Limnol. Oceanogr., 58(6), 2013, 2037–2045

doi:10.4319/lo.2013.58.6.2037

Copper requirements of the ammonia-oxidizing archaeon *Nitrosopumilus maritimus* SCM1 and implications for nitrification in the marine environment

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Abstract

Ammonia oxidizing archaea (AOA) have recently been recognized as the primary nitrifiers in the marine environment; they thus play an important role in the nitrogen cycle. Available genome sequences of AOA indicate that numerous Cu-dependent enzymes are essential for both ammonia oxidation and electron transfer, suggesting a particularly high requirement for copper. However, our knowledge of the copper requirements of AOA and their response to copper limitation in the ocean is nonexistent. Here, we examine the copper requirements of the chemolithoautotrophic AOA *Candidatus Nitrosopumilus maritimus* SCM1 using a combination of the metal chelators ethylenediaminetetraacetic acid and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid and show that ammonia oxidation is limited at free cupric ion concentrations $< 10^{-12.7}$ mol L^{-1}, which are higher than concentrations frequently reported for many coastal and oceanic regimes. Prolonged exposure of cells to copper starvation for up to 6 d had no effect on the recovery of ammonia oxidation by *N. maritimus.* In addition, we present evidence that *N. maritimus* does not produce a copper-binding ligand (chalkophore) under copper limitation and therefore probably relies mainly on acquisition of copper ions from surrounding media. Copper limitation may be an important constraint on archaeal ammonia oxidation throughout the marine environment.

Ammonia oxidation is the first step of nitrification and is a critical component of the nitrogen cycle. In the oceans, autotrophic ammonia oxidation is carried out by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) and in anoxic environments by bacteria mediating the anammox process (Ward et al. 2011). In the marine environment, presumptive AOA usually outnumber AOB (Bouskill et al. 2012), suggesting that the previously unrecognized AOA play a major role in the nitrogen cycle (Konneke et al. 2005). The first isolation and description of an AOA, the mesophilic, chemolithoautotroph *Candidatus Nitrosopumilus maritimus* SCM1 (referred to henceforth as *N. maritimus*), has led to numerous efforts to culture additional AOA members and understand their metabolism and physiology (Konneke et al. 2005). To date, several different AOA from a variety of environments have been cultured (reviewed in Stahl and De La Torre 2012). Among these, *N. maritimus* remains the only described marine AOA available in pure culture and as such is becoming a model organism for developing an understanding of AOA physiology.

AOA and AOB derive energy from the oxidation of NH_3 to NO_2^-. The first step in the reaction sequence, the oxidation of ammonia to hydroxylamine, is catalyzed by the membrane-bound enzyme ammonia monooxygenase (AMO) encoded by the *amoABC* gene cluster. No crystal structure has been reported for AMO; therefore, most of its mechanistic and structural features have been deduced from the homologous metalloenzyme, the particulate methane monooxygenase (pMMO) of methanotrophs (Arp et al. 2002). Like pMMO, AMO is believed to contain mono- and di-nuclear copper centers, one of which is believed to be the site of substrate binding (Lieberman and Rosenzweig 2005). Thus, copper is a required cofactor for the oxidation of ammonia in both AOA and AOB (Walker et al. 2010). The AOA also possess an unusually high number of Cu-containing metalloenzymes, some of which are believed to be involved in electron transfer. For example, the genome of *N. maritimus* encodes eight putative multi-copper oxidases (MCO) and numerous copper-rich halo- and plastocyanin enzymes (Walker et al. 2010). The genomes of two other AOA, *Cenarchaeum symbiosum* and *Nitrosoarchaeum limnia,* also encode multiple putative proteins belonging to the plastocyanin and blue copper protein families (Hallam et al. 2006; Blainey et al. 2011). The alleged reliance on copper-based electron transfer enzymes in AOA contrasts with the use of Fe-rich *c*-type cytochromes by AOB in the respiratory pathway for the oxidation of hydroxylamine, the product of the AMO. This difference suggests a higher copper requirement for AOA. However, to date, no study has examined the copper requirements of AOA nor demonstrated the effect of copper limitation or toxicity on ammonia oxidation by AOA.

In addition to its involvement in ammonia oxidation, copper is a required trace metal in numerous general and specialized physiological functions, including electron transfer, oxygen transport, superoxide detoxification, and denitrification. Yet, like other essential trace metals such as iron, it is present in scarce quantities in the ocean and is highly complexed by organic ligands that reduce the inorganic dissolved free-metal concentration to $<$ pmol L^{-1} (Coale and Bruland 1988; Moffett and Dupont 2007).

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Table 1. Synthetic seawater medium composition used for trace-metal limitation of *Candidatus Nitrosopumilus maritimus* SCM1. Units are mol L^{-1} throughout.

	Final concentration	Final concentration used in this study	Final composition used previously
Salts			
NaCl	0.44	—	—
$MgSO_4 \cdot 7H_2O$	0.0202	—	—
$MgCl_2 \cdot 6H_2O$	0.025	—	—
$CaCl_2 \cdot 2H_2O$	0.0102	—	—
$NaHCO_3$	0.002	—	—
KBr	8.4×10^{-4}	—	—
H_3BO_3	5.0×10^{-7}	—	—
Nutrients			
KH_2PO_4	1.47×10^{-5}	—	—
NH_4Cl	0.001	—	—
Buffer			
HEPES (pH 7.5)	0.01	—	—
Chelators and trace metals			
TETA	—	1.0×10^{-6}	—
EDTA*	—	7.50×10^{-6}	7.50×10^{-6}
$FeCl_3 \cdot 6H_2O$*	—	7.50×10^{-6}	7.50×10^{-6}
$CuCl_2 \cdot 2H_2O$†	—	1.0×10^{-7}	1.1×10^{-9}
$NiCl_2 \cdot 6H_2O$	—	1.0×10^{-7}	1.0×10^{-7}
$ZnSO_4 \cdot 7H_2O$	—	5.0×10^{-7}	5.0×10^{-7}
$CoCl_2 \cdot 6H_2O$	—	8.0×10^{-7}	8.0×10^{-7}
$MnCl_2 \cdot 4H_2O$	—	5.0×10^{-7}	5.0×10^{-7}
$Na_2MoO_4 \cdot 2H_2O$	—	1.5×10^{-7}	1.5×10^{-7}

* Iron and ethylenediaminetetraacetic acid (EDTA) were added as an Fe-EDTA stock.
† Copper was added either as a Cu-EDTA stock when no tetraacetic acid (TETA) was added or as a Cu-TETA stock when TETA was used.

Copper can also be toxic at high concentrations as it catalyzes formation of reactive oxygen species.

Here we examine the copper requirements of the mesophilic chemolithoautotroph *N. maritimus* utilizing a trace-metal ion buffer system that combines the chelators ethylenediaminetetraacetic acid (EDTA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) to circumvent inhibitory effects induced by high concentrations of EDTA. In addition, we examine the ability of *N. maritimus* to withstand prolonged copper starvation scenarios and its effect on ammonia oxidation. Finally, we investigate the ability of *N. maritimus* to produce a copper-binding ligand under copper-limiting and copper-replete conditions.

Methods

Archaeal growth and culture manipulations—*Nitrosopumilus maritimus* SCM1 was maintained at 30°C in the dark in synthetic seawater medium (Table 1). Cultures were regularly monitored for bacterial contamination by checking for bacterial growth in Zobell marine broth (Zobell 1941) and by using Sybr Green I staining followed by microscopy. Because *N. maritimus* cells are small (typically 0.4–0.6 μm in length), cells were visually distinguishable from bacteria (typically 1 μm in length).

Nitrite measurements and cell counts—Growth was measured either by measuring nitrite concentrations spectrophotometrically (Strickland and Parsons 1972) or by performing cell counts on the day of sampling using Sybr Green I staining and epi-fluorescent microscopy (Nikon Eclipse 80i) as described previously (Lunau et al. 2005).

Media preparation—Milli-Q water (> 18.2 MΩ cm) was used for all rinsing and media preparation. To reduce copper contamination, polycarbonate bottles were used to culture *N. maritimus* and autoclaving was avoided for all procedures. Instead, filter-sterilization was used to sterilize all stocks and media as described below. Although using microwave radiation to sterilize media for trace-metal limitation studies is common, we found that *N. maritimus* is sensitive to microwave treatment. Therefore, we only used microwave radiation to sterilize culture bottles.

All components listed in Table 1, with the exception of chelators and trace metals, were dissolved, combined, and passed through a column packed with Chelex-100 resin (Bio-Rad) to remove contaminating trace metals (Price et al. 1988/1989). Chelexed media were filter-sterilized using sterile filtration units (Nalgene) with 0.1 μm pore-size polyethersulfone (PES) membranes, which were pre-soaked with 1.0 mol L^{-1} HCl (Optima grade; Fisher) and washed thoroughly with sterile Milli-Q water. Media were stored at 4°C in polycarbonate bottles that were soaked for 24–48 h in 10% v : v HCl (Trace Metal grade; Fisher), rinsed three times with Milli-Q water, soaked for 24 h in 1 mol L^{-1} HCl (Optima grade), and rinsed again with Milli-Q water to remove surface-bound metals. Bottles were then filled with Milli-Q water and sterilized by microwave radiation.

Trace-metal and chelator stocks were filter-sterilized using 0.2 μm pore-size syringe filters (PES membrane) that were pre-washed with 1 mol L^{-1} HCl (Optima grade) and rinsed with Milli-Q water and media. Stored media were dispensed into polycarbonate bottles prepared as described above and chelators and trace metals were added 24 h prior to inoculation to ensure reaching equilibrium (Price et al. 1988/1989). Copper was added as the Cu-TETA complex when TETA was used for limitation or as the Cu-EDTA complex in experiments lacking TETA. Iron and EDTA were added as a pre-complexed concentrated stock and the rest of the trace metals were combined and added as a concentrated stock. The final pH of the medium was 7.5. Trace-metal speciation was calculated using MINEQL (Environmental Research Software) with the incorporation of stability and protonation constants for TETA (Clarke and Martell 1991*a,b;* Chaves et al. 1992).

Developing an optimal EDTA-TETA buffer system—The synthetic seawater medium originally developed to culture *N. maritimus* contains 7.5 μmol L^{-1} of EDTA as the sole metal ion buffer (Martens-Habbena et al. 2009). EDTA is supplemented as a ferric-EDTA complex, with no excess EDTA added to control iron speciation or buffer other important trace metals such as copper (Table 1). We first attempted to use higher EDTA concentrations to better control trace-metal speciation. However, initial experiments that varied total concentrations of EDTA (11–100 μmol L^{-1}) while adjusting the free-metal concentrations to prevent metal limitation showed strong inhibition of ammonia oxidation as a function of increasing $[EDTA]_{total}$ (data not shown). We instead used the strong metal chelator TETA as an additional trace-metal ion buffer (Moffett et al. 2012), while keeping EDTA as in the original recipe. TETA consists of a tetraazamacrocycle that resembles a porphyrin ring with four near co-planar nitrogen donor atoms, making it a more effective metal chelator than EDTA due to the macrocylic effect (Lippard and Berg 1994). In addition, the macrocycle in TETA is functionalized with four terminal carboxylate moieties that increase the solubility of the ligand and further facilitate metal complexation (Clarke and Martell 1991*a,b*; Chaves et al. 1992). These structural features allowed us to only add 1 μmol L^{-1} TETA in addition to the 7.5 μmol L^{-1} Fe-EDTA present originally in the medium to attain low free cupric ion concentrations (Table 1).

At the lowest $[Cu]_{total}$ added (1 nmol L^{-1}), MINEQL predicts that $\sim$ 90% of the Cu is complexed by TETA. This TETA-complexed fraction decreases to 80% at the highest $[Cu]_{total}$ (750 nmol L^{-1}), with the remainder of the Cu being complexed with EDTA in both cases. This distribution changes because Fe also has a high affinity for TETA, although most of the Fe remains complexed by EDTA. Ideally, we would have a significant excess of TETA over the total metal concentrations in the media, so that the distribution of metals between EDTA and TETA would not change. A large excess of TETA would also ensure that the other metals in the media are strongly complexed by TETA. In practice we found that, like with large excess of EDTA, *N. maritimus* would not grow at higher TETA concentrations (data not shown), even when Cu was adjusted to maintain Cu-replete conditions. We do not know if this is because these chelators were inhibiting ammonium oxidation or were binding the other metals in the media, such as zinc and cobalt, and inducing deficiencies there. Chelator-mediated inhibition of ammonium oxidation is widely reported (Hu et al. 2003), even for weak chelators like ethylenediamine, and it appears that this is a chronic problem when working with strongly buffered media.

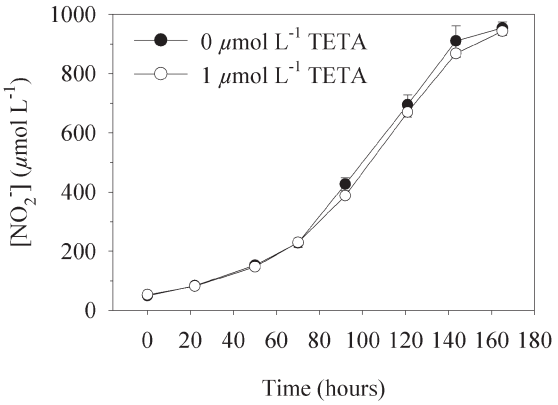

Fig. 1. The effect of TETA on ammonia oxidation of *Candidatus Nitrosopumilus maritimus* SCM1. Cultures were grown in traditional medium containing either no TETA addition (closed symbols) or 1 μmol L^{-1} TETA with 100 nmol L^{-1} Cu (open symbols). Error bars represent the range of duplicate treatments.

Although there is a possibility that other trace metals in the media that are not chelated in our system could act antagonistically under Cu-limiting conditions and cause a toxic effect, we believe this is an unlikely possibility. To our knowledge, there is no precedence of any of the unchelated transition metals in our media acting antagonistically to affect Cu requirements of microbes. Cu^{2+} is at the top of the Irving-Williams Series, leading Cu^{2+} to form very tight complexes with peptides relative to all other first-row divalent transition metals (Lippard and Berg 1994). Consequently, Cu^{2+} transporters are highly selective for Cu^{2+} over other divalent metals such as Mn^{2+} (Tottey et al. 2008).

In order to evaluate whether the modified media containing 1 μmol L^{-1} TETA had side effects of its own, we added 100 nmol L^{-1} copper (Table 1) to obtain a free cupric ion concentration, $[Cu^{2+}]$, of $10^{-11.7}$ mol L^{-1}, similar to Cu-replete growth in other microorganisms (Sunda and Huntsman 1995; Moffett and Brand 1996). TETA appears to have no effect on the growth of *N. maritimus*, as shown in Fig. 1.

To achieve copper limitation, cells were grown in the presence of a range of total copper concentrations (Table 2). Batch cultures were acclimated to the different copper concentrations by transferring 1 mL inoculum of cultures in the exponential growth phase to new media (100 mL). Acclimation was achieved when the specific growth rate (measured as nitrite production) of three consecutive cultures did not vary by $> 10\%$.

Copper starvation—All cultures were inoculated from cells grown in trace-metal clean media without TETA and

Table 2. Total and free cupric ion concentrations and specific growth rates (μ) for n samples of *N. maritimus* cultures acclimated to different copper concentrations using TETA and EDTA as trace-metal ion buffers. Free cupric ion concentrations were calculated using MINEQL. Values reported are means ± standard deviation.

Total added Cu (nmol L^{-1})	Free Cu (mol L^{-1})	μ (d^{-1})	n
0	$\leq 1.0\times10^{-15}$	0.0	2
1	1.7×10^{-14}	0.34±0.01	6
5	8.5×10^{-14}	0.47±0.02	4
10	1.7×10^{-13}	0.57±0.01	3
25	4.4×10^{-13}	0.60±0.01	3
50	9.1×10^{-13}	0.62±0.01	3
100	2.0×10^{-12}	0.61±0.01	9
250	6.3×10^{-12}	0.60±0.01	3
500	1.9×10^{-11}	0.58±0.01	3
750	4.4×10^{-11}	0.56±0.01	3

with 0.5 nmol L^{-1} Cu-EDTA. To reduce copper carryover from the inoculant into the copper-deficient cultures, all cultures were inoculated with a relatively low inoculum (0.25 mL instead of 1 mL inoculum per 100 mL media). Cu-starved cultures were grown in the same media as the inoculum but with no Cu-EDTA. To each set of Cu-starved cultures, 11 nmol L^{-1} Cu-EDTA were added at the indicated times.

Ligand production analysis—Cultures were grown in trace-metal clean media without TETA and with either 0.5 nmol L^{-1} Cu-EDTA (Cu-limited) or 11 nmol L^{-1} Cu-EDTA (Cu-replete). TETA was omitted because it interferes with the electrochemical analysis. Cells were filtered onto sterile filtration units (Nalgene) with 0.1 μm pore-size PES membranes pre-washed with 1.0 mol L^{-1} HCl (Optima) at mid-exponential growth for Cu-limited cultures ($\sim$ 150 μmol L^{-1} NO_2^-) or at the same cell density as Cu-limited for Cu-replete cultures. The supernatant from the cultures was stored at 4°C in 1 liter fluorinated high-density polyethylene (FLPE, Nalgene) bottles that were consecutively soaked for 24 h in 5% Citranox acid detergent bath (Alconox), 24 h in a 10% HCl bath (Baker Instra-Analyzed; VWR), filled with 10% HCl and baked at 60°C for 48 h, and filled with clean 0.1 mol L^{-1} quartz-distilled HCl (Optima, Fisher) and baked at 60°C again for 48 h. Bottles were thoroughly rinsed at least five times with Milli-Q water in between each step. Media with no cell cultures that were treated similarly were used as negative controls.

The [Cu^{2+}] concentrations, ligand concentrations ([L]), and conditional stability constants (K) were determined using a competitive ligand exchange–adsorptive cathodic stripping voltammetry (CLE–ACSV) method (Buck and Bruland 2005; Moffett and Dupont 2007). Salicylaldoxime (SA; ≥ 98%; Aldrich) was used as the competing ligand. The concentration of SA in these measurements was adjusted depending on the strength of Cu complexation. Only 2.5 μmol L^{-1} SA was used to measure [Cu^{2+}] and [L] for the Cu-replete samples, where the Cu was not strongly bound. In the original media and Cu-limited samples, 10 μmol L^{-1} and 5 μmol L^{-1} SA, respectively, were used because the ambient Cu in the media was highly refractory (*see* below). The measurements were carried out on a BioAnalytical Systems (BAS*i*) Controlled Growth Mercury Electrode set to the Static Mercury Drop setting (drop size: 14) and interfaced with a BAS*i* Epsilon ε2 voltammetric analyzer. The titration data were analyzed using a single ligand model that was a nonlinear fit to a Langmuir adsorption isotherm (Gerringa et al. 1995), and we then used nonlinear regression analysis (Moffett and Dupont 2007) to solve for K, [L], and [Cu^{2+}].

Measurement and speciation of residual Cu in media—After cleaning the media with Chelex-100, a residual Cu concentration of 5 nmol L^{-1} was determined by inductively coupled plasma mass spectrometry (ICP–MS), as described previously (Jacquot et al. 2013). We investigated the speciation of this residual Cu by CSV and were not able to discern a measurable peak, even at [SA] = 10 μmol L^{-1}. Therefore [Cu^{2+}] was below our detection limit of 10^{-15} mol L^{-1}. These findings implied that the residual Cu did not contribute to bioavailable Cu in the media because it was not exchangeable with the CSV ligand and likewise would not have been removed by the Chelex-100 treatment. Therefore, we did not include this residual Cu in subsequent speciation calculations. A titration of the media revealed no evidence of any excess ligand in the media, so it seems likely that the ambient copper was associated with some refractory, filterable phase, such as a colloid, rather than being complexed by strong ligands that might have been introduced to the salts we used during manufacturing.

Results

Effect of copper limitation on ammonia oxidation rates and cell growth—Cultures of *N. maritimus* grown in our modified media, which consists of the standard EDTA medium and supplemented with TETA and $10^{-11.7}$ mol L^{-1} [Cu^{2+}] (Table 1), showed no difference in ammonia oxidation rates (measured as nitrite production) relative to cultures grown without TETA (Fig. 1). Using this modified media, *N. maritimus* cultures were grown with trace metal-clean techniques (*see* Methods) under three conditions: Cu-replete (100 nmol L^{-1} Cu_{Total}), Cu-limited (5 nmol L^{-1} Cu_{Total}), and Cu-deficient (0 nmol L^{-1} Cu_{Total}; refer to Table 2 for [Cu^{2+}] values corresponding to these total concentrations), the latter condition being a measure of background Cu that could affect observed Cu-limited ammonia oxidation rates. Ammonia oxidation and cell density showed strong responses to copper bioavailability (Fig. 2A). Cu-replete cultures consumed all the ammonia in the media to produce 1 mmol L^{-1} nitrite within 200 h. The same cultures exhibited a maximum cell density of 5.1×10^7 cells mL^{-1} and a specific growth rate, μ, of 0.64 ± 0.01 d^{-1}. In contrast, Cu-limited cultures produced < 0.3 mmol L^{-1} nitrite within the same timeframe; they reached a maximum cell density of 2.0×10^7 cells mL^{-1} and μ of 0.44 ± 0.02 d^{-1}, after which the nitrification rate and increase in cell density slowed. Background copper contamination did not contribute to the observed ammonia

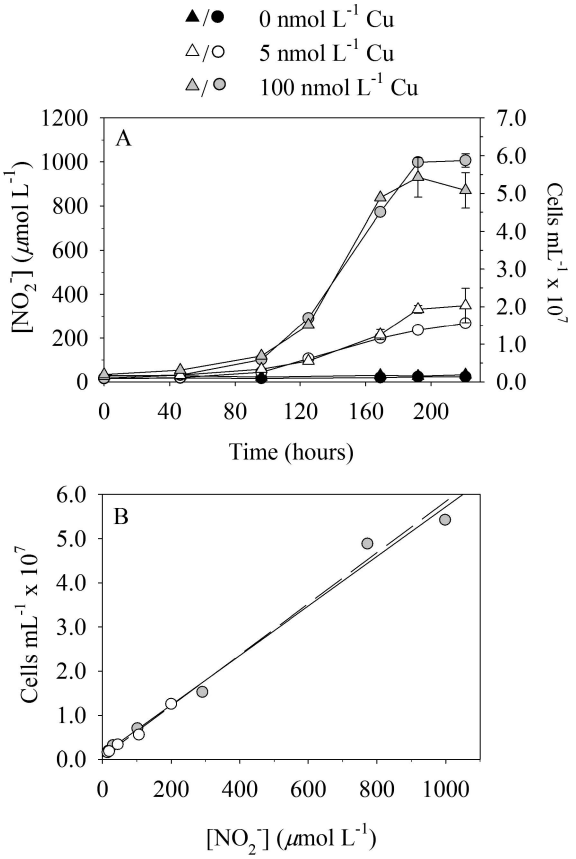

Fig. 2. The effect of copper limitation on ammonia oxidation rates and cell counts of *N. maritimus*. Batch cultures were acclimated to 0, 5, or 100 nmol L^{-1} total Cu (refer to Table 2 for the corresponding $[Cu^{2+}]$). (A) Nitrite concentration (circles) and cell counts (triangles) are shown for each Cu concentration. Error bars represent the range of duplicate treatments. (B) Correlation and corresponding linear regression between cell counts and nitrite concentrations for 5 nmol L^{-1} Cu (open circles, dashed line) and 100 nmol L^{-1} Cu (grey circles, solid line) with correlation coefficients of 0.95 and 0.99, respectively.

oxidation rates because Cu-deficient cultures that only contained background concentrations of copper failed to produce any significant amounts of nitrite while cell number dropped slightly for the duration of the experiment (Fig. 2A). Cell densities correlated with nitrite concentrations throughout most of the lifetime of the cultures under both Cu-replete ($r^2 = 0.99$) and Cu-limited conditions ($r^2 = 0.95$; Fig. 2B). This strong correlation is consistent with prior studies that have used nitrite concentrations in lieu of cell counts to measure specific growth rates (Martens-Habbena et al. 2009).

Effect of free cupric ion concentrations on ammonia oxidation—To examine the copper requirements of *N. maritimus* and whether free cupric ion concentrations ($[Cu^{2+}]$) alter ammonia oxidation rates, cultures were acclimated to different $[Cu^{2+}]$ (Table 2). Overall, growth of *N. maritimus* cultures, even at the lowest $[Cu^{2+}]$ examined, showed consistent reproducibility among replicates and across several acclimations (data not shown).

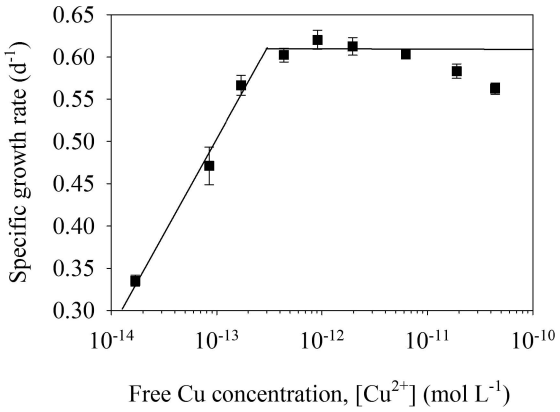

Fig. 3. The effect of free cupric ion concentrations on ammonia oxidation rates of *N. maritimus*. Batch cultures were acclimated to different $[Cu^{2+}]$ (*see* Methods). Specific growth rates were calculated from nitrite production. Free cupric ion concentrations were calculated using MINEQL. Error bars represent the standard deviation (SD) of biological replicates from Table 2.

Free cupric ion concentrations had a major effect on ammonia oxidation of *N. maritimus* (Fig. 3). Specific growth rates decreased significantly from 97% to 55% of maximum specific growth rate, μ_{max}, with decreasing free cupric ion concentrations between $10^{-12.3}$ and $10^{-13.7}$ mol L^{-1}, respectively. Increasing $[Cu^{2+}]$ above $10^{-12.3}$ up to $10^{-11.2}$ mol L^{-1} had no significant effect on μ, indicating that *N. maritimus* was Cu-replete in this regime. Higher $[Cu^{2+}]$ than $10^{-11.2}$ and up to $10^{-10.3}$ mol L^{-1} caused a decrease in μ to 90% of μ_{max}, indicating a toxicity effect (Table 2; Fig. 3).

Effect of copper starvation on ammonia oxidation—AOA likely encounter variable pulses of copper in the marine environment that may cause them to experience prolonged periods of copper limitation. To examine whether AOA ammonia oxidation can recover after prolonged periods of copper starvation, we exposed *N. maritimus* to copper-deficient conditions and supplemented cultures after 0, 2, 4, or 6 d with 11 nmol L^{-1} Cu-EDTA. In all cases, ammonia oxidation recovered within 24 h of copper addition. Specific growth rates for the 0, 2, 4, and 6 d Cu additions after recovery from starvation did not vary appreciably and were 0.62, 0.59, 0.59, and 0.58 d^{-1}, respectively (Fig. 4). Specific growth rates do not resolve the question of whether all starving cells retain the capacity to initiate growth following copper addition. However, the time interval between copper addition and ammonia depletion (stationary phase), which is nearly constant between the 2, 4, and 6 d treatments, indicates that all starving cells do retain the capacity to oxidize ammonia following copper addition (Fig. 4).

Cu-binding ligand production as a function of copper availability—The basic premise behind our culture work is that the free cupric ion concentration is a good index of Cu bioavailability. However, there is a large body of evidence that copper can be acquired by chalkophores (analogous to siderophores) that are produced by microbes and form

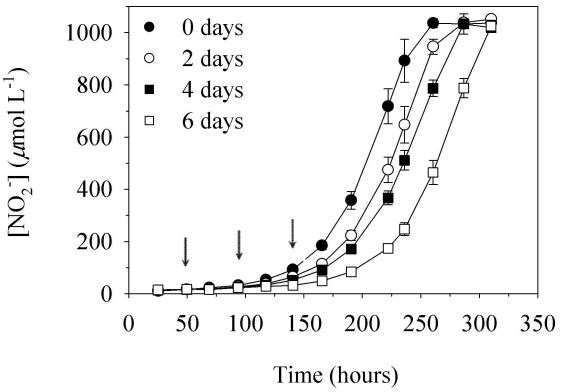

Fig. 4. The effect of prolonged copper starvation on ammonia oxidation of *N. maritimus.* Cells were inoculated into Cu-deficient media and 11 nmol L^{-1} Cu-EDTA were added to different cultures every 48 h. Arrows indicate the time Cu-EDTA was added to cultures after 48 h (open circles), 96 h (closed squares), and 144 h (open squares) except for '0 days,' which had Cu-EDTA added at time 0 (closed circles). Error bars represent the range of duplicate treatments.

biologically available complexes. So far, this Cu acquisition mechanism has been fully characterized only for methane-oxidizing bacteria (Kim et al. 2004). We decided to examine our spent-culture media for evidence of a Cu-binding ligand produced by *N. maritimus,* particularly under copper limitation. Spent media from *N. maritimus* cultures grown under Cu-replete or Cu-limiting conditions as well as blank media were analyzed for the presence of Cu(II)-binding ligands using CLE–ACSV. The spent media of Cu-limited cultures were indistinguishable from the blank media. In both cases, the residual copper in the media ($\sim$ 5 nmol L^{-1}; *see* Methods) was inert to exchange with the competing ligand, SA, under our experimental conditions. In contrast to the residual Cu, all of the added Cu in the titration was 100% complexed by SA, even at the lowest SA concentrations we used, a finding identical to that in the original media. These findings indicate that there was no production of a ligand within the culture and thus there is no evidence that *N. maritimus* produces any Cu(II)-binding ligands under copper limitation. In contrast, under Cu-replete conditions, *N. maritimus* produced a Cu(II)-binding ligand with an average log stability constant of 13.1 and a total ligand concentration ranging from 11 nmol L^{-1} to 17 nmol L^{-1} (equivalent to 2–3 amol $cell^{-1}$; Table 3).

Discussion

Initial attempts to control copper speciation with EDTA (11–100 μmol L^{-1}) proved lethal to *N. maritimus,* likely due to an unexpected toxic effect of EDTA. Because the free metal concentrations were adjusted to avoid metal limitation of *N. maritimus,* the toxic effect of EDTA may be related to scavenging copper from surface-bound enzymes such as AMO, thus affecting ammonia oxidation. Others have noted the secondary negative effects of high concentrations of EDTA on microorganisms (Muggli and Harrison 1996). EDTA and other ethylenediamine-based chelators have also been reported to inhibit ammonia oxidation of AOB in nitrifying enrichment reactors, albeit in the millimolar concentration range (Hu et al. 2003).

Table 3. Ligand concentrations and conditional stability constants, (log *K*), of *N. maritimus* cultures grown under different copper conditions. Ranges were obtained from biological duplicates.

N. maritimus growth condition	log *K*	[*L*]* (nmol L^{-1})	SA concentration† (μmol L^{-1})
Cu-limited	14.1–14.5	6.3–6.9	5.0
Cu-replete	12.8–13.4	11–17	2.5
Blank media	14.1–14.3	5.2–5.6	5.0

* Cultures were harvested at similar cell densities to allow comparison of [*L*] values between the two treatments.

† Salicylaldoxime (SA) concentrations highlight different detection windows used for each sample, which indicate that each growth condition was dominated by a different class of ligands. Window of detection is defined here as the optimal [SA] to obtain the lowest detection limit of electrode signal with minimal error propagation in calculating [Cu^{2+}].

In order to assess the importance of copper limitation to AOA in the environment and to compare copper limitation of *N. maritimus* against other microorganisms, we define a [Cu^{2+}] of $10^{-13.0}$ mol L^{-1} (equivalent to 76% of μ_{max}) as the Cu-limiting threshold for *N. maritimus.* This Cu-limiting threshold is significantly higher than some denitrifying bacteria ($< 10^{-16}$ mol L^{-1}) that—like *N. maritimus*—possess copper metalloenzymes essential to their energy production and denitrifying activity (Moffett et al. 2012). The *N. maritimus* Cu-limiting threshold is also higher than that of the coastal diatoms *Chaetoceros decipiens* and *Thalassiosira weissflogii* and the coccolithophore *Emiliania huxleyi,* which grow at 77%, 76%, and 88%, respectively, of their μ_{max} at [Cu^{2+}] $\sim 10^{-15}$ mol L^{-1} (Sunda and Huntsman 1995; Annett et al. 2008). In contrast, the oceanic diatom *T. oceanica*, achieves 65% of its μ_{max} at [Cu^{2+}] $\sim 10^{-13.9}$ mol L^{-1} (Peers et al. 2005), a value that is similar to *N. maritimus.* The disparity of copper requirements between coastal and oceanic species of diatoms is due to the requirement of the latter for plastocyanin, a Cu-containing protein involved in electron transfer between cytochrome b_6/f and Photosystem I. Plastocyanin is absent from coastal species that instead use cytochrome c_6. A reliance on copper enzymes allows oceanic species to thrive at lower iron concentrations than coastal species and is thought to be an adaptation of oceanic diatoms to the low iron bioavailability characteristic of high-nutrient–low-chlorophyll regions (Peers and Price 2006).

Like oceanic diatoms, *N. maritimus* and other AOA contain a large number of Cu-containing proteins involved in electron transfer and lack the typical Fe-containing *c*-type cytochrome proteins present in AOB. For example, *N. maritimus* contains numerous putative periplasmic MCOs and plastocyanin and blue copper domain proteins that are hypothesized to function in lieu of the missing Fe-containing *c*-type cytochromes from the electron transfer chain (Walker et al. 2010). The lack of *c*-type cytochromes and the enrichment of MCOs and other Cu-containing proteins have also been noted in two other AOA genomes (Hallam et al. 2006; Blainey et al. 2011). It is likely that

Table 4. Examples of sites in the marine environment where free cupric ion concentrations may be limiting to AOA.

Location	Depth (m)	log [Cu^{2+}]	Reference
Oceanic			
Northeast Pacific	surface	−13.8	Coale and Bruland 1988
North Pacific (multiple sites)	20–150	~−14.0	Coale and Bruland 1990
Sargasso Sea	60	−15.4	Van Den Berg and Donat 1992
North Sea	8	−13.7	Van Den Berg and Donat 1992
Bering Sea	1500	−13.7	Moffett and Dupont 2007
Coastal and estuaries			
Tamar estuary, UK	surface	−16.2–−18.2	Van Den Berg et al. 1990
San Francisco Bay (multiple sites), CA	—	−13.8–−15.5	Buck and Bruland 2005
Antarctic Peninsula region	surface	<−14.0	Bundy et al. 2013
Waquoit Bay, MA	surface	−13.8	Moffett et al. 1997
Hood Canal, WA	3–18 m	−13.7–−14.5	J. Jacquot unpubl.

AOA have a higher copper requirement than AOB similar to oceanic vs. coastal diatoms. Whether iron bioavailability drives the different copper requirements of AOA and AOB is not clear. The fact that AOA and AOB are prevalent throughout the marine environment in coastal and open-ocean regions as well as marine sediments, estuaries, and soils that have strikingly different iron concentrations suggests that the reliance of *N. maritimus* on copper metalloenzymes may not necessarily be an adaptation to low iron bioavailability.

The Cu-limiting threshold for *N. maritimus* reported here ($10^{-13.0}$ mol L^{-1}) is higher than [Cu^{2+}] measured in many oceanic environments, which suggests that copper bioavailability can potentially limit archaeal nitrification. For example, [Cu^{2+}] measured in surface waters of the Northeast Pacific, at multiple depths in the North Pacific, in the North Sea, and the Sargasso Sea were found to be significantly lower than $10^{-13.0}$ mol L^{-1} (Table 4; Coale and Bruland 1988, 1990; Van Den Berg and Donat 1992). In addition, [Cu^{2+}] measured in various estuaries were also lower than $10^{-13.0}$ mol L^{-1}, despite the presence of high levels of total copper (Table 4; Van Den Berg et al. 1990; Moffett et al. 1997; Buck and Bruland 2005). These [Cu^{2+}] are similar to the values reported in Hood Canal, a fjord in Washington that is dominated by AOA (Table 4; J. Jacquot unpubl.). These data indicate that AOA are potentially limited by copper bioavailability in both oceanic and estuarine basins, particularly within the euphotic zone.

Levels of [Cu^{2+}] that exceed the $10^{-13.0}$ mol L^{-1} threshold have also been reported from other coastal and oceanic regimes, including the euphotic zone of the Sargasso Sea (Moffett 1995) and Narragansett Bay (Bruland et al. 2000). Moreover, [Cu^{2+}] can vary significantly in regimes exhibiting seasonal variability in primary production, such as the Sargasso Sea (Moffett 1995). Therefore, though we anticipate that copper limitation may be widespread, we also anticipate considerable spatial and temporal variability in its effect upon ammonia oxidation. In addition, *N. maritimus* was isolated from an aquarium that likely was not Cu-limiting (Stahl and De La Torre 2012). Therefore, the Cu-limiting threshold for *N. maritimus* may not necessarily apply to all AOA populations, particularly in the open ocean. These populations may have evolved high-affinity Cu-uptake systems that could offset the low copper levels they encounter.

There was no evidence for production of strong ligands such as chalkophores in the Cu-limited media. The ligand produced by *N. maritimus* under copper-replete conditions is unlikely to be a true chalkophore, because by analogy with siderophores, production of chalkophores should be repressed under replete conditions. The production of Cu-binding ligands has been demonstrated in cultures of marine prokaryotic and eukaryotic phytoplankton (Moffett and Brand 1996) and conditional stability constants are similar to those reported here (~ 10^{12}–10^{13}), significantly lower than chalkophores. The high and variable ligand concentrations found in the Cu-replete media (Table 3) suggest it is a general metabolic byproduct produced by *N. maritimus* during normal cell growth. The role of this biomaterial in copper acquisition is unclear though previous studies have reported the production of such material, mainly exopolysaccharides (EPS), by phytoplankton in response to limitation by another trace metal, iron (Hassler et al. 2011). Acidic EPS production by bacteria and phytoplankton have long been hypothesized to play a role in trace-metal speciation and acquisition owing to the affinity this heterogeneous pool of ligands has for metals such as iron and copper (Hassler et al. 2011).

N. maritimus possesses two putative divalent heavy-metal cation transporters (Nmar_1130 and Nmar_1662), each adjacent to a multi-copper oxidase (Nmar_1131 and Nmar_1663) in two separate operon-like arrangements in the genome, implicating these transporters in Cu^{2+} assimilation. Together, these observations argue against ligand production under copper-replete scenarios when diffusion limitation is unlikely to occur. Although *N. maritimus* does not appear to produce a chalkophore under Cu limitation, other AOA populations, particularly in Cu-limited regions may be capable of producing chalkophores and/or acquire Cu from organic ligands. Future research should focus on characterizing potential similarities or differences between *N. maritimus* and other AOA isolates with respect to copper acquisition mechanisms.

These findings have wide implications for the nitrogen cycle, particularly where copper limitation can be alleviated by anthropogenic and/or aeolian inputs as occurs in coastal

zones and estuaries. These observations also raise the possibility that copper bioavailability may be an overlooked controlling factor of nitrification in the euphotic zone of estuaries and in high-nutrient–low-chlorophyll regions of the oceans, regions in which low rates of ammonia oxidation have been previously attributed primarily to photo-inhibition of ammonia oxidizers. Future research should focus on characterizing the response of other AOA representatives to copper bioavailability, the influence of light and copper on ammonia oxidation by AOA in the euphotic zone of copper-limiting environments, and novel copper acquisition mechanisms that AOA may possess to alleviate potential copper limitation in the oceans.

Acknowledgments

The authors would like to thank two anonymous reviewers whose insight and suggestions greatly improved the paper.

This work was funded in part by the U.S. National Science Foundation grants Molecular and Cellular Biosciences-0920741 and Ocean Sciences-1046017.

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Associate editor: Mary I. Scranton

Received: 13 February 2013
Accepted: 23 July 2013
Amended: 31 July 2013