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ORIGINAL ARTICLE

Ammonia oxidation kinetics and temperature sensitivity of a natural marine community dominated by Archaea

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Archaeal ammonia oxidizers (AOAs) are increasingly recognized as prominent members of natural microbial assemblages. Evidence that links the presence of AOA with *in situ* ammonia oxidation activity is limited, and the abiotic factors that regulate the distribution of AOA natural assemblages are not well defined. We used quantitative PCR to enumerate *amoA* (encodes α-subunit of ammonia monooxygenase) abundances; AOA *amoA* gene copies greatly outnumbered ammonia-oxidizing bacteria and *amoA* transcripts were derived primarily from AOA throughout the water column of Hood Canal, Puget Sound, WA, USA. We generated a Michaelis–Menten kinetics curve for ammonia oxidation by the natural community and found that the measured K_m of 98 ± 14 nmol l^{-1} was close to that for cultivated AOA representative *Nitrosopumilus maritimus* SCM1. Temperature did not have a significant effect on ammonia oxidation rates for incubation temperatures ranging from 8 to 20 °C, which is within the temperature range for depths of measurable ammonia oxidation at the site. This study provides substantial evidence, through both *amoA* gene copies and transcript abundances and the kinetics response, that AOA are the dominant active ammonia oxidizers in this marine environment. We propose that future ammonia oxidation experiments use a K_m for the natural community to better constrain ammonia oxidation rates determined with the commonly used $^{15}NH_4^+$ dilution technique.

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Introduction

Aerobic ammonia oxidation is the first step in nitrification in oxygenated waters and is an essential part of the nitrogen (N) biogeochemical cycle in the oligotrophic open and coastal ocean. Recent studies suggest that nitrification is a more significant process in the euphotic zone than previously thought, possibly controlling the form of nitrogen available for assimilation in N-limited waters (Dore and Karl, 1996; Ward, 2005; Yool *et al.*, 2007; Clark *et al.*, 2008; Beman *et al.*, 2012) and contributing to significant N_2O production (Santoro *et al.*, 2011). Nevertheless, water column ammonia oxidation is not well constrained. Molecular surveys in the oligotrophic open and coastal ocean based on the detection of the gene and transcripts encoding the α-subunit of ammonia monooxygenase (AMO) enzyme, indicate that marine group I *Crenarchaeota* (Archaea; phylum Thaumarchaeota; henceforth called ammonia-oxidizing Archaea or AOA) can be the numerically dominant ammonia oxidizers, greatly exceeding the ammonia-oxidizing bacteria (AOB; Caffrey *et al.,* 2007; Mincer *et al.*, 2007; Agogue *et al.*, 2008; Kalanetra *et al.*, 2009; Beman *et al.*, 2010; Church *et al.*, 2010; Newell *et al.*, 2011; Sintes *et al.*, 2013).

Numerical abundance has been generally associated with functional importance. This has been indicated by a few studies that demonstrated a correlation between ammonia oxidation rates and the depth profile of AOA *amoA* gene or transcript copies, but no such correlations are seen with *amoA* from AOB (Beman *et al.*, 2008, 2012). In contrast, neither Newell *et al.* (2011) nor Santoro *et al.* (2010) found a direct correlation of either AOA or AOB *amoA* abundances with ammonia oxidation rate depth profiles. Santoro *et al.* (2010) hypothesized

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that a lack of correlation between *amoA* abundances and rates could result from either water column physiochemical differences or different rates per cell by different clades of AOA.

In the absence of additional biochemical and ecophysiological data, the co-occurrence patterns between AOA and AOB abundances with ammonia oxidation rates must be interpreted with caution. The detection of the *amoA* gene and its transcript does not necessarily signal that an organism is carrying out ammonia oxidation (Prosser and Nicol, 2008; Stahl and de la Torre, 2012; Veuger *et al.*, 2012). The function of the AOA AMO has not yet been established; therefore, it is possible that the AOA homolog of the AOB AMO enzyme may be multifunctional. For instance, the AOB AMO enzyme can oxidize a variety of reduced compounds such as methane and carbon monoxide (Prosser and Nicol, 2008). Alternatively, it could have an entirely different function, such as the closely related butane monooxygenase of a butane-oxidizing bacterium (Sayavedra-Soto *et al.*, 2011). The functional diversity of monooxygenase enzymes (Tavormina *et al.,* 2011) is the reason that the initial annotation of a crenarchaeal (Thaumarchaeal) gene, identified in a marine metagenomic library, as an ammonia monooxygenase should have included these caveats. In this study, we take advantage of physiological characteristics of the one available cultivated marine AOA, *Nitrosopumilus maritimus* SCM1 (an obligate ammonia-oxidizing chemoautotroph, Könneke *et al.,* 2005), to guide interpretation of ammonia oxidation in natural assemblages.

Nitrosopumilus maritimus SCM1 is closely related to the numerically dominant marine AOA based on similar gene content and sequence (Walker *et al.*, 2010) and also closely related to AOA recovered in enrichment cultures (Wuchter *et al.*, 2006; Agogue *et al.*, 2008; Santoro *et al.*, 2010; Santoro and Casciotti, 2011). The K_m for AOA cultivated representative *N. maritimus* is at least 60× lower (133 nmol l^{-1} total $NH_3 + NH_4^+$; Martens-Habbena *et al.*, 2009) than the lowest reported K_m for a cultivated marine AOB (*Nitrosococcus oceani,* 8 μmol l^{-1}; Ward, 1987) and several orders of magnitude less than many AOB (Martens-Habbena *et al.*, 2009). This physiological property indicates that the substrate (NH_3) availability may in part determine the niche partitioning of AOA and AOB, such that AOA abundance and activity are higher in low NH_3 environments (Martens-Habbena *et al.*, 2009).

Recognition of the extremely high affinity for ammonia by AOA in culture also introduced a methodological problem. The current methods used to measure ammonia oxidation rates in the marine environment can cause significant deviations from *in situ* rates. Most marine ammonia oxidation rate studies use the $^{15}NH_4^+$ isotope dilution technique (reviewed in Ward, 2011), where a 'trace' amount of $^{15}NH_4^+$ is added to a sample and the sample is incubated at a specific light intensity and temperature. Under substrate-limiting conditions, the $^{15}NH_4^+$ dilution technique will increase the ammonia oxidation rate of the sample by the addition of substrate, thus overestimating the *in situ* rate. The low K_m of *N. maritimus* SCM1 (133 nmol l^{-1}) is comparable to typical NH_4^+ concentrations in well-oxygenated open ocean sites (for example, typical NH_4^+ at BATS station = 0–100 nmol l^{-1}; Lipschultz, 2001), which suggests that AOA may be substrate limited at *in situ* NH_4^+ concentrations. With a well-defined K_m for natural assemblages of marine AOA, ammonia oxidation rates for low NH_4^+ environments can be corrected to account for the addition of $^{15}NH_4^+$.

Another possible source of error in experimentally determined rates originates from the difference between the *in situ* temperature and the *ex situ* temperature applied during incubation. In this case, a Q_{10} correction factor is needed to account for the effect of the incubation temperature. There is scant data available for the temperature response of natural assemblages or cultures of AOA, as is needed for correction when the incubation temperature used to determine the rate differs from the *in situ* temperature.

In order to mitigate the influence of these experimental limitations on data interpretation, we investigated the temperature sensitivity of ammonia oxidation and applied kinetic corrections to the calculation of *in situ* ammonia oxidation rates in a well-characterized site in Hood Canal, a fjord-like basin in Puget Sound, Washington, USA (http://hoodcanal.washington.edu). We demonstrated that an *in situ* K_m is comparable to that of *N. maritimus* SCM1, and this now implicates the AOA as major contributors to ammonia oxidation in this coastal system. Ammonia oxidation rates measured in our incubations were not sensitive to temperature changes within the temperature range at the field site. Together, our data provide compelling evidence in support of the functional dominance of AOA in this field site, and have established an ecophysiological framework for more general studies of factors that control the distribution of AOA and AOB in the marine environment.

Materials and methods

Sample collection and core measurements

All samples were collected at a long-term mooring site near Hoodsport in Hood Canal, Washington, USA (47° 25.309 N, 123° 6.755 W; Oceanic Remote Chemical Analyzer, http://orca.ocean.washington.edu). Sampling dates and cruise numbers can be found in Supplementary Table 1. For the June 2011 field work, we used a 5-l Niskin bottle to collect water samples from a small research vessel. For all other studies, we collected seawater aboard the R/V *Clifford A. Barnes* using a conductivity–temperature–depth (CTD) rosette fitted with 10-l

Niskin bottles and equipped with a Seabird SBE CTD sensor package. Oxygen concentrations were measured with the CTD sensor package (SBE-43) on the Hoodsport buoy mooring, and were calibrated against Winkler dissolved oxygen determinations ($R^2 > 0.84$).

Within 2 h of sample collection, NH_4^+ concentrations were measured using the o-phthaldialdehyde fluorescence method (standard concentration range: 0–1000 nmol l^{-1}, $n = 6$ standards, $R^2 > 0.97$; Holmes *et al.*, 1999) and NO_2^- spectrophotometrically (standard concentration range: 0–800 nmol l^{-1}, $n = 6$ standards, $R^2 > 0.99$; Grasshoff *et al.*, 1999). We determined that the precision for the replicate samples with the o-phthaldialdehyde method is approximately 5%, and our detection limit was 10 nmol l^{-1}. Samples for NO_3^- were filtered (0.22 μm) and frozen at −20 °C until shore-based analysis (UNESCO, 1994).

Quantification of amoA gene and transcript copies using quantitative PCR (q-PCR)

Samples for q-PCR (2 l) and reverse transcription q-PCR (4 l) were collected from each depth on the same cast as samples for NH_4^+ oxidation rate measurements and filtered on 0.22-μm Sterivex-GP (Millipore Corporation, Billerica, MA, USA) filters with a peristaltic pump. DNA extracts were obtained as previously described (Urakawa *et al.*, 2010), and crude RNA extracts were isolated with a hot phenol extraction method (MacGregor *et al.*, 1997). RNA samples were treated twice with DNA-free kit (Invitrogen, Grand Island, NY, USA) to remove DNA contamination for reverse transcription q-PCR analysis. RNA samples were run with LightCycler FastStart DNA Master SYBR green I kit (Roche, Indianapolis, IN, USA; see below) to confirm the efficiency of DNase treatment in RNA purification. GlycoBlue (Invitrogen) was added to the DNA and RNA samples to increase yields. Nucleic acid concentration was measured using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The concentration and quality of RNA were further determined using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

AOB and AOA a*moA* gene copy numbers were quantified using the LightCycler FastStart DNA Master SYBR Green I kit (Roche) and capillary system (LightCycler; Roche). AOA and AOB *amoA* transcript abundances were determined by one-step reverse transcription q-PCR using the LightCycler RNA Master SYBR Green I kit (Roche) and capillary system (Roche). RNA samples were also run with LightCycler FastStart DNA Master SYBR green I kit (no reverse transcription reactions) to confirm the efficiency of DNase treatment in RNA purification. The carryover genomic DNA contamination was negligible (<0.01%) in all samples of this study. AOA *amoA* genes and transcripts were detected using the primer set CrenAmoAQ-F and CrenAmoAModR (Mincer *et al.*, 2007) with *N. maritimus* genomic DNA in 10-fold dilutions ranging from 6×10^5 to 6×10^1 copies per reaction. β-AOB *amoA* genes and transcripts were quantified using the primer set amoA-1F and amoA-2R (Rotthauwe *et al.*, 1997) with *Nitrosomonas europaea* genomic DNA in 10-fold dilutions ranging from 7×10^5 to 7×10^1 copies per reaction. The melting curve analysis was performed following amplification to evaluate the stringency of q-PCR products. The products were also tested on 1.5% agarose gel to evaluate nonspecific amplification. R^2 values of all standard curves for gene and transcript quantification were higher than 0.997. The amplification efficiencies were 87.3%, 93.5% and 95.2%, 92.3% for AOA and AOB *amoA* transcripts and AOA and AOB *amoA* gene copies, respectively. In order to estimate the possible inhibitory effects on q-PCR from nucleic acid extracts, copy numbers were determined for a dilution series of over four orders of magnitude of DNA and RNA templates. No inhibition was detected.

Ammonia oxidation rate measurements

Ammonia oxidation rates were measured through the addition of ^{15}N-labeled NH_4^+. This method measures the appearance of the labeled ^{15}N in the dissolved $NO_2^- + NO_3^-$ pool relative to the initial NH_4^+ pool. Duplicate or triplicate 125-ml 10% HCl-washed and Milli-Q water-rinsed polycarbonate bottles were rinsed three times with Niskin-collected water and filled with whole seawater after three overflowing volumes. $^{15}NH_4^+$ (99 atom percent, at%; Cambridge Isotope Laboratories, Andover, MA, USA) was added before sealing the bottle with as little headspace as possible (final concentrations are noted in assay-specific details below). We sampled for initial $\delta^{15}N$ of the $NO_2^- + NO_3^-$ pool for each depth immediately after the addition of $^{15}NH_4^+$. In order to maintain dissolved gas concentrations throughout the incubation for experiments with multiple time points, a single bottle was sampled at each time point, and no bottle was sampled more than once. All incubations were terminated by flash-freezing 50-ml aliquots in a dry-ice ethanol slurry and stored at −20 °C until analysis.

Temperature amendment assays

Experiments to determine the effect of temperature upon ammonia oxidation were conducted in a shore-based laboratory (CB966, CB974, CB980) or shipboard (CB985). Water was collected from 50 m, and the *in situ* water temperature was approximately 8.5 °C. We added 50 nmol l^{-1} $^{15}NH_4^+$ to all bottles. For the laboratory-based experiments, we incubated the samples in the dark (black trash bags) in temperature-controlled incubators (incubators keep the temperature to within 1 °C of the set temperature). For the August 2012 shipboard

experiment, we collected water in darkened polycarbonate bottles (wrapped in electrical tape) and incubated the samples either in the refrigerator (4 °C treatment; range = 3–5 °C), attached to a free-floating array at the study site (8, 12.5, 15 °C treatments), or in surface seawater incubators (18 °C). Repeated CTD rosette casts during the time course of incubation indicated that the temperature profile, hence, the temperature of incubation on the free-floating array, was stable throughout the incubation period. Samples were collected at 5 and 10 h for CB966 (23–28 September 2011), at 3.5, 6.5 and 10 h for CB974 (7–13 May 2012) and a single end point was taken for CB980 (16–22 July 2012) and CB985 (24–30 August 2012). Duplicate samples for each time point were taken for CB966 and CB974, quadruplicate samples for CB980 and triplicate samples for CB985.

Ammonia oxidation kinetics assays

We conducted the assay for ammonia oxidation kinetics with water from 50 m ($[NH_4^+] = 60\ nmol\ l^{-1}$) in June 2011. Seawater was transported from the study site to the laboratory in a darkened carboy and maintained at near ambient temperature (8 °C). We measured the effect of substrate on the ammonia oxidation rate by varying the concentration of added $^{15}NH_4^+$ (15.6–250 $nmol\ l^{-1}$). The experiment was conducted in the shore-based laboratory, samples were incubated in the dark at near ambient temperature (8 °C) and replicate samples for $\delta^{15}N$—$NO_2^- + NO_3^-$ analysis were taken at 6, 11 and 24 h.

For both the temperature and kinetics assays, the 'wet cadmium-azide' method was used to reduce $NO_2^- + NO_3^-$ to N_2O, and ^{15}N label in recovered N_2O was determined by mass spectrometry (McIlvin and Altabet, 2005). Briefly, wet cadmium was added to 10 ml of sample and the samples were shaken vigorously overnight to reduce NO_3^- to NO_2^-. Two-milliliter aliquots were transferred into a crimp seal vial (22 ml capacity) with butyl rubber septa. We added 0.6 ml of an acetic acid/sodium azide solution (1:1 solution of 20% acetic acid and 2 $mol\ l^{-1}$ sodium azide) through a syringe to reduce the NO_2^- to N_2O (McIlvin and Altabet, 2005), and 0.3 ml of 6 $mol\ l^{-1}$ NaOH was added through a syringe to stop the reaction. Immediately after this step, headspace $^{44/45}N_2O$ isotopic ratios were measured on a Thermo-Finnigan Delta Plus continuous flow-isotope ratio mass spectrometer (Bremen, Germany). Standards of N_2O were run every five samples to assess instrument drift. $\delta^{15}N$-N_2O precision, as determined by replicate measurements of 15 randomly selected samples, was 2.8% (SD = 2.4%). Rates of $^{15}NH_4^+$ oxidation were calculated with modifications of equations presented in Ward *et al.* (1989), and is as follows

$$rNH_{4-ox} = 2 * N_{N+N} * \frac{(n_tNO_x) - (n_oNO_x)}{\Delta t * (N_T/N_{TA})} \quad (1)$$

Where $rNH_{4\text{-}ox}$ is the ammonia oxidation rate ($nmol\ l^{-1}\ d^{-1}$), N_{N+N} is ambient $NO_2^- + NO_3^-$ concentration, n_tNO_x is the atom percent (at%) $^{15}NO_2^- + {}^{15}NO_3^-$ at time t, n_oNO_x is the measured initial at% $^{15}NO_2^- + {}^{15}NO_3^-$, Δt is the change in time, N_T is concentration of $^{15}NH_4^+$ added, N_{TA} is tracer + ambient NH_4^+ concentration. We assumed that the initial ^{15}N at% of unlabeled NH_4^+ was 0.3663. We multiply the product on the right by 2 because the N_2O has one N atom from the azide and one N atom from the $^{15}NO_2^-$ pool. We calculated the ammonia oxidation rate using the enrichment of ^{15}N into the $NO_2^- + NO_3^-$ pool only; we did not account for any isotopic change of the substrate during the incubation (we assumed low regeneration) nor did we quantify uptake into the particulate fraction.

Determination of Q_{10}

We conducted a one-way analysis of variance (ANOVA) to determine whether different incubation temperatures had an effect upon ammonia oxidation rates. A Tukey's *post-hoc* test ($\alpha = 0.05$) was used to determine whether there was a difference in the ammonia oxidation rate between the control treatment (8 °C, approximately the *in situ* temperature) and the temperature-amended treatment. We used the following equation to determine Q_{10} for the temperature pair:

$$Q_{10} = \left(\frac{Rate_{8C}}{Rate_T}\right)^{\frac{10}{8-T}} \quad (2)$$

where T is the temperature significantly different than the control, $Rate_{8C}$ is the ammonia oxidation rate for the control treatment (8 °C) and $Rate_T$ is the ammonia oxidation treatment for temperature T.

Determination of K_m for ammonia oxidation kinetic-corrected ammonia oxidation rates

The experiment yielded three Michaelis–Menten curves (one for each time interval), and they were treated as replicates for the nonlinear regression. We applied nonlinear regression (Simple enzyme kinetics module, one-site saturation, SigmaPlot 10.0) to the Michaelis–Menten kinetics curves to calculate K_m for the experiment.

We used the following equation (based on Rees *et al.*, 1999; Diaz and Raimbault, 2000) to calculate a revised ammonia oxidation rate that accounts for the rate enhancement brought about by the addition of tracer:

$$r_{corr}NH_{4-ox} = \frac{r_oNH_{4-ox}}{N_{TA}/(K_m + N_{TA}) * (K_m + N_A)/N_A} \quad (3)$$

Where $r_{corr}NH_{4\text{-}ox}$ is the ammonia oxidation rate ($nmol\ l^{-1}\ d^{-1}$) revised for the addition of $^{15}NH_4^+$, $r_oNH_{4\text{-}ox}$ is the original ammonia oxidation rate from Equation 1 ($nmol\ l^{-1}\ d^{-1}$), N_{TA} is concentration of tracer + ambient NH_4^+, K_m is the ammonia oxidation half-saturation constant and N_A is concentration of ambient NH_4^+. At some depths, N_A was below the

detection limit. For these instances, we calculated a maximum ammonia oxidation rate by substituting the detection limit for N_A.

Results

Environmental conditions and nutrient profiles

Hood Canal is a seasonally stratified water column with a shallow sill near the mouth of the canal that restricts water exchange with Puget Sound (HCDOP IAM, 2011). Temperatures, as measured daily by the Hoodsport ORCA buoy, at the site are highly variable and depth dependent. Data collected with the ORCA buoy from 2005 to 2013 indicate that temperatures range 5.28–22.23 °C at the surface (3.4 m), 6.97–17.43 °C at 10.4 m and 8.03–11.50 °C at 50 m (http://orca.ocean.washington.edu). A temperature and salinity profile from May 2012 (Figure 1a) typifies spring and summer conditions. In this profile, water temperatures range from 8–18 °C, and there is a prominent subsurface chlorophyll maximum (Figure 1b) generally located 3–6 m above the primary nitrite maximum (Figure 1c, open circles and hashed line). Following the spring bloom, nutrient levels in the mixed layer are highly depleted (Figure 1c) and low NH_4^+ concentrations are found throughout the water column (Figure 1c, filled circles and solid line), except at the chlorophyll maximum. For the depth profile, NH_4^+ ranged from 0 to 646 nmol l^{-1}; at 50 m, the depth at which kinetics and temperature amendment experiments were conducted, NH_4^+ ranged from 0 to 60 nmol l^{-1}.

AOA and β-AOB amoA gene copies and transcript abundances

Throughout the spring and summer, AOA *amoA* gene copies and transcript abundances were to minimal in surface waters, increased with depth down to 20 m, and were relatively constant at depths below 20 m (Figure 1d). The abundance of β-AOB *amoA* gene copies was similar through the entire depth profile (Figure 1d), and ranged from 2.3 times (9 m) to 60 times (115 m) less abundant than AOA *amoA* gene copies. β-AOB *amoA* transcript abundances were below detection limits for all depths (data not shown).

The effect of temperature upon ammonia oxidation rates

We tested the effect of temperature upon ammonia oxidation rates on four separate occasions by manipulating temperature during rate incubations. For CB974 (May 2012) and CB980 (July 2012), temperature had a significant effect on ammonia oxidation rates (ANOVA; Table 1). For CB974, ammonia oxidation rates increased significantly when the temperature was increased from the 8 °C control temperature to 23 °C (Tukey's *post-hoc* test). For CB980, ammonia oxidation rates at 4 °C and 22 °C were significantly lower than rates measured at 8 (control), 13 and 18 °C temperatures (ANOVA with Tukey's *post-hoc* test; Figure 2). No significant differences in ammonia oxidation rates with temperature were observed for experiments conducted on either CB966 (September 2011) or CB985 (August 2012; ANOVA; Table 1; Figure 2).

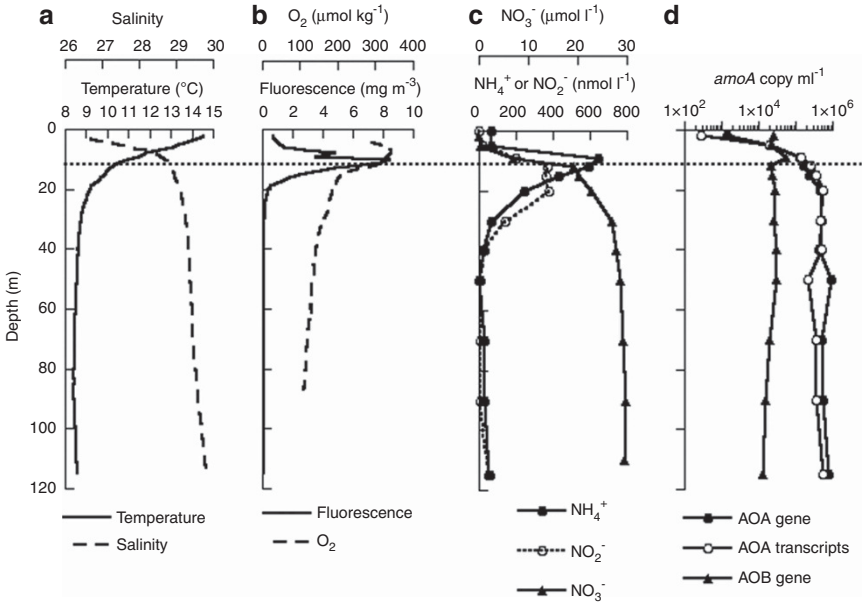

Figure 1 Representative CTD data and inorganic N vertical profiles for Hoodsport study site (CB974, May 2012, data are shown). (**a**) Temperature and salinity; (**b**) fluorescence and oxygen; (**c**) NO_3^-, NO_2^- and NH_4^+. (**d**) q-PCR *amoA* abundance profile. Dotted horizontal line is 1% surface PAR (approximately 11 m). Data are the mean of replicate q-PCR measurements for AOA *amoA* gene copies, AOA *amoA* transcripts and β-AOB *amoA* gene copies.

Table 1 Calculation of Q_{10} for four separate ammonia oxidation experiments at the Hoodsport site

Date	*Cruise ID*	*ANOVA P-value*	*Treatment (°C)*	Q_{10}
May 2012	CB974	<0.0001	4	1.00
			13	1.00
			18	1.00
			23	1.31
July 2012	CB980	<0.0001	4	1.84
			13	1.00
			18	1.00
			22	0.87
August 2012	CB985	0.577	4–18	1.0
September 2011	CB966	0.069	4–20	1.0

Abbreviation: ANOVA, analysis of variance.
Ambient temperature at time of sampling for all experiments was approximately 8.5 °C. If the ANOVA for all temperature amendments was significant, then a Q_{10} was calculated for temperature treatments that were significantly different than the control treatment (8 °C).

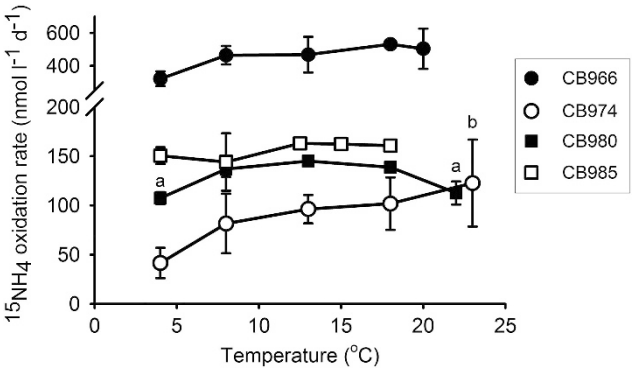

Figure 2 The effect of temperature amendment on $^{15}NH_4$ oxidation rate for water collected at 50 m. Data are presented for four cruises (inset legend). Data are mean ± 1 s.d. ($n = 3$ or 4). Letters (a, b) indicate treatments which had significantly different rates than the *in situ* temperature treatment.

Because of the varied response of ammonia oxidation rates to temperature, we calculated a Q_{10} for the temperature treatment as compared with the control (Table 1). Q_{10} was 1.0 for temperature treatments between 8 and 20 °C, which is within the 8-year temperature range (6.97–17.43 °C) for depths, which had measurable ammonia oxidation rates (9 m and deeper). Q_{10} was greater than or less than 1.0 only for temperatures outside of the 8-year temperature range (Table 1).

Kinetics of Archaeal ammonia oxidation

We investigated the effect of substrate concentration on ammonia oxidation rates in low NH_4^+ ($[NH_4^+] = 60$ nmol l^{-1}) water collected from 50 m and incubated at near *in situ* temperature (8 °C). Labeled product ($\delta^{15}N$–$^{15}NO_2^- + {}^{15}NO_3^-$) increased linearly over time with increasing substrate concentration (Figure 3a), regardless of the time interval used to calculate the rate. The Michaelis–Menten kinetics curves (substrate versus ammonia oxidation rate) for

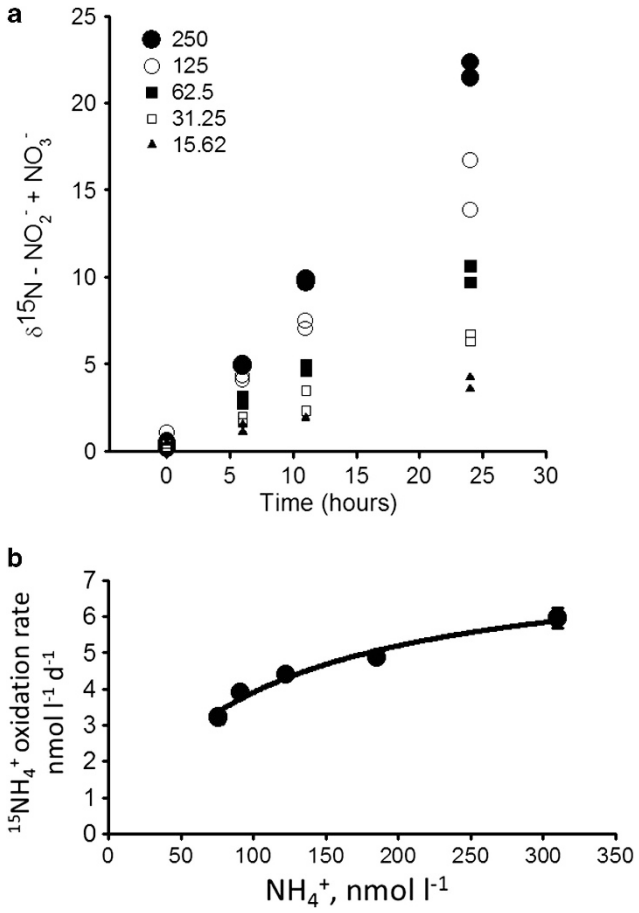

Figure 3 Kinetics of ammonia oxidation. (**a**) The increase in the $\delta^{15}N$ of the $NO_2^- + NO_3^-$ pool is linear for $^{15}NH_4^+$ oxidation rate incubations. Data points are individual bottle incubations. Legend indicates $^{15}NH_4^+$ added (nmol l^{-1}). (**b**) Regression fit for Michaelis–Menten kinetics model. The mean and 95% confidence interval for the rate are indicated for the substrate concentration.

each of the three time intervals of incubation were similar and were used to fit a single Michaelis–Menten kinetics curve (Figure 3b). The calculated K_m value for the fit was 98 nmol l^{-1} (s.e. = 14; 95% confidence interval: 67–130 nmol l^{-1}; $P = 0.0001$; $R^2 = 0.949$).

Corrections to ammonia oxidation rates

We determined a high-resolution ammonia oxidation rate profile in May 2012 (CB974; Figure 4a, open circles), and added 50 nmol l^{-1} $^{15}NH_4^+$ as substrate. The temperature of the surface seawater incubators was 14 °C, which was within the temperature range not requiring a Q_{10} correction. We applied the kinetic correction equation (Equation 3) to all depths, which incorporated our calculated K_m and accounted for the impact of substrate additions on *in situ* ammonia oxidation rates (Figure 4a, filled circles). The NH_4^+ concentration at 50 m was below the detection limit of 10 nmol l^{-1}. We therefore used 10 nmol l^{-1} as the ambient NH_4^+ concentration in Equation 3 to calculate a maximum rate. Use of

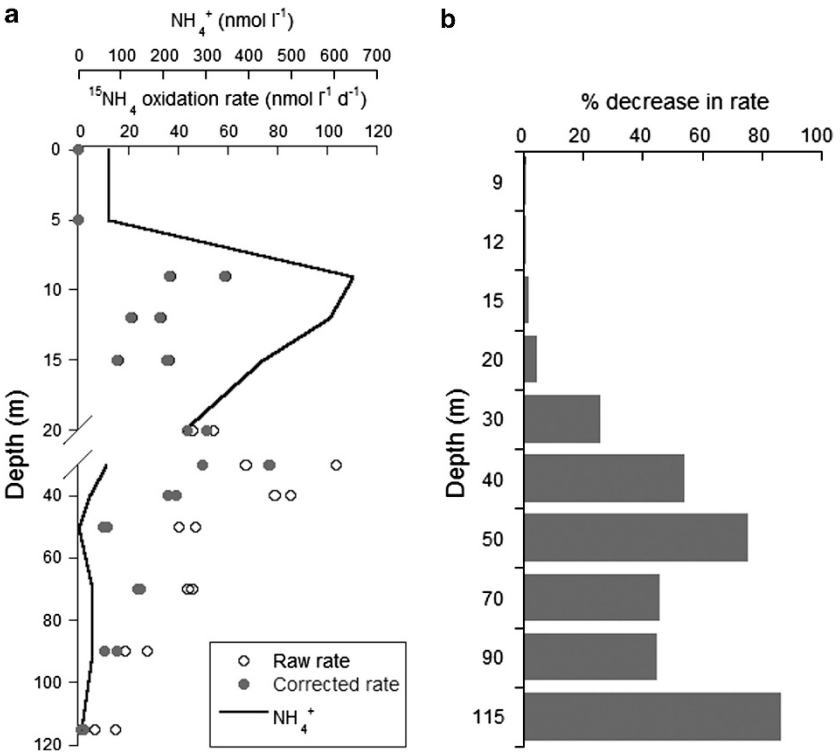

Figure 4 Ammonia oxidation rate profile and a kinetics correction for cruise CB974 (May 2012). (**a**) A comparison of the raw $^{15}NH_4^+$ oxidation rate (raw rate; hidden by closed circles for depths 0–15 m) and a rate corrected for kinetic effects in the bottle incubation (corrected rate). Ambient NH_4^+ is indicated with the solid line. Each data point is the rate from an individual bottle. (**b**) Percent decrease in ammonia oxidation rate resulting from a correction for the addition of substrate.

the kinetics correction decreased the measured ammonia oxidation rates by up to 86% for depths where the ambient NH_4^+ concentrations were low ($<100\,nmol\,l^{-1}$; Figure 4b). The kinetic-corrected ammonia oxidation rate profile (12 depths) was significantly correlated with AOA *amoA* transcripts, although the correlation was not strong (linear regression, $P=0.038$, $R^2=0.18$). Rates were not correlated with AOA *amoA* gene copies (linear regression, $P=0.908$, $R^2<0.01$).

Discussion

Variability in ammonia oxidation rates

We measured ammonia oxidation rates at several depths and at several times of the year. Ammonia oxidation rates, uncorrected for enzyme kinetics, varied widely in space and time (0–550 $nmol\,l^{-1}\,d^{-1}$) and the uncorrected ammonia oxidation rates for the depth profile were similar to rates recently measured in the water column at various oceanic sites (Beman *et al.,* 2008, 2012; Santoro *et al.,* 2010). There was considerable variability in ammonia oxidation rates at 50 m (range = 5–550 $nmol\,l^{-1}\,d^{-1}$). High variability in ammonia oxidation rates at a single site has also been recorded in the sediments of a small California estuary, presumably because of physical forcing (500-fold variation in rates; Caffrey *et al.*, 2003). The inland waters of Hood Canal are known to have a high degree of small-scale patchiness and are greatly influenced by physical forcing, such as storms, mixing and annual bottom water flushing events (HCDOP IAM, 2011). We suggest that such a high degree of physical forcing makes our study site highly dynamic, which can cause variability in ammonia oxidation rates. The effects of physical forcing most likely had a strong influence in the September 2011 (CB966) results, where ammonia oxidation rates were abnormally high at 50 m; at this time, the waters of Hood Canal were undergoing a deep-water flushing event.

Molecular and kinetic evidence for Archaeal ammonia oxidation

Our molecular study, which focused on *amoA*, is some evidence that AOA likely dominate ammonia oxidation in the Hood Canal fjord of Puget Sound, WA, USA. In Hood Canal, AOA *amoA* gene copies outnumbered those from β-AOB by 2.3 to 60-fold at all depths where ammonia oxidation occurred. Detected *amoA* transcripts were derived from AOA as no β-AOB *amoA* transcripts were found at any depth. High ratios of AOA/AOB *amoA* gene copy have also been detected in marine water column depth profiles, such as the Gulf of California (AOA/AOB = 37–217; Beman *et al.*, 2008), the California Current (AOA/AOB = 10–10 000; Santoro *et al.*, 2010) and North Pacific Subtropical Gyre (AOA/AOB = up to 100; Mincer *et al.*, 2007). In addition,

ammonia oxidation rates were significantly correlated with *amoA* transcript copies. These molecular-based results suggest that AOA are the dominant organisms carrying out active ammonia oxidation during our study.

Further evidence that AOA are the major contributors to ammonia oxidation at Hood Canal comes from a comparison of the physiological constants for the natural assemblage and the closely related marine AOA *N. maritimus* SCM1. The K_m for ammonia oxidation in the natural assemblage was 98 nmol l^{-1}, which is comparable to that of *N. maritimus* SCM1 (133 nmol l^{-1}; Martens-Habbena *et al.*, 2009). The lowest reported K_m for ammonia oxidation for cultivated AOB representatives is around 8 μmol l^{-1} (Knowles *et al.*, 1965; Ward, 1987), but the typical range for cultivated and natural assemblages of AOB is 0.05–14 mmol l^{-1} (Stehr *et al.*, 1995; Martens-Habbena *et al.*, 2009; Martens-Habbena and Stahl, 2011). Interestingly, the K_m for Hood Canal is similar to the K_m estimated by Olson (1981; <100 nmol l^{-1}) and Hashimoto *et al.* (1983; 150 nmol l^{-1}) for natural assemblages of ammonia oxidizers at oceanic sites. The similarity of the ammonia oxidation kinetics to *N. maritimus* SCM1 further supports the hypothesis that the vast majority of ammonia oxidation in the Hood Canal natural assemblage is being carried out by AOA.

Temperature sensitivity of AOA

The determination of ammonia oxidation rates requires an incubation step. At sea, this is sometimes conducted in on-deck incubators maintained with surface seawater that can be significantly warmer than the deep water being incubated (Ward and Kilpatrick, 1990; Clark *et al.*, 2008; Santoro *et al.,* 2010). We therefore examined the temperature sensitivity of ammonia oxidation rates by a natural assemblage. In all four temperature-manipulation experiments, the ammonia oxidation rate was not significantly different for amendment treatments in the 8–20 °C range, which is similar to the 8-year temperature range for depths of measurable ammonia oxidation at the site. Thus, no temperature correction was required for our rate profile. In two of the experiments (CB974, May 2012, and CB980, July 2012), ammonia oxidation rates were significantly affected only when incubation temperatures were well above and below the 8-year temperature range. Collectively, these results suggest that ammonia oxidation by AOA in our incubations is well regulated within the range of temperatures the organisms experience *in situ* (see Ward (2008) for discussion). Thus, we do not expect temperature to regulate ammonia oxidation rates at depth at any time of the year.

Ours is the first report of the impacts of experimental temperature on a natural assemblage dominated by marine water column AOA. Our results differ from the temperature response of some cultivated AOB and soil communities. The Q_{10} of 1.0 determined for Hood Canal AOA is much lower than that measured in the cultivated β-AOB *Nitrosomonas* sp. ($Q_{10} = 3.3$, temperature range: 10–20 °C; Helder and De Vries, 1983), in sandy soils ($Q_{10} = 5.0$, temperature range: 5–20 °C; Russell *et al.*, 2002) and in coastal marine sediments ($Q_{10} = 2.5$, temperature range 2–22 °C; Hansen *et al.*, 1981). Berounsky and Nixon (1990) reported higher Q_{10} ($Q_{10} = 6.8$–17.6, depending on the site, temperature range: 5–24 °C) for natural assemblage of ammonia oxidizers in Narragansett Bay. Our study results are most similar to that of Bianchi *et al.* (1997), who found no correlation between temperature and ammonia oxidation rates on a transect in the Indian sector of the Southern Ocean.

The lack of a significant influence of temperature upon marine ammonia oxidation rates may result from a combination of other limiting factors in the marine environment (Ward, 2008). It was not likely a result of either NH_4^+ depletion or kinetic substrate limitation. There was no evidence of substrate depletion in any of our incubations, based on calculations of total NH_4^+ oxidized during the incubation period (from concentrations in the incubation bottle and ammonia oxidation rate). Moreover, during the September 2011 (CB966) temperature experiment, ambient [NH_4^+] was 250 nmol l^{-1}, which was well over the K_m; even in this case of abundant substrate, there were no significant effects of temperature upon ammonia oxidation rates. Neither light nor salinity should have had an effect because the samples were collected from the aphotic zone and incubated in the dark, and salinity was similar to oceanic values and the same for all experiments (salinity = 29). Oxygen has the potential to limit ammonia oxidation rates, but we do not believe that oxygen was low enough to limit rates. Recent studies have detected AOA *amoA* gene copies and transcripts and ammonia oxidation rates in the presence of lower oxygen concentrations than found at our study site (Newell *et al.,* 2011; Bouskill *et al.,* 2012).

We suspect that low pH or trace metal concentrations at our study site may contribute to a lack of temperature sensitivity. pH can cause significant declines in nitrification (Beman *et al.,* 2011). Because the pH in Hood Canal (7.32–7.75; Feely *et al.,* 2010) is much lower than those pH values reported by Beman *et al.* (2011) to cause declines in rates, pH may be a factor that limits a temperature response. Also, the genome of cultivated *N. maritimus* revealed a high number on copper-dependent systems (Walker *et al.,* 2010), so it is possible that low free copper may be another factor that limits the response of ammonia oxidation rates to temperature.

Kinetic correction to ammonia oxidation rates

The establishment of K_m for the natural population allowed us to adjust measured ammonia oxidation

rates to account for potential deviations from *in situ* conditions during incubations, such as the increase in the size of the NH_4^+ pool resulting from $^{15}NH_4^+$ tracer addition. In our study, ammonia oxidation rates were reduced by 1–86% (Figure 4) when the suggested kinetic correction was applied, with the highest decrease in rate occurring at depths with low ambient NH_4^+. For ammonia oxidation rate studies, Ward (2011) suggested a $^{15}NH_4^+$ final concentration of 50–100 nmol l^{-1} for low NH_4^+ marine samples, and some studies have added up to 500 nmol l^{-1}. The corrected rate changes as a function of the $^{15}NH_4^+$ added and ambient NH_4^+ concentration. For example, if the concentration of added $^{15}NH_4^+$ is 50 nmol l^{-1}, rates will be reduced by 6.5% when ambient NH_4^+ is 200 nmol l^{-1} (approximately 2 * K_m), 16.5% when NH_4^+ is 100 nmol l^{-1} and 53% when NH_4^+ is 25 nmol l^{-1}. The effect is more pronounced when the concentration of $^{15}NH_4^+$ is higher; if the concentration of added $^{15}NH_4^+$ is 200 nmol l^{-1}, rates will be reduced by 16% when ambient NH_4^+ is 200 nmol l^{-1}, 33% when NH_4^+ is 100 nmol l^{-1} and 70% when NH_4^+ is 25 nmol l^{-1}. Conversely, if ambient NH_4^+ is higher (over 500 nmol l^{-1}), ammonia oxidation rates are negligibly affected by $^{15}NH_4^+$ tracer, as in the case at depths of 9, 12, 15, and 20 m in our study. Extensive studies of NH_4^+ concentration distributions in the ocean are rare, but most studies indicate that NH_4^+ concentrations vary from below detection to several hundred nmol l^{-1}. In three recent studies of oceanic ammonia oxidation, NH_4^+ was quite low, from below the detection limit (100 nmol l^{-1}) to 195 nmol l^{-1} (Beman *et al.,* 2008, 2012; Bouskill *et al.,* 2012). Assuming that most of the ammonia oxidation is carried out by AOA and that NH_4^+ concentrations are low in the ocean, many published ammonia oxidation rates are likely overestimates.

The application of Equation 3 to ammonia oxidation rates is important to determine more accurate rates, but the suggested method does have some potential limitations. First, K_m is expected to change with temperature or pH, provided that there are no other limiting factors. Second, an accurate measurement of low-level ambient NH_4^+ is essential in order to apply the kinetic correction to sites with low ambient NH_4^+. Finally, as seen in the 50-m depth at our study site in May 2012, the calculated ammonia oxidation rate is zero when ambient NH_4^+ is zero (or below detection limit). We do not believe that this indicates the absence of ammonia oxidation activity in these instances; instead, we propose that a rate calculated with the NH_4^+ detection limit represents an upper limit of the *in situ* rate.

Martens-Habbena *et al.* (2009) suggested that niche partitioning between AOA and AOB may result from the clade-specific enzyme kinetics of AMO, where AOA are capable of active ammonia oxidation in low NH_4^+ environments but AOB require higher ambient NH_4^+. Our study confirms this finding in a natural microbial assemblage, and we provided experimental evidence that the environmental factors that control the distribution of ammonia oxidation by natural assemblages of marine AOA and AOB may differ. We generated a Michaelis–Menten kinetics curve and determined that K_m was somewhat lower than pure cultures of cultivated AOA *N. maritimus* SCM1 and much lower than representative AOB cultures. Unlike AOB, temperature does not appear to control the distribution and activity of marine AOA at our study site. Given the recent discovery of natural populations of AOA and that salinity, light, oxygen concentration and pH all affect ammonia oxidation in AOB cultures and AOB-rich natural assemblages, we believe that future experiments to assess the controls of environmental factors over marine AOA activity and distribution may yield exciting new discoveries.

Conflict of interest

The authors declare no conflict of interest.

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