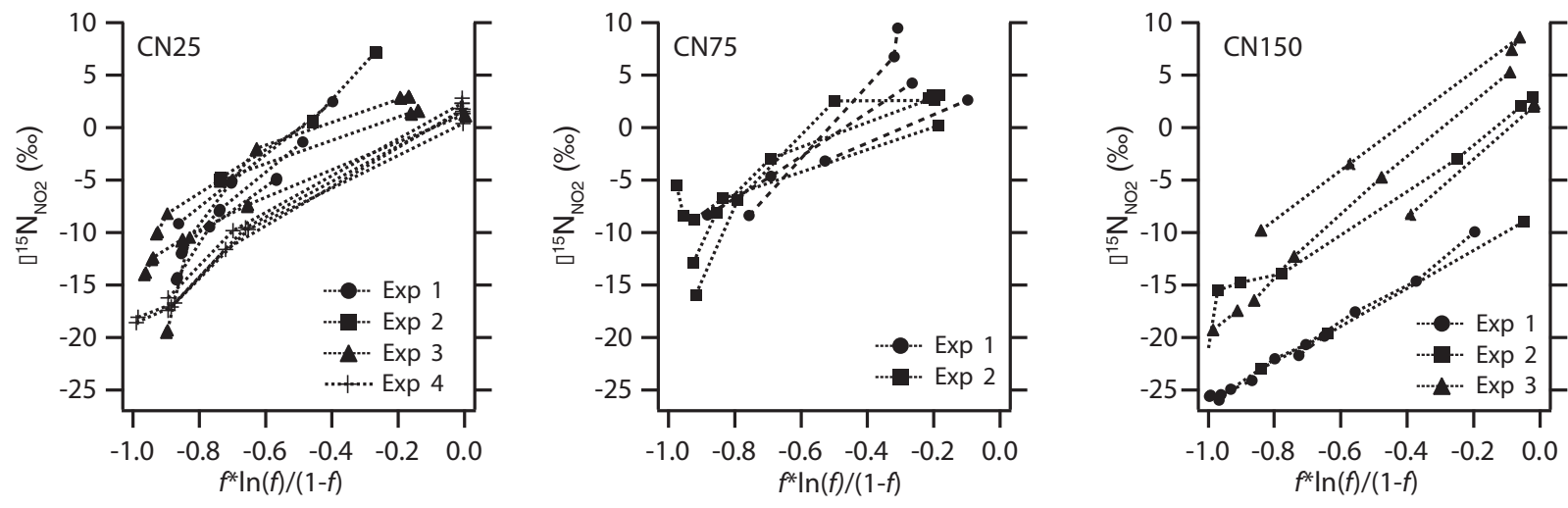


Fig. 5

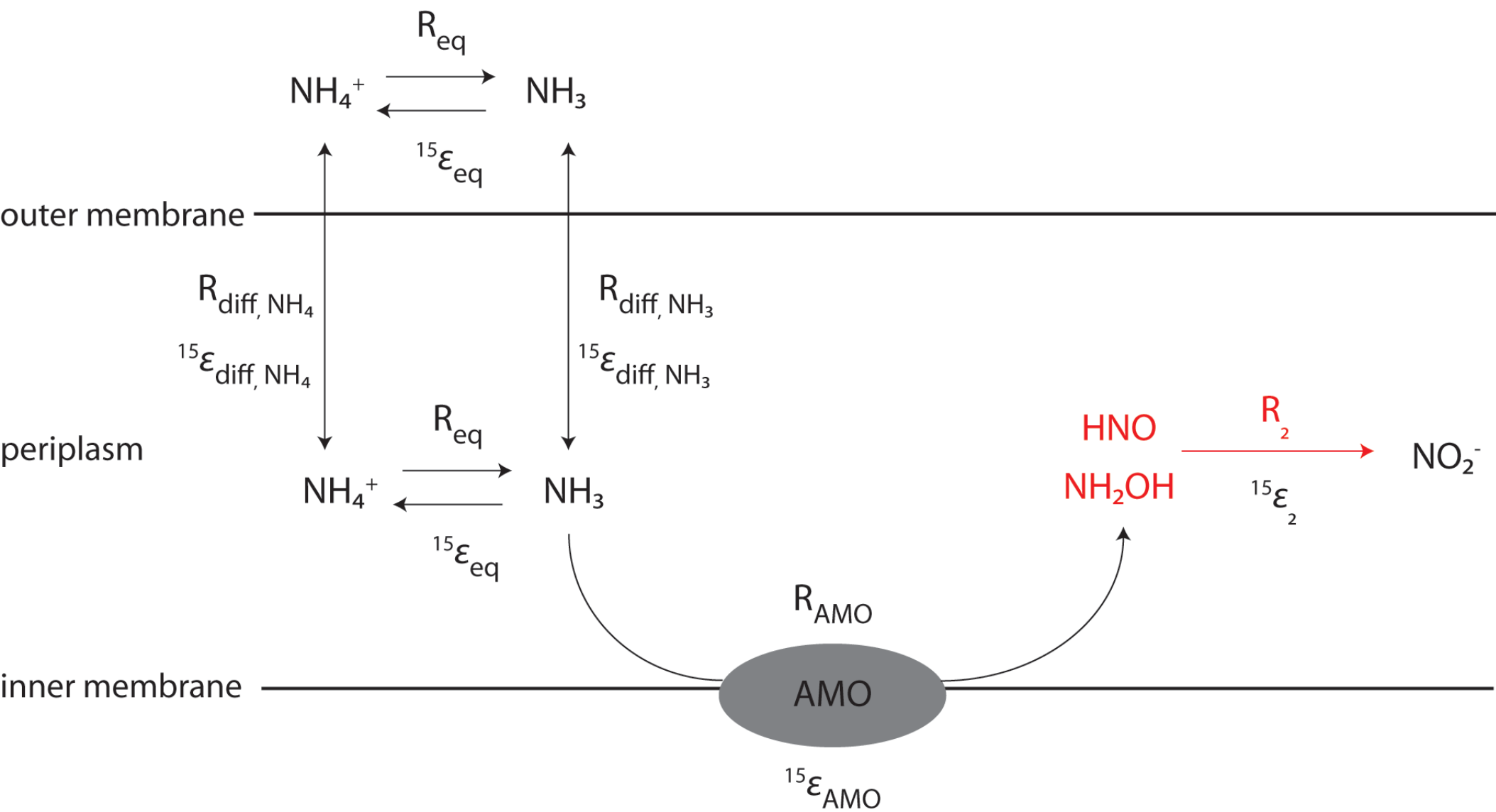


Fig.6

