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Original Article

Copper limiting threshold in the terrestrial ammonia oxidizing archaeon *Nitrososphaera viennensis*

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ABSTRACT

Ammonia oxidizing archaea (AOA) inhabiting soils have a central role in the global nitrogen cycle. Copper (Cu) is central to many enzymes in AOA including ammonia monooxygenase (AMO), the enzyme involved in the first step of ammonia oxidation. This study explored the physiological response of the AOA soil isolate, *Nitrososphaera viennensis* (EN76^T) to Cu-limiting conditions in order to approach its limiting threshold under laboratory conditions. The chelator TETA (1,4,8,11-tetraazacyclotetradecane N, N′, N″, N‴-tetraacetic acid hydrochloride hydrate) with selective affinity for Cu^{2+} was used to lower bioavailable Cu^{2+} in culture experiments as predicted by thermodynamic speciation calculations. Results show that *N. viennensis* is Cu-limited at concentrations $\leq 10^{-15}$ mol L^{-1} free Cu^{2+} compared to standard conditions (10^{-12} mol L^{-1}). This Cu^{2+} limiting threshold is similar to pure cultures of denitrifying bacteria and other AOA and AOB inhabiting soils, freshwaters and sewage ($<10^{-16}$ mol L^{-1}), and lower than pure cultures of the marine AOA *Nitrosopumilus maritimus* ($<10^{-12.7}$ mol L^{-1}), which also possesses a high amount of Cu-dependent enzymes.

1. Introduction

Ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB) and comammox bacteria contribute to the global nitrogen biogeochemical nitrogen cycle via nitrification [1–7]. They all contain, the copper (Cu)-dependent enzyme ammonia monooxygenase, which is the key enzyme of ammonia oxidation, the first step of nitrification [1,8,9]. In particular it is hypothesized that AOA contain a large number of Cu-dependent enzymes including plastocyanins and multicopper oxidases based on their abundance in AOA genomes [10,11] and proteomes [12–14]. This is in contrast to AOB, whose energy metabolism (besides AMO) and respiratory chain requires mostly iron (Fe) [15].

In general, metals such as Cu are reversibly bound to soil particles and only a small bioavailable fraction exists in solution [16]. In natural systems, Cu predominantly exists in the monovalent and divalent redox states. Cu(I) is unstable in aqueous solution and is converted to Cu(II) if it is stabilized as a component of a mineral phase or in a complex [17] such as a Cu(I) specific metallophore [18]. Even in suboxic environments the Cu(II) redox state can be sufficiently stabilized by complexation to humic acids to lessen or prevent Cu^{2+} reduction [19]. Generally, it is assumed that only soluble Cu^{2+} is available for biological uptake and is therefore used as a proxy for the bioavailability of Cu [16] unless a high affinity uptake mechanism involving chalkopohores is used [20]. The presence of inorganic ligands (Cl^-, PO_4^{3-}, SO_4^{2-}, Fe, Mn, carbonates),

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clays and organic substances, like humic acids [19], which complex free Cu^{2+} in soil solution (i.e. liquid water found within soils) can all lower its bioavailability [21–23]. In soils that approach neutral pH conditions, free Cu can adsorb to oxides and clays [24]. Thus, environmental factors such as pH, Eh, soil type, water hardness and organic content can influence the bioavailability of Cu [25]. A recent study by Bayer et al. [26] showed that certain marine AOA appear to depend highly on Cu [26] based on their Cu cell content. Perhaps this is also true of other AOA, including terrestrial species. If so, differences in the bioavailability of Cu in turn could influence whether AOA or rather AOB are more active in soils [27].

Reduced bioavailability of Cu can lead to Cu limitation for many microorganisms. The effects of Cu limitation on cell physiology, metabolism or uptake have been studied using pure cultures of denitrifying bacteria [28], methane oxidizing archaea [29] and bacteria [30], some marine diatoms [31–33] and more recently also the marine AOA *Nitrosopumilus maritimus* SCM1 [13,34]. In some of these studies, cultures grew slower at free Cu^{2+} concentrations of $<10^{-12.7}$ mol L^{-1} (*N. maritimus* SCM1) [34], $<10^{-14}$ mol L^{-1} (oceanic diatoms) [33], and $<10^{-16}$ mol L^{-1} (*Pseudomonas stutzeri*) [28], *Nitrosoarchaeum koreense*, *Nitrosomonas europaea*, *Ca.* Nitrosotenuis chungbukensis and *Ca.* Nitrosocosmicus oleophilus [35]) compared to Cu-replete conditions. In environmental studies, Cu bioavailability to Archaea in Hood Canal fjord [36] and to Thaumarchaeota in a salt marsh estuary [37] has also been studied. In the former study, free Cu^{2+} concentrations from 1.3×10^{-12} M to 6×10^{-15} M were reported to limit AOA abundances in Hood Canal, whereas in the latter study, Thaumarchaeota were found to thrive at free Cu^{2+} concentrations of 0.4×10^{-15} M.

In this study, the physiological response of the soil representative soil AOA isolate, *Nitrososphaera viennensis* [38,39] to Cu-limiting conditions was explored. The hypothesis that Cu limitation would hinder the ability of *N. viennensis* to oxidize ammonia was tested in batch cultures. The chelator TETA (1,4,8,11-tetraazacyclotetradecane-N, N′, N″, N‴-tetraacetic acid hydrochloride hydrate) with selective affinity for Cu^{2+}, was used to control bioavailable Cu^{2+} in culture experiments.

2. Methods

2.1. Media preparation

Ultrapure water (18.2 MΩ cm, 2 ppb TOC) was used to prepare all media and to rinse polypropylene plastic bottles using water from a MilliQ®-Elix® and ELGA PureLab Chorus purification system. To reduce Cu contamination all media components were prepared in plastic bottles that had been washed with a mild detergent and then acid washed for 2–4 weeks, first in 3.8% (v/v) HCl, and then in 7.4% (v/v) nitric acid. In between acid washes and following the nitric acid wash, bottles were rinsed five times with ultrapure water. Preparation of all media components and handling of cultures was done in a trace metal free clean room environment with a HEPA filtration system. One liter of FWM medium [1 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.2 g KH_2PO_4, 0.5 g KCl (Emsure®, ACS, ISO Reagents)], amended with 1 mL trace element solution [HCl 100 mM (2X distilled HCl), H_3BO_3 0.5 mM, $MnCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, 0.01 mM $CuCl_2 \cdot 2H_2O$ (Emsure®, ACS, ISO Reagents)], 1 mL vitamin solution [0.08 mM biotin (≥99% pure); 0.05 mM folic acid (≥97% pure), 0.48 mM pyrodoxin HCl (≥99% pure), 0.14 mM thiamine HCl (≥99% pure), 0.13 mM riboflavin (≥97% pure), 0.4 mM nicotinic acid (≥98% pure), 0.2 mM D-L-panthothenic acid (≥97% pure), 0.36 mM p-aminobenozic acid (≥99% pure), 1.4 mM choline chloride (≥98% pure), 7.3×10^{-6} M vitamin B12 (Millipore®)], 7.5 μM ferric sodium EDTA (Fe(III)-chloride Emsure®, ACS, ISO Reagent, EDTA Analytical Reagent), 2 mM NH_4Cl (≥99.7% pure), 2 mM $NaHCO_3$ (≥99.5% ACS, ISO Reagent) (as carbon source) and 1 mM sodium pyruvate (≥99% pure) (as a free radical scavenger), was used for growth. The medium was buffered with 10 mM HEPES (Pufferan® ≥99.5% pure)/6 mM NaOH (Reagent Grade) to pH 7.5 (Table 1). FWM medium, trace element mixture, vitamin solution, NH_4Cl, $NaHCO_3$, sodium pyruvate and HEPES buffer were filter sterilized with a sterile 0.2 μm PVDF filter (Fisher Scientific SCGVU05RE) and stored in the attached polystyrene bottles at 4 °C.

2.2. Culture conditions

N. viennensis (strain EN76^T) was isolated from garden soil as previously described [38,39]. Its growth was initiated from a 0.5 mL 40% glycerol stock by first spinning down the cells at 16,168×*g* for 30 min (4 °C), removing the supernatant and then resuspending the cell pellet in 0.5 mL Fresh Water Medium (FWM). The re-suspended cells were used to inoculate a 1 L volume glass bottle with a blue plastic cap, containing 500 mL FWM medium supplemented with kanamycin (100 μg mL^{-1}) (≥750 I.U/mg, biochemistry grade) to prevent bacterial contamination [38,39]. *N. viennensis* cultures were incubated at 42 °C in the dark without shaking. Growth was monitored by following NH_4^+ consumption and NO_2^- production using two modified colorimetric assays [40,41] described below. Following consumption of 1 mM NH_4^+, the culture was split into

Table 1
FWM medium composition used to grow Cu-limited and Cu-replete cultures of *N. viennensis*.

	Final Concentration mol/L
Salts	
NaCl	0.017
$MgCl_2$ $6H_2O$	0.0019
$CaCl_2$ $2H_2O$	6.8×10^{-3}
KCl	0.0067
$NaHCO_3$	0.002
Nutrients	
NH_4Cl	0.002
$KHPO_4$	0.0014
Na-pyruvate	1.0×10^{-4}
Buffer	
HEPES (7.5)	0.01
FeNaEDTA solution	7.5×10^{-6}
Non Chelated trace element mixture	
HCl	1×10^{-3}
H_3BO_3	5×10^{-7}
$MnCl_2$ $4H_2O$	5×10^{-7}
$CoCl_2$ $6H_2O$	8×10^{-7}
$NiCl_2$ $6H_2O$	1×10^{-7}
$ZnSO_4$ $7H_2O$	5×10^{-7}
Na_2MoO_4 $2H_2O$	1.5×10^{-7}
$CuCl_2$ H_2O[a]	1.0×10^{-8}
Antibiotics	
Carbenicillin	2.6×10^{-4}
Kanamycin	2.6×10^{-4}
Vitamins	
Biotin	8×10^{-8}
Folic acid	5×10^{-8}
Pyrodoxine HCl	4.8×10^{-7}
Thiamine HCl	1.4×10^{-7}
Riboflavin	1.3×10^{-7}
Nicotinic acid	4.0×10^{-7}
DL Panthothenic acid	2.0×10^{-7}
p-aminobenzoic acid	3.6×10^{-7}
Choline chloride	1.4×10^{-6}
Vitamin B12	7.3×10^{-12}

[a] For Cu-limited cultures, Cu was excluded from the trace metal solution.

subcultures by transferring growing cells to 20 mL of FWM in 30 mL polystyrene tubes (Greiner Bio-One; 201172). An inoculation volume of 0.25% was used to start 20 mL cultures. FWM media was supplemented with kanamycin (100 μg mL^{-1}) and carbenicillin (100 μg mL^{-1}) (≥88% pure, biochemistry grade). Subcultures were continuously transferred every seven days for several weeks before PCR testing the cultures for bacterial contamination and initiating the Cu limitation experiments.

2.3. Cell counts and growth rates

200 μL of cell culture were fixed with 4 μL glutaraldehyde (25%) for at least 10 min before freezing cells at −20 °C. Prior to analysis, samples were diluted (1:10 or 1:50) in 0.2 μm PVDF filtered Tris-EDTA buffer [1 M Tris-HCl, 0.1 M EDTA, pH 8] and stained with SybrGreen I (1:20,000×). Samples were incubated in the dark for ~10 min and then analyzed with a FACSAria II flow cytometer (BD Biosciences) as previously described [42]. Nitrite production was previously shown to follow biomass production [38]. Thus, NO_2^- concentrations were used to calculate the specific growth rate (μ). NO_2^- concentrations were plotted on a logarithmic scale to determine the linear portion of the growth curves. These data were selected, re-plotted on a normal scale and the value of the slope of the trend-line used as the growth rate.

2.4. Nucleic acid extraction and PCR testing

Nucleic acids were extracted from 1 mL culture in exponential growth phase (determined by measuring the NO_2^- production). Cells were centrifuged at 16,168×*g* (4 °C) for 30 min. The supernatant was discarded and cells were resuspended in SDS Extraction Buffer [0.14 M NaCl, 0.02 M Na_2SO_3, 0.1 M Tris/HCl, 0.5 M EDTA pH 8 and 1% SDS]. This mixture was transferred to sterile 2 mL microtubes (Sarstedt 72.693.005) containing ~ 0.5 g of 0.1 mm zirconia beads (Carol-Roth N033.1) that had been baked at 180 °C for at least 3 h. Phenol-chloroform-isoamyl alcohol (0.5 mL) [PCI, 25:24:1 (vol:vol:vol); pH 6.7–8] was first added to facilitate DNA recovery. Cells were lysed by bead beating at 4 m/s for 30 s using a Fast Prep-24 instrument (MP Biomedicals). After bead beating and removal of PCI, chloroform-isoamyl-alcohol (0.5 mL) [CI, 24:1 (vol:vol)] was added to samples. During the PCI and CI extraction steps, tubes were centrifuged at 16,168×*g* (4 °C) for 10 min. Between PCI and CI steps, before centrifugation, tubes were shaken heavily. Finally, nucleic acids were precipitated from the aqueous phase by adding 1 μL of glycogen (Thermo Fisher EP0701) and 2 volumes of 40% polyethylene glycol (molecular weight 5000–7000, Sigma 81255) at 4 °C overnight, followed by centrifugation at 16,168×*g* (4 °C) for 30 min. Pellets were washed with 1 mL of 4 °C cold 70% ethanol, air dried for 3–5 min under vacuum [Eppendorf Vacufuge Plus, 30 °C/AQ setting] and resuspended in 50 μL of DNase-free water. SDS Extraction Buffer, PEG and ethanol solutions were made using DNase-free water. Total DNA concentration was measured using the NanoDrop Spectrophotometer ND-1000 (PeqLab VWR International GmbH) following the manufacturer's instructions.

DNA of the culture samples was analysed by performing a PCR using 2 different archaea primer sets including: 0.2 μM of primers Arc109f [5′-ACKGCTCAGTAACACGT-3′] [43] and Arc1492r [5′-GYYACCTTGTTACGACTT-3′] [44], CamoA-19f [5′-ATGGTCTGGYTWAGACG-3′] originally published by Tourna et al. [38] and modified by Pester et al. [45], and TamoA-692r [5′-TGGCANTAYMGATGGATGGC-3′] [46]. Possible bacterial contamination was assessed using 0.2 μM of bac27f [5′-AGAGTTTGATCCTGGCTCAG-3′] and bac1492r [5′-GGTTACCTTGTTACGACTT-3′] [47]. 2 μL of DNA sample was used along with the following: Thermo Fisher 200 μM dNTP mix (R0241), 0.002 mg mL^{-1} BSA (B14), 0.15 μL Dream-Taq DNA polymerase (EP0701), 1X Dream-Taq buffer and DNA free water in a 25 μL reaction volume. The following conditions were used for 16S rRNA primers: 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. The following conditions were used for *amoA* primers: 95 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s, and a final elongation step at 72 °C for 10 min. PCR reactions were checked on a 1% low melting agarose gel.

2.5. PhreeqC modelling

Free concentration of metals including Cu were calculated using the PhreeqC [48] software package (version 3.1.7–9213) with stability constants from the minteq.v4 database. Stability constants for TETA input were from Anderegg et al. [49] and those for kanamycin were from Szczepanik et al. [50]. Both stability constants were corrected for zero ionic strength i.e. $\mu = 0$. TETA binds Cu^{2+} with a stability constant of $10^{23.5}$.

2.6. TETA toxicity tests

Under Cu-replete conditions, *N. viennensis* is predicted to have ~10^{-12} M Cu available in FWM with added supplements as described above. To test whether TETA was toxic to cells, 0.28 μM $CuCl_2 \cdot 2H_2O$ was added to FWM in addition to 1 μM of TETA (≥95% pure), to achieve a free Cu^{2+} concentration of ~10^{-12} M.

2.7. Establishing Cu-limited cultures

The trace element solution used in the Cu-limited sub-cultures was prepared form ACS/ISO analytical-grade chemicals. Attempts were made to further purify the nutrient solutions using chelating resins. However, the cultures showed clear toxicity responses after Cu was added to the medium prepared with chelated solutions. Therefore, we used salts as received. The total concentration of Cu in the FWM with additives was measured using ICP-MS and no Cu was detected in the medium. In the presence of Cu and TETA, Cu concentration is buffered and tightly controlled. Therefore, changes in free Cu activities throughout the growth curve should be extremely low.

Cu-limited cultures were initiated by transferring a 0.25% inoculation volume to 20 mL of FWM in polystyrene tubes. The trace element solution for Cu-limited cultures did not contain any Cu. TETA and $CuCl_2 \cdot 2H_2O$ were individually added from stocks to cultures at various concentrations as described in Table 2 to establish the following free Cu^{2+} concentrations: experiment Cu7 (3×10^{-13} mol L^{-1}), Cu9 (3×10^{-14} mol L^{-1}), Cu10 (6×10^{-15} mol L^{-1}) and Cu12 (7×10^{-16} mol L^{-1}). Cu7 through Cu12 refer to arbitrary sample names. A control with no Cu and no TETA was also included. These cultures were continuously transferred and monitored for growth by measuring the NO_2^- concentration. Cu limitation was assessed as a decrease in NH_4^+ consumption and NO_2^- production relative to the Cu-replete culture. Furthermore, Cu-limited cultures were amended with 5 nM of Cu^{2+} during exponential phase to determine if they could recover their ability to produce NO_2^- at levels comparable to Cu-replete cultures.

2.8. Ammonium and nitrite measurements

Ammonium concentrations were determined based on a spectrophotometric method modified after Kandeler et al. [40]. Briefly 200 μL of sample was combined with 400 μL of FWM and to this 300 μL of Color Reagent [5.18 mM sodium salicylate, 2.15×10^{-5} M sodium nitroprusside, 0.1 M NaOH] and 120 μL Oxidation Solution

Table 2
Estimated free Cu^{2+} present in FWM using PhreeqC in the presence and absence of TETA.

Total TETA added [uM]	Total Cu added [μM]	[free Cu^{2+}] calculated [mol L^{-1}]	Experiment	No. of Experiments	Biological replicates
1	0[b]	$\leq 5 \times 10^{-21}$	–	–	–
1	0.29	3×10^{-12}	Cu-TETA	2	4
0	0.01[a]	3×10^{-12}	Cu-Replete	3	7
1	0.05	3×10^{-13}	Cu7	2	6
1	0.005	3×10^{-14}	Cu9	3	7
1	0.006	3×10^{-14}	Cu10 + spike	2	5
1	0.001	6×10^{-15}	Cu10/Cu-Limited	7	3
1	0.0001	7×10^{-16}	Cu12/Cu-Limited	2	4
0	0[b]	$<7 \times 10^{-16}$	No Cu, No TETA	3	7

[a] 0.01 μM is the total Cu normally added to the standard FWM (see Table 1).
[b] 0.9×10^{-9} mol L^{-1} Cu is the limit of quantification of our instrument and was the value used as input to calculate the free Cu^{2+} concentration.

[3.91×10^{-5} M of dichloroisocyanuric acid] was added. After mixing, samples were stored in the dark at room temperature for 30 min. The absorbance of 200 μL samples was measured in a 96-well plate (Greiner Bio-one; 65501) with a UV spectrophotometer [TECAN Sunrise Spectrophotometer] at 660 nm wavelength. NO_2^- concentrations were determined by using a spectrophotometric method modified after Griess [41]. Briefly, 20 μL of sample was combined with 780 μL FWM and 200 μL of sulfanilamide/NED Reagent [0.058 M sulfanilamide, 1.9 mM N-(1-Napthyl)-ethylendiamin dihydrochloride, 2.22 M ortho-phosphoric acid 85%] was added. After mixing, the samples were stored in the dark at room temperature for 30 min before measuring the absorbance at 545 nm wavelength.

2.9. LC-ICP-MS supernatant analysis for Cu speciation

To measure potential Cu speciation changes, samples were analyzed by Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry (LC-ICP-MS). Samples consisted of incubated culture supernatants or blank media which were prepared by filtration (0.2 μm syringe filters) and addition of $CuCl_2$ to a final concentration of 1 μM and 100 μM to look for strong but low abundance ligands as well as possibly weaker ligands at higher concentrations. The LC-ICP-MS platform consisted of a liquid chromatography system (Ultimate 3000, ThermoFisher) coupled to an ICP-MS (iCAP RQ, ThermoFisher). Reversed phase chromatography was done with a C18 column (ACE Excel 1.7 SuperC18, 2.1 × 100 mm). Injected samples (25 μL) were separated at pH 7 under a gradient of solutions A and B (solution A: water + 5 mM ammonium acetate, pH 7; solution B: acetonitrile + 5 mM ammonium acetate; gradient 2–100% B, flow rate 0.4 mL min^{-1}). The column outflow was split using a flow-splitter (Analytical Scientific Instruments, 600-PO10-06) to introduce 0.1 mL min^{-1} into the ICP-MS with a zero dead volume PFA micro-nebulizer (Elemental Scientific). Oxygen was used as an added gas in the ICP-MS (flow 8.75 mL min^{-1}) to minimize carbon deposition on the ICP-MS cones (Pt cones) at high organic buffer concentrations. The instrument was operated in KED mode with He as a collision gas to analyze the ^{63}Cu signal.

2.10. HR-LC-MS supernatant metabolite analysis

High-Resolution Liquid-Chromatography Mass-Spectrometry (HR-LC-MS) was used to analyze metabolites, such as potential Cu chelating exudates. Samples consisted of incubated culture supernatants or blank media and were analyzed after filtration (0.2 μm syringe filters) and acidification (0.1% formic acid). To one incubated sample, we added $CuCl_2$, to a final concentration of 100 μM, to search for potential Cu chelating metabolites by screening for Cu specific isotope patterns. The remaining samples were analyzed without added Cu^{2+}. HR-LC-MS analyses were performed on a high mass accuracy and resolution, reversed phase HPLC-MS platform, using a C18 column (Waters Acquity UPLC BEH C18 1.7 μm, 2.1 × 100 mm) coupled to an Orbitrap ID-X mass spectrometer (ThermoFisher). Injected samples (25 μL) were separated under a gradient of solutions A and B (solution A: water + 0.1% formic acid; solution B: acetonitrile + 0.1% formic acid; gradient 2–100% B, flow rate 0.4 mL min^{-1}). Full-scan mass spectra were acquired in positive-ion mode (m/z = 85–1000) with a resolving power of R = 100,000 (at m/z = 400). MS/MS spectra were simultaneously acquired using CID in the Orbitrap. The column outflow was diverted to waste for the first 1.5 min to desalt the sample before introduction into the mass spectrometer. The HR-LC-MS data acquired was analyzed using mzMine2 [51] to generate a list of features defined by m/z, retention time, and peak intensity. The major features that were differentially present in blank media and samples could be assigned to the added antibacterial compounds and compounds generated by abiotic reaction or metabolic modifications of the antibiotics (Table S1). The samples with added Cu were mined for potential Cu chelators by (1) filtering for the ^{63}Cu-^{65}Cu isotope pattern and by (2) searching for co-eluting free ligands of Cu complexes by their exact mass difference (Δm ($-2H^+ + Cu^{2+}$) = 60.9139; Δm ($-H^+ + Cu^+$) = 61.9218) as described previously [52,53]. We observed Cu chelation by the added antibiotics and their related compounds.

3. Results

3.1. Modelling of free Cu^{2+} concentrations

The background concentration of Cu in FWM without Cu additions was below the limit of quantification of our instrument (0.9×10^{-9} mol L^{-1} for total Cu). Based on PhreeqC calculations, we estimate the standard FWM with 1 μM TETA and no additional Cu has a free Cu^{2+} concentration of $\leq 5 \times 10^{-21}$ mol L^{-1}. Cu-replete cultures are predicted to have a free Cu^{2+} concentration of 3×10^{-12} mol L^{-1}. Cultures studied in the presence of 1 μM TETA and variable Cu concentrations (Table 2) are predicted to have the following free Cu^{2+} concentrations available to them: Cu7 (3×10^{-13} mol L^{-1}), Cu9 (3×10^{-14} mol L^{-1}), Cu10 (6×10^{-15} mol L^{-1}) and Cu12 (7×10^{-16} mol L^{-1}), No Cu, No TETA ($\leq 7 \times 10^{-16}$ mol L^{-1}). It is known that the antibiotics used in culture solutions (i.e., kanamycin and carbenicillin) can chelate copper [50,54,55]. Mass spectrometry confirmed the presence of such complexes in the culture solution after addition of excess Cu (vide infra). However, modelling results showed that in the presence of kanamycin, the free Cu^{2+} concentration was the same order of magnitude as predicted in the standard FWM with 1 μM TETA and no additional Cu. Non-conditional stability constants were not available for carbenicillin to calculate its effect on Cu speciation

under the experimental conditions. While we assume that the affinity of the Cu-specific ligand TETA for Cu is significantly higher than the antibiotics, it should be noted that the calculated free Cu^{2+} activity constitutes an upper limit as it may be further lowered by the formation of complexes with antibiotics.

3.2. Analysis of Cu chelation and metabolites in culture supernatants

In order to verify if biogenic Cu-binding ligands in the spent medium changed Cu speciation and availability, speciation analysis was done by liquid chromatography with inductively coupled plasma mass spectrometry (LC-ICP-MS). Potential Cu binding metabolites in the media were analyzed by high resolution electrospray ionization mass spectrometry (HR-LC-MS) [56]. LC-ICP-MS provides a quantitative analysis of metal complexes that can be separated by the chromatographic column (reversed phase C-18 column) while HR-LC-MS can be used to discover and characterize metal chelating metabolites [52,53]. We compared added Cu concentrations of 1 μM and 100 μM to titrate ligands and resolve possible strong chelators present at low concentrations from weaker ligands at higher concentrations (Fig. S1 and S2). After adding Cu to a final concentration of 1 μM, Cu was mainly bound to a hydrophilic compound in agreement with chelation to TETA. After adding Cu to a final concentration of 100 μM, we observed Cu binding by the added antibiotics (260 μM kanamycin and carbenicillin) as expected. HR-LC-MS analysis detected a reaction product between carbenicillin and kanamycin and two other compounds related to carbenicillin reactions (Table S1). It is known that these antibiotics are not toxic to *N. viennensis* [38,39] and could potentially be partially degraded by enzymes present in their proteome [12]. The added antibiotics were most likely metabolized in both Cu-limited and Cu-replete spent media, which showed more than 10× lower carbenicillin peaks with simultaneous increases in the concentration of putative metabolic by-products of the antibiotics' degradation (Table S1). However, no strong biogenic Cu-binding compounds were detected in the spent media.

3.3. TETA toxicity tests

When *N. viennensis* was exposed to 1 μM TETA and excess Cu (0.29 μM), to provide it with the standard total Cu concentration (0.01 μM, Table 2), this culture was able to oxidize ~ 2 mM NH_4^+ and produce ~ 1800 μM NO_2^-. This is comparable to the standard culture (Fig. 1), demonstrating that TETA was not toxic to *N. viennensis* cells.

3.4. Ammonia oxidation activity and growth of Cu-limited cultures

When grown in the presence of TETA, the Cu-limited cultures Cu10 and Cu12 showed a decrease in their ability to oxidize NH_4^+ compared to Cu7, Cu9 and the Cu-replete cultures (Fig. 2). At day 12, Cu7, Cu9 and the Cu-replete cultures had produced ~1800 μM of NO_2^- and Cu10 had produced ~400–700 μM of NO_2^-. At day 11, Cu12 had produced ~200 μM NO_2^-. When a 5 nM spike of Cu was introduced into Cu10 at day 5 of growth, an increase in NO_2^- production was observed concomitant with an increase in NH_4^+ consumption (Fig. 2). At stationary phase, the spiked Cu10 culture had produced ~1800 μM of NO_2^- comparable to Cu7, Cu9 and the Cu-replete cultures. Cell numbers showed the same trend as NO_2^- production (Fig. 3). At Day 12, Cu10 showed low cell numbers (~1 × 10^7 cells mL^{-1}) compared to Cu7, Cu9 and Cu-replete cultures (~3–5 × 10^7 cells mL^{-1}). Control incubations without addition of Cu and TETA showed the lowest cell numbers (<1 × 10^7 cells mL^{-1}), confirming that contamination of Cu in nutrient solutions, if present, were low enough not to interfere with the growth

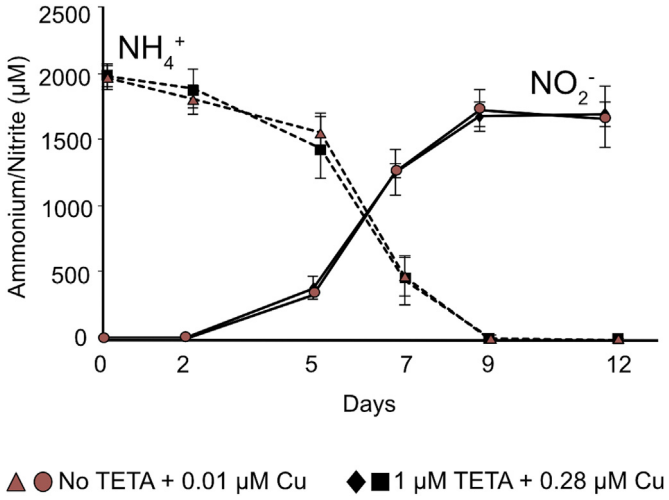

Fig. 1. Aerobic ammonia oxidation in Cu-replete batch cultures of *N. viennensis* grown with and without TETA. The data and error bars represent the average and standard deviation of 4 biological replicates (n = 4) from two experiments. Dotted lines represent ammonium concentrations and solid lines nitrite concentrations. Elevated Cu in 1 μM TETA medium ensured sufficient Cu was available to cells and compensated for Cu-TETA complex formation.

experiments. Growth rates for each culture also displayed a similar trend as NO_2^- production and cell numbers. Cu7, Cu9 and Cu-replete cultures had the highest specific growth rates (~0.024 h^{-1}), whereas Cu10 and Cu12 cultures showed a decreased growth rate (~0.008 h^{-1}) and (~0.004 h^{-1}) (Fig. 4).

4. Discussion

Results of this study show that the ability of *N. viennensis* to oxidize ammonia and grow is hindered when Cu levels are at or below 10^{-15} mol L^{-1} free Cu^{2+} and that it recovers this ability upon reintroduction of Cu. These results are similar to Cu thresholds observed for *N. viennensis* and other AOA and AOB tested in laboratory studies using histidine as a Cu-binding ligand instead of TETA [35]. However, results from this study differ from the marine AOA, *N. maritimus* SCM1 whose ammonia oxidation ability was impeded below 10^{-12} mol L^{-1} free Cu^{2+} in laboratory based studies [34]. It is possible that *N. viennensis* could take up Cu using a different mechanism than *N. maritimus* thereby explaining this difference in Cu-limitation threshold.

The ability of *N. viennensis* to tolerate lower levels of copper than *N. maritimus* could be partially attributed to the larger amount of copper transporters and regulatory genes found in the genome [12,57]. The genome of *N. viennensis* encodes several proteins that could participate in copper acquisition. These include two copC/D genes (NVIE_014300 and NVIE_014310), two copT genes (NVIE_012920 and NVIE_014090), two copA genes (NVIE_008380 and NVIE_012900), a copZ homolog (NVIE_012910), and a gene encoding a putative copper storage protein (NVIE_028200). CopC/D appears to encode a fusion protein composed of CopC, a putative periplasmic Cu chaperone [58] and CopD, a cytoplasmic membrane protein [59]. In *Bacillus subtilis* YcnJ, a CopC/D fusion protein, has been hypothesized to bind Cu and transport it into the cytoplasm [60,61]. Extensive bioinformatics analysis by Lawton et al. [62] of bacterial and archaeal CopC protein sequences, showed that the CopC/D fusion protein was the most prevalent type of CopC in bacteria and archaea included in the analysis. CopA is a P1B-type, CPTx type ATPase, that could be involved in the import of Cu in *Listeria monocytogenes* [63], *Pseudomonas aeruginosa* Q9I147 [64]

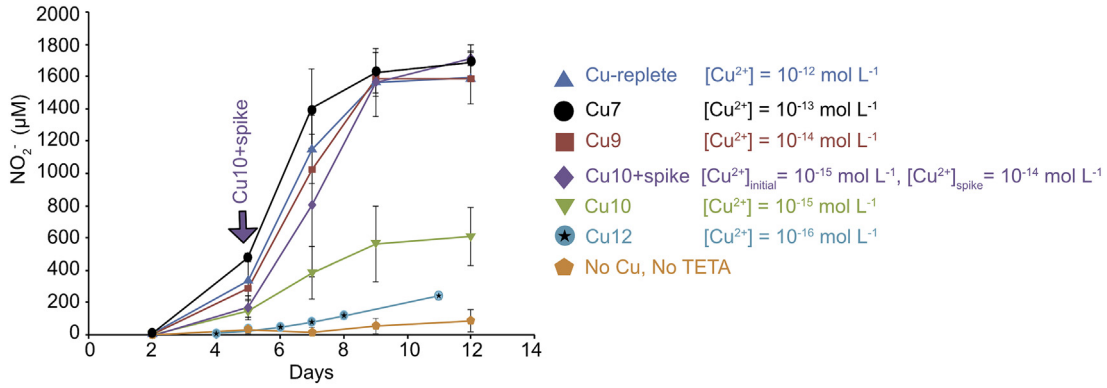

Fig. 2. Nitrite concentrations in batch cultures of *N. viennensis* grown under Cu-replete and Cu-limited conditions as a function of the calculated free Cu^{2+} species concentration. The data and error bars represent the average and standard deviation of multiple biological replicates (≥4) from several experiments (see Table 2).

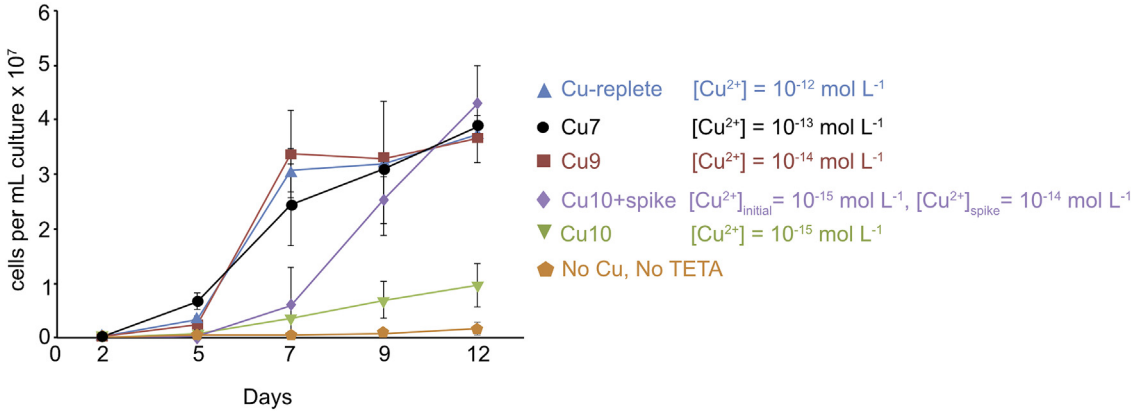

Fig. 3. The effect of Cu-limitation on growth of *N. viennensis*. Cultures were grown in batch at various Cu concentrations and the Cu-limited culture was spiked with 5 nM of Cu at day 5 of exponential growth. The data points and error bars represent averages and standard deviations of three biological replicates from a single experiment.

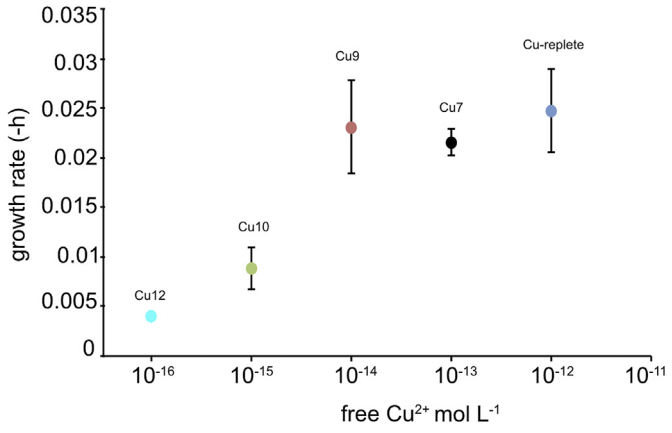

Fig. 4. The effect of Cu-limitation on growth rates of *N. viennensis*. The data points and error bars represent averages and standard deviations of multiple biological replicates (≥4) from several experiments (see Table 2).

and/or export of copper in various bacteria [65,66] and archaea [67,68], while copT [69] and copZ [70] are thought to act as transcriptional regulators and chaperone proteins respectively. One of these copper genes, copC/D (NVIE_014310), was a member of genes that compose the core genome of AOA as described by Kerou et al. [12]. The high number of genes encoding proteins that regulate Cu uptake in *N. viennensis* implies a strong transcriptional regulatory network e.g. CopT and CopZ, to combat copper stress. Contrastingly, *N. maritimus* contains only one copC/D gene (Nmar_1652) and no known homologs of copA, copT, copZ, or a copper storage protein. The copC/D found in *N. maritimus* (Nmar_1652) is also the corresponding copC/D found in the core genome of AOA.

Another possibility why the Cu threshold may differ between these two species is that they might possess Cu transporters with different binding affinities for free and inorganically complexed Cu. This idea was proposed by Gwak et al. [35] in order to explain Cu-threshold differences between various terrestrial, freshwater AOA and AOB. However, this has yet to be experimentally shown.

The low copper levels used in this study are comparable to soil environments with low copper. Copper (II) activities $\leq 10^{-14}$ have been determined for sandy soils from Nadec, Saudi Arabia [71] and other soil types (W. Schenkeveld pers. comm.) through soil chemical analysis and surface complexation modelling. Since the Cu-limiting threshold for *N. viennensis* is estimated to be $\leq 10^{-15}$ mol L^{-1}, it is possible that AOA could encounter Cu concentrations in line with our experiments in such environments. Exposure of AOA such as *N. viennensis*, that rely on Cu containing enzymes for their metabolism, to soils with low free Cu^{2+} concentrations could be less efficient at oxidizing NH_3/NH_4^+ compared to soils where Cu is more readily available. However, complementary field based studies, with different soil types, would have to be made to test this hypothesis. It is also likely that AOA adapt to Cu availabilities and thus exhibit different genotypes and ecophysiologies depending on their habitats. For example, Whitby et al. [37]

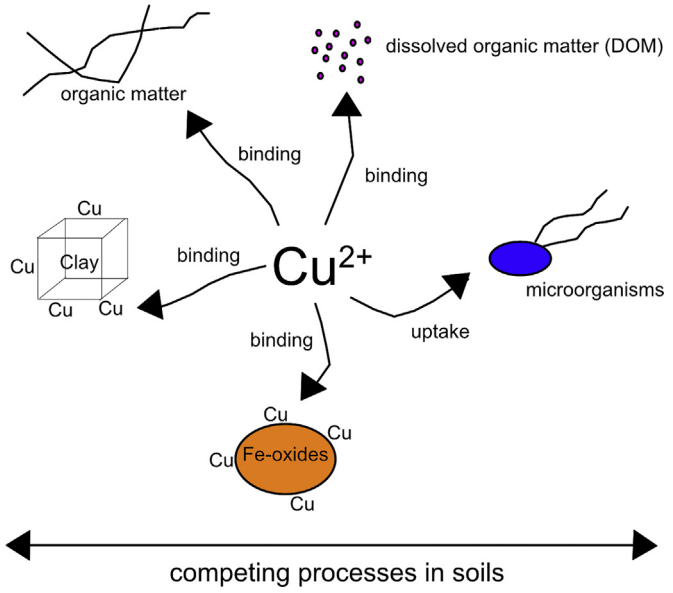

Fig. 5. Conceptual model of how Cu^{2+} could become limited in soil environments through binding and uptake processes.

found AOA similar to *N. maritimus* were thriving in marine waters with free Cu^{2+} concentrations lower than the laboratory-based concentrations measured by Amin et al. [34] for the pure culture, which stemmed from an aquarium. Nevertheless, results from this study and that of Amin et al. [34] serve as model systems to understand how individual factors such as free Cu^{2+} concentrations can affect cell physiology and growth of different species of AOA.

Types of soils where Cu and other metals are limited include neutral and alkaline soils. Under high pH conditions, increased binding of metals to solid, organic matter [72] and inorganic soil particles (e.g. clays and metal oxides) [73], and to dissolved organic molecules (e.g. humic and fulvic acids) [72] or dissolved inorganic molecules (e.g. sulfate, nitrate, phosphate) is known to occur. Decreased availability of Cu and Fe in such soil environments could be factors influencing AOA and AOB abundances or activities (Fig. 5).

In contrast to alkaline soil environments, the affinity of Cu to bind to soil particles is reduced and therefore it should be more bioavailable [74]. In a study by Schaik et al. [75] Fe was shown to outcompete Cu for binding sites to different types of soil organic matter at low pH. Thus, in low pH soils, Fe could displace Cu from certain types of organic matter making it more bioavailable. Perhaps acidic soil environments where Cu is more bioavailable than Fe, along with a physiological specialization of AOA under acidic conditions [76–82] could offer a competitive advantage to certain AOA over AOB. Increased Cu concentrations have been shown to stimulate denitrification in wetland sediments [83], thereby supporting the idea that Cu concentrations can influence the abundance or activity of N cycling microorganisms with Cu reliant enzymes.

Soils that are heavily weathered and eroded are also environments where metals such as Cu might be limiting. A recent study found that in highly weathered soils from Western Australia, AOA abundances were low compared to AOB [27]. However, it is not clear if a lack of Cu was the only factor contributing to these differences in abundances.

In summary, results from this study show that *N. viennensis* is adapted to very low Cu concentrations. The organism showed decreased ammonia oxidation ability at a free Cu^{2+} threshold as low as 10^{-15} mol L^{-1}. This threshold is considerably lower than the one estimated for *N. maritimus* under similar experimental conditions perhaps due to different uptake mechanisms between the two species. In consequence, *N. viennensis* and related AOA can be active even in alkaline soil environments where Cu is expected to be less bioavailable. Future laboratory experiments should focus on testing whether naturally occurring soil ligands such as humic acids have a similar effect on the cell physiology of *N. viennensis*, i.e. reduce the bioavailability of Cu such that *N. viennensis* becomes limited. More integrated microbiology and geochemical studies are needed to characterize the *in situ* activities of AOA and AOB in Cu-limited soils.

Declaration of Competing Interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.resmic.2020.01.003.

References

[1] Stahl DA, de la Torre JR. Physiology and diversity of ammonia-oxidizing archaea. Annu Rev Microbiol 2012;66:83–101. https://doi.org/10.1146/annurev-micro-092611-150128.
[2] Schleper C, Nicol GW. Ammonia-oxidising archaea – physiology, ecology and evolution. Adv Microb Physiol 2010;57:1–41. https://doi.org/10.1016/B978-0-12-381045-8.00001-1.
[3] Hatzenpichler R. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Appl Environ Microbiol 2012;78:7501–10. https://doi.org/10.1128/AEM.01960-12.
[4] Stein LY. Insights into the physiology of ammonia-oxidizing microorganisms. Curr Opin Chem Biol 2019;49:9–15. https://doi.org/10.1016/j.cbpa.2018.09.003.
[5] Hink L, Gubry-Rangin C, Nicol GW, Prosser JI. The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. ISME J 2018;12:1084–93. https://doi.org/10.1038/s41396-017-0025-5.
[6] Daims H, Lebedeva EV, Pjevac P, Han P, Herbold C, Albertsen M, et al. Complete nitrification by Nitrospira bacteria. Nature 2015;528:504–9. https://doi.org/10.1038/nature16461.
[7] van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, Op den Camp HJM, Kartal B, et al. Complete nitrification by a single microorganism. Nature 2015;528:555–9. https://doi.org/10.1038/nature16459.
[8] Kozlowski JA, Stieglmeier M, Schleper C, Klotz MG, Stein LY. Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. ISME J 2016;10:1836–45. https://doi.org/10.1038/ismej.2016.2.
[9] Klotz MG, Stein LY. Nitrifier genomics and evolution of the nitrogen cycle. FEMS Microbiol Lett 2008;278:146–56. https://doi.org/10.1111/j.1574-6968.2007.00970.x.
[10] Walker CB, de la Torre JR, Klotz MG, Urakawa H, Pinel N, Arp DJ, et al. Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proc Natl Acad Sci U S A 2010;107:8818–23. https://doi.org/10.1073/pnas.0913533107.
[11] Jung M-Y, Kim J-G, Sinninghe Damsté JS, Rijpstra WIC, Madsen EL, Kim S-J, et al. A hydrophobic ammonia-oxidizing archaeon of the *Nitrosocosmicus* clade isolated from coal tar-contaminated sediment. Environ Microbiol Rep 2016;8:983–92. https://doi.org/10.1111/1758-2229.12477.

[12] Kerou M, Offre P, Valledor L, Abby SS, Melcher M, Nagler M, et al. Proteomics and comparative genomics of Nitrososphaera viennensis reveal the core genome and adaptations of archaeal ammonia oxidizers. Proc Natl Acad Sci 2016;113:E7937–46. https://doi.org/10.1073/pnas.1601212113.
[13] Qin W, Amin SA, Lundeen RA, Heal KR, Martens-Habbena W, Turkarslan S, et al. Stress response of a marine ammonia-oxidizing archaeon informs physiological status of environmental populations. ISME J 2018;12:508–19. https://doi.org/10.1038/ismej.2017.186.
[14] Santoro AE, Dupont CL, Richter RA, Craig MT, Carini P, McIlvin MR, et al. Genomic and proteomic characterization of "Candidatus Nitrosopelagicus brevis": an ammonia-oxidizing archaeon from the open ocean. Proc Natl Acad Sci USA 2015;112:1173–8. https://doi.org/10.1073/pnas.1416223112.
[15] Chain Patrick, Lamerdin Jane, Larimer Frank, Regala Warren, Lao Victoria, Land Miriam, et al. Complete genome sequence of the ammonia-oxidizing bacterium and obligate Chemolithoautotroph Nitrosomonas europaea. J Bacteriol 2003:2759–73. https://doi.org/10.1128/JB.185.9.2759–2773.2003.
[16] Sauvé S, McBride MB, Norvell WA, Hendershot WH. Copper solubility and speciation of in situ contaminated soils: effects of copper level, pH and organic matter. Water Air Soil Pollut 1997;100:133–49. https://doi.org/10.1023/A:1018312109677.
[17] Oorts K. Chemical behavior of copper in soils. In: Alloway BJ, editor. Heavy Met. Soils Trace Met. Met. Soils their Bioavailab. Third. Dordrecht: Springer Science & Business Media; 2012. p. 374.
[18] Kraemer SM, Duckworth OW, Harrington JM, Schenkeveld WDC. Metallophores and trace metal biogeochemistry. Aquat Geochem 2015;21:159–95. https://doi.org/10.1007/s10498-014-9246-7.
[19] Maurer F, Christl I, Fulda B, Voegelin A, Kretzschmar R. Copper redox transformation and complexation by reduced and oxidized soil humic acid. 2. Potentiometric titrations and dialysis cell experiments. Environ Sci Technol 2013;47:10912–21. https://doi.org/10.1021/es4024095.
[20] Kim HJ, Graham DW, DiSpirito AA, Alterman MA, Galeva N, Larive CK, et al. Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria. Science (80-) 2004;305:1612–5. https://doi.org/10.1126/science.1098322.
[21] Logan EM, Pulford ID, Cook GT, McKenzie aB. Complexation of Cu2+ and Pb2+ by peat and humic acid. Eur J Soil Sci 1997;48:685–96. https://doi.org/10.1046/j.1365-2389.1997.00123.x.
[22] Minnich MM, McBride MB. Copper activity in soil solution: I. Measurement by ion-selective electrode and donnan dialysis 1. Soil Sci Soc Am J 1987;51:568. https://doi.org/10.2136/sssaj1987.03615995005100030003x.
[23] Pendias AK. Elements of group 11: previously group Ib. In: Pendias AK, editor. Trace Elem. Soils Plants. 4th ed. Boca Raton: CRC Press Taylor and Francis Group; 2010. p. 584.
[24] Adriano DC. Copper in Soils. Trace Elem. Terr. Environ. Berlin, Heidelberg: Springer-Verlag; 1986. p. 533.
[25] Flemming CA, Trevors JT. Copper toxicity and chemistry in the environment: a review. Water Air Soil Pollut 1989;44:143–58.
[26] Bayer B, Vojvoda J, Reinthaler T, Reyes C, Pinto M, Herndl GJ. Nitrosopumilus adriaticus sp. nov. and Nitrosopumilus piranensis sp. nov., two ammonia-oxidizing archaea from the Adriatic Sea and members of the class Nitrososphaeria. Int J Syst Evol Microbiol 2019;69:1892–902. https://doi.org/10.1099/ijsem.0.003360.
[27] Jenkins SN, Murphy DV, Waite IS, Rushton SP, O'Donnell AG. Ancient landscapes and the relationship with microbial nitrification. Sci Rep 2016;6:30733. https://doi.org/10.1038/srep30733.
[28] Moffett JW, Tuit CB, Ward BB. Chelator-induced inhibition of copper metalloenzymes in denitrifying bacteria. Limnol Oceanogr 2012;57:272–80. https://doi.org/10.4319/lo.2012.57.1.0272.
[29] Glass JB, Orphan VJ. Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. Front Microbiol 2012;3:61. https://doi.org/10.3389/fmicb.2012.00061.
[30] Stanley SH, Prior SD, Leak DJ, Dalton H. Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: studies in batch and continuous cultures. Biotechnol Lett 1983;5:487–92. https://doi.org/10.1007/BF00132233.
[31] Guo J, Annett AL, Taylor RL, Lapi S, Ruth TJ, Maldonado MT. Copper-uptake kinetics of coastal and oceanic diatoms. J Phycol 2010;46:1218–28. https://doi.org/10.1111/j.1529-8817.2010.00911.x.
[32] Walsh MJ, Goodnow SD, Vezeau GE, Richter LV, Ahner BA. Cysteine enhances bioavailability of copper to marine phytoplankton. Environ Sci Technol 2015;49:12145–52. https://doi.org/10.1021/acs.est.5b02112.
[33] Annett AL, Lapi S, Ruth TJ, Maldonado MT. The effects of Cu and Fe availability on the growth and Cu:C ratios of marine diatoms. Limnol Oceanogr 2008;53:2451–61. https://doi.org/10.4319/lo.2008.53.6.2451.
[34] Amin SA, Moffett JW, Martens-Habbena W, Jacquot JE, Han Y, Devol A, et al. Copper requirements of the ammonia-oxidizing archaeon Nitrosopumilus maritimus SCM1 and implications for nitrification in the marine environment. Limnol Oceanogr 2013;58:2037–45. https://doi.org/10.4319/lo.2013.58.6.2037.
[35] Gwak Joo-Han, Jung M, Hong H, Quan JKZ, Spasov E, et al. Archaeal nitrification is constrained by copper complexation with organic matter in municipal wastewater treatment plants. ISME J 2019. https://doi.org/10.1038/s41396-019-0538-1.
[36] Jacquot JE, Horak REA, Amin SA, Devol AH, Ingalls AE, Armbrust EV, et al. Assessment of the potential for copper limitation of ammonia oxidation by Archaea in a dynamic estuary. Mar Chem 2014;162:37–49. https://doi.org/10.1016/j.marchem.2014.02.002.
[37] Whitby H, Hollibaugh JT, van den Berg CMG. Chemical speciation of copper in a salt marsh estuary and bioavailability to Thaumarchaeota. Front Mar Sci 2017;4:178. https://doi.org/10.3389/fmars.2017.00178.
[38] Tourna M, Stieglmeier M, Spang A, Könneke M, Schintlmeister A, Urich T, et al. Nitrososphaera viennensis, an ammonia oxidizing archaeon from soil. Proc Natl Acad Sci USA 2011. https://doi.org/10.1073/pnas.1013488108.
[39] Stieglmeier M, Klingl A, Alves RJE, Rittmann SKMR, Melcher M, Leisch N, et al. Nitrososphaera viennensis gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum Thaumarchaeota. Int J Syst Evol Microbiol 2014. https://doi.org/10.1099/ijs.0.063172-0.
[40] Kandeler E, Gerber H. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 1988;6:68–72. https://doi.org/10.1007/BF00257924.
[41] Griess P. Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt "Ueber einige Azoverbindungen". Berichte Der Dtsch Chem Gesellschaft 1879;12:426–8. https://doi.org/10.1002/cber.187901201117.
[42] Marie D, Partensky F, Vaulot D, Brussaard C, Marie D, Partensky F, et al. Enumeration of phytoplankton, bacteria, and viruses in marine samples. Curr. Protoc. Cytom.. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2001. p. 11.11.1–11.11.15. https://doi.org/10.1002/0471142956.cy1111s10.
[43] Grosskopf R, Janssen PH, Liesack W. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl Environ Microbiol 1998;64:960–9.
[44] Delong EF. Archaea in coastal marine environments. Proc Natl Acad Sci USA 1992;89:5685–9. https://doi.org/10.1073/pnas.89.12.5685.
[45] Pester M, Rattei T, Flechl S, Gröngröft A, Richter A, Overmann J, et al. amoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions. Environ Microbiol 2012;14:525–39. https://doi.org/10.1111/j.1462-2920.2011.02666.x.
[46] Arce MI, von Schiller D, Bengtsson MM, Hinze C, Jung H, Alves RJE, et al. Drying and rainfall shape the structure and functioning of nitrifying microbial communities in riverbed sediments. Front Microbiol 2018;9:2794. https://doi.org/10.3389/fmicb.2018.02794.
[47] Eden PA, Schmidt TM, Blakemore RP, Pace NR. Phylogenetic analysis of Aquaspirillum magnetotacticum using polymerase chain reaction-amplified 16S rRNA-specific DNA. Int J Syst Bacteriol 1991;41:324–5. https://doi.org/10.1099/00207713-41-2-324.
[48] Parkhurst DL. User's guide to PHREEQC, a computer program for speciation, reaction-path, advective-transport, and inverse geochemical calculations. Water Resour Investig Rep 1995. Report 95-4227.
[49] Anderegg Giorgio, Arnaud-Neu Francoise, Delgado Rita, Felcman Judith, Popov K. Critical evaluation of stability constants of metal complexes of complexones for biomedical environmental applications 2005;8. https://doi.org/10.1351/pac200577081445.
[50] Szczepanik W, Czarny A, Zaczyńska E, Jezowska-Bojczuk M. Preferences of kanamycin A towards copper(II). Effect of the resulting complexes on immunological mediators production by human leukocytes. J Inorg Biochem 2004;98:245–53. https://doi.org/10.1016/j.jinorgbio.2003.10.013.
[51] Pluskal T, Castillo S, Villar-Briones A, Orešič M. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinf 2010;11:395. https://doi.org/10.1186/1471-2105-11-395.
[52] Baars O, Morel FMM, Perlman DH. ChelomEx: isotope-assisted discovery of metal chelates in complex media using high-resolution LC-MS. Anal Chem 2014;86:11298–305. https://doi.org/10.1021/ac503000e.
[53] Baars O, Zhang X, Morel FMM, Seyedsayamdost MR. The Siderophore metabolome of Azotobacter vinelandii. Appl Environ Microbiol 2016;82:27–39. https://doi.org/10.1128/AEM.03160-15.
[54] Szczepanik W, Kaczmarek P, Sobczak J, Bal W, Gatner K, Jezowska-Bojczuk M. Copper(II) binding by kanamycin A and hydrogen peroxide activation by resulting complexes. New J Chem 2002;26:1507–14. https://doi.org/10.1039/b203812a.
[55] Sher A, Veber M, Marolt-Gomišček M. Spectroscopic and polarographic investigations: copper(II)-penicillin derivatives. Int J Pharm 1997;148:191–9. https://doi.org/10.1016/S0378-5173(96)04844-2.
[56] Boiteau RM, Fitzsimmons JN, Repeta DJ, Boyle EA. Detection of iron ligands in seawater and marine Cyanobacteria cultures by high-performance liquid chromatography-inductively coupled plasma mass spectrometry. Anal Chem 2013;85:4357–62.
[57] Offre P, Kerou M, Spang A, Schleper C. Variability of the transporter gene complement in ammonia-oxidizing archaea. Trends Microbiol 2014;22:665–75. https://doi.org/10.1016/j.tim.2014.07.007.
[58] Zhang Lianyi, Koay Melissa, Maher Megan J, Zhiguang Xiao, Wedd AG. Intermolecular transfer of copper ions from the CopC protein of Pseudomonas syringae. Crystal Struct Fully Loaded CuICuII Forms 2006. https://doi.org/10.1021/JA058528X.
[59] Zhang X-X, Rainey PB. Regulation of copper homeostasis in *Pseudomonas fluorescens* SBW25. Environ Microbiol 2008;10:3284–94. https://doi.org/10.1111/j.1462-2920.2008.01720.x.

[60] Hirooka K, Edahiro T, Kimura K, Fujita Y. Direct and indirect regulation of the ycnKJI operon involved in copper uptake through two transcriptional repressors, YcnK and CsoR, in Bacillus subtilis. J Bacteriol 2012;194:5675–87. https://doi.org/10.1128/JB.00919-12.
[61] Chillappagari S, Miethke M, Trip H, Kuipers OP, Marahiel MA. Copper acquisition is mediated by YcnJ and regulated by YcnK and CsoR in Bacillus subtilis. J Bacteriol 2009;191:2362–70. https://doi.org/10.1128/JB.01616-08.
[62] Lawton TJ, Kenney GE, Hurley JD, Rosenzweig AC. The CopC family: structural and bioinformatic insights into a diverse group of Periplasmic copper binding proteins. Biochemistry 2016;55:2278–90. https://doi.org/10.1021/acs.biochem.6b00175.
[63] Francis MS, Thomas CJ. Mutants in the CtpA copper transporting P-type ATPase reduce virulence of Listeria monocytogenes. Microb Pathog 1997;22:67–78. https://doi.org/10.1006/mpat.1996.0092.
[64] Lewinson O, Lee AT, Rees DC. A P-type ATPase importer that discriminates between essential and toxic transition metals. Proc Natl Acad Sci 2009;106:4677–82. https://doi.org/10.1073/pnas.0900666106.
[65] Arguello JM. Identification of ion-selectivity determinants in heavy-metal transport P 1B -type ATPases. J Membr Biol 2003;195:93–108. https://doi.org/10.1007/s00232-003-2048-2.
[66] González-Guerrero M, Raimunda D, Cheng X, Argüello JM. Distinct functional roles of homologous Cu+ efflux ATPases in Pseudomonas aeruginosa. Mol Microbiol 2010;78:1246–58. https://doi.org/10.1111/j.1365-2958.2010.07402.x.
[67] Coombs JM, Barkay T. New findings on evolution of metal homeostasis genes: evidence from comparative genome analysis of bacteria and archaea. Appl Environ Microbiol 2005;71:7083–91. https://doi.org/10.1128/AEM.71.11.7083-7091.2005.
[68] Srivastava P, Kowshik M. Mechanisms of metal resistance and homeostasis in Haloarchaea. Archaea 2013;2013. https://doi.org/10.1155/2013/732864.
[69] Ettema TJG, Brinkman AB, Lamers PP, Kornet NG, de Vos WM, van der Oost J. Molecular characterization of a conserved archaeal copper resistance (cop) gene cluster and its copper-responsive regulator in Sulfolobus solfataricus P2. Microbiology 2006;152:1969–79. https://doi.org/10.1099/mic.0.28724-0.
[70] González-Guerrero M, Argüello JM. Mechanism of Cu+-transporting ATPases: soluble Cu+ chaperones directly transfer Cu+ to transmembrane transport sites. Proc Natl Acad Sci U S A 2008;105:5992–7. https://doi.org/10.1073/pnas.0711446105.
[71] Schenkeveld WDC, Weng LP, Reichwein AM, Temminghoff EJM, van Riemsdijk WH. Evaluation of the potential impact of Cu competition on the performance of o,o-FeEDDHA in soil applications. Eur J Soil Sci 2015;66:277–85. https://doi.org/10.1111/ejss.12226.
[72] Villaverde P, Gondar D, Antelo J, López R, Fiol S, Arce F. Influence of pH on copper, lead and cadmium binding by an ombrotrophic peat. Eur J Soil Sci 2009;60:377–85. https://doi.org/10.1111/j.1365-2389.2009.01132.x.
[73] Mengel Konrad, Kirkby Ernest A, Kosegarten Harald, Appel T. Soil copper. In: Princ. Plant Nutr. 5th ed. Dordrecht: Kluwer Academic Publishers; 2001. p. 849.
[74] Nriagu JO. Copper in the Environment Part 1: Ecological Cycling. Wiley-Blackwell; 1979.
[75] van Schaik JWJ, Kleja DB, Gustafsson JP. Acid-base and copper-binding properties of three organic matter fractions isolated from a forest floor soil solution. Geochim Cosmochim Acta 2010;74:1391–406. https://doi.org/10.1016/j.gca.2009.11.007.
[76] Macqueen DJ, Gubry-Rangin C. Molecular adaptation of ammonia monooxygenase during independent pH specialization in Thaumarchaeota. Mol Ecol 2016;25:1986–99. https://doi.org/10.1111/mec.13607.
[77] Lehtovirta-Morley LE, Stoecker K, Vilcinskas A, Prosser JI, Nicol GW. Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. Proc Natl Acad Sci U S A 2011;108:15892–7. https://doi.org/10.1073/pnas.1107196108.
[78] Nicol GW, Leininger S, Schleper C, Prosser JI. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ Microbiol 2008;10:2966–78. https://doi.org/10.1111/j.1462-2920.2008.01701.x.
[79] Che J, Zhao XQ, Zhou X, Jia ZJ, Shen RF. High pH-enhanced soil nitrification was associated with ammonia-oxidizing bacteria rather than archaea in acidic soils. Appl Soil Ecol 2014;85:21–9. https://doi.org/10.1016/j.apsoil.2014.09.003.
[80] He J-Z, Hu H-W, Zhang L-M. Current insights into the autotrophic thaumarchaeal ammonia oxidation in acidic soils. Soil Biol Biochem 2012;55:146–54. https://doi.org/10.1016/J.SOILBIO.2012.06.006.
[81] Zhang M, Alves RJE, Zhang D, Han L, He J, Zhang L. Soil Biology & Biochemistry Time-dependent shifts in populations and activity of bacterial and archaeal ammonia oxidizers in response to liming in acidic soils. Soil Biol Biochem 2017;112:77–89. https://doi.org/10.1016/j.soilbio.2017.05.001.
[82] Stempfhuber B, Engel M, Fischer D, Neskovic-Prit G, Wubet T, Schöning I, et al. pH as a driver for ammonia-oxidizing archaea in forest soils. Microb Ecol 2015;69:879–83. https://doi.org/10.1007/s00248-014-0548-5.
[83] Giannopoulos Georgios, Hartop Katherine, Bonnie Brown RF, Giannopoulos G, Hartop K, Brown B, et al. Response relationships between CO2, CH4 and N2O emissions and microbial functional groups in wetland sediments after trace metal addition. BioRxiv 515809 2019:1–21. https://doi.org/10.1101/515809.