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„Characterization of Thaumarchaeota isolated from the Northern Adriatic Sea“

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Abstract

The discovery of ubiquitous ammonia-oxidizing Thaumarchaota in aquatic and terrestrial environments has led to an increased interest to further understand their physiology and ecological function. Here we report the physiological and genomic characterization of two *Nitrosopumilus* isolates (D3C and NF5) originating from the surface waters of the Northern Adriatic Sea. In addition to genes required for ammonia-driven chemolithoautotrophy, D3C possesses genes for the degradation and transport of urea and cultivation approaches confirmed its ability to utilize urea as an alternative substrate for ammonia oxidation. The extremely low ammonia half-saturation constants observed for both isolates (K_m= 54 nM and 34 nM NH_3+NH_4 for D3C and NF5, respectively) indicate adaptations to even more oligotrophic conditions than previously reported for *Nitrosopumilus maritimus* (Martens-Habbena *et al.* 2009). Additionally, a high number of motility-associated genes were identified in the NF5 isolate, and both genomes contained a gene encoding photolyase. The finding of phylogenetically close but functionally distinctive members of the Nitrosopumilus genus in the Northern Adriatic Sea indicates a larger functional diversity of marine mesophilic Thaumarchaeota than previously assumed and points towards a possible niche differentiation among several closely related species.

Keywords: Thaumarchaeota, ammonia oxidation, urease, Adriatic Sea, photolyase

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Introduction

Archaea represent one of the three domains of life and were initially thought to be restricted to extreme environments including geothermal hot and acidic springs, salt lakes and strictly anoxic ecosystems (Woese *et al.* 1978; Jones *et al.* 1987). In the early 1990ies, 16S rRNA gene fragments of *Archaea* were found in various marine environments (DeLong 1992; Fuhrman *et al.* 1992), constituting up to 20% of the picoplankton (Karner *et al.* 2001). Their global distribution, however, has only been predicted by PCR-based surveys and the ecological function of mesophilic *Archaea* remained elusive.

^{13}C label experiments demonstrated that pelagic marine *Archaea* are capable of light-independent bicarbonate uptake, suggesting archaeal autotrophy (Wuchter *et al.* 2003). The identification of putative archaeal ammonia monooxygenase (*amo*) genes (the primary enzyme for the oxidation of ammonia) in terrestrial and marine habitats provided first evidence that at least some mesophilic *Archaea* might be capable of autotrophic ammonia oxidation (Treusch *et al.* 2005). This was supported by the isolation of the marine archaeon, *Nitrosopumilus maritimus*, the first cultured representative of ammonia-oxidizing Archaea (AOA) able to grow chemolithoautotrophically, utilizing ammonia as sole energy source (Koenneke *et al.* 2005).

Aerobic ammonia oxidation is the first, rate-limiting step of nitrification and the only process known which converts reduced inorganic nitrogen compounds into their oxidized form. Ammonia-oxidizing Bacteria (AOB) have long been thought to be the sole drivers of nitrification, however, the ubiquity of archaeal *amo*A genes throughout the water column, and their numerical dominance over that of Bacteria, challenged this view (Prosser & Nicol 2008). AOA seem to be among the most abundant organisms in marine ecosystems and likely play a major role in the global nitrogen and carbon cycling.

Ammonia oxidation has been experimentally demonstrated for cultivated AOA, however, the extremely low ammonium concentrations (< 10 nM) in most parts of the meso- and bathypelagic ocean are inconsistent with archaeal *amo* gene abundances in those regions (Church *et al.* 2010), indicating the utilization of possible alternative energy sources. Although archaeal *amo*A genes are established marker genes for ammonia oxidation, the protein family of copper-containing membrane-bound monooxygenases

(CuMMO), to which archaeal ammonia monooxygenases belong, have a wide substrate range, including methane and alkanes (Pester *et al.* 2011). In some Bacteria, monooxygenases exhibit even nearly equal substrate specificities for ammonia and methane (Lontoh *et al.* 2000), indicating that archaeal monooxygenases might as well have the potential to oxidize different substrates. Additionally, the sponge-associated symbiont *Cand*. Cenarchaeum symbiosum as well as arctic AOA have been found to contain genes for urea degradation and transport (Hallam *et al.* 2006; Alonso-Saez *et al.* 2012), tentatively suggesting that urea might be utilized as a substrate for nitrification in at least some members of AOA.

Phylogenetic analyses indicated the affiliation of AOA to the novel deep-branching phylum Thaumarchaeota rather than to the Crenarchaeota as initially assumed (Brochier-Armanet *et al.* 2008), supported by the genomic information of *Cand.* Cenarchaeum symbiosum, *Nitrosopumilus maritimus* and the terrestrial archaeon *Cand.* Nitrososphaera gargensis, which share >250 unique proteins that are not present in members of the Crenarchaeota and Euryarchaeota (Gupta & Shami 2011).

Thus far, all characterized Thaumarchaeota have been found to contain genes encoding a variant of the 3-Hydroxypropionate/4-Hydroxybutyrate (3HP/4HB) cycle (Berg *et al.* 2010), enabling the fixation of inorganic carbon. However, genomic analyses also pointed towards a remaining potential for mixotrophy (Walker *et al.* 2010; Spang *et al.* 2012), whereas up to 60% of marine planktonic Archaea seem to be able to take up dissolved amino acids, suggesting heterotrophy (Ouverney & Fuhrman 2000). Furthermore, the pure culture of the soil-inhabiting *Cand.* Nitrososphaera viennensis has been shown to incorporate considerable amounts of ^{13}C labeled pyruvate into biomass when it was added to the growth medium (Tourna *et al.* 2011).

Advances in molecular ecological approaches and metagenomics have led to an increased understanding of archaeal diversity and the possible ecological function of Thaumarchaeota in marine and terrestrial environments. However, metagenomic approaches bear the inherent risk of mis-assembling the genome of closely related members from natural communities (Tringe *et al.* 2005; Kunin *et al.* 2008). The availability of pure and enriched cultures makes it possible to characterize genomes more comprehensively and to combine and link genomic information with detailed physiological studies, thereby providing essential information on the ecological role of AOA in the environment.

The aim of this study was the physiological and genomic characterization of two *Nitrosopumilus* species isolated from the surface waters of the Northern Adriatic Sea. The isolates D3C and NF5 are both ammonia oxidizers, able to grow in completely inorganic medium, and seem to be adapted to even more extreme oligotrophic conditions than *N. maritimus* (Martens-Habbena *et al.* 2009). The utilization of alternative substrates was investigated for both isolates and complemented with genomic data. The identification of phylogenetically close but functionally distinct Thaumarchaeota provides insight into the functional diversity within the Nitrosopumilus genus and points towards a possible niche differentiation among closely related AOA species.

Material and Methods

Cultivation and enrichment of Thaumarchaeota isolates

Cultures were grown in Synthetic Crenarchaeota Medium (SCM) (Koenneke *et al.* 2005) consisting of NaCl (26 g l^{-1}), $MgCl_2 \cdot 6H_2O$ (5 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (5 g l^{-1}), $CaCl_2$ (1.5 g l^{-1}) and KBr (0.1 g l^{-1}). After autoclaving, 1 ml non-chelated trace element solution, 1 ml Fe-NaEDTA solution, 10 ml KH_2PO_4 solution (4 g l^{-1}), 1 ml selenite-tungstate solution, 1 ml sodium bicarbonate (1 M), and 1 ml ammonium chloride (1 M) were added to 1 L of media. The media contained final ammonium chloride and sodium bicarbonate concentrations of 1 mM and 2 mM, respectively. The pH was adjusted to 7.1-7.3 by adding 10 ml l^{-1} of HEPES buffer (1 M HEPES, 0.6 M NaOH). Cultures were incubated in 20 ml flasks aerobically at 30°C without shaking. Ammonia consumption and nitrite production were measured spectrophotometrically using the OPA reagent (Holmes *et al.* 1999) and Griess reagent (Griess 1879), respectively. When ammonium concentrations dropped below 300 µM, cultures were transferred into new medium (5% or 10% inoculum). The state of enrichment was regularly checked via CARD-FISH (details see below).

Catalyzed Reporter Deposition Fluorescence *In Situ* Hybridization (CARD-FISH)

Samples were fixed overnight with formaldehyde (3% final conc.) at 4°C and subsequently filtered onto 0.2 µm polycarbonate GTTP membranes (Millipore). CARD-FISH was carried out according to established protocols (Teira *et al.* 2004) with the following modifications: The permeabilization mix contained 10 mg ml^{-1} lysoyzme (Sigma L6876) or 10.9 mg ml^{-1} proteinase K (Sigma P4850), 0.01 M Tris-HCl (pH 8) and 0.4 M EDTA. The hybridization buffer contained 0.05% (v/v) TritonX instead of 0.02% (v/v) SDS. Filters were hybridized with horseradish peroxidase (HRP)-labeled oligonucleotide probe mixtures using Cren537 + Cren557 probes to target archaeal and EUBI-III probes to target bacterial 16S rRNA gene sequences. After the amplification step, filters were incubated (in the dark on ice for 3 min) with DAPI (final conc. 1 µg ml^{-1}), subsequently washed with ethanol (80% v/v) and Milli-Q water and mounted in PBS-Vectashield-Citifluor (0.5 µg ml^{-1} PBS, 1 µg ml^{-1} Vectashield, 5.5 µg ml^{-1} Citifluor). Slides were examined at 1250x magnification under an epifluorescence microscope (Axio Imager.M2, Zeiss).

Quantitative PCR analysis (qPCR)

qPCR analysis was carried out to estimate the percentage of enrichment based on bacterial 16S rRNA gene copy numbers compared to archaeal *amo*A gene copy numbers. DNA from at least 15 ml culture was extracted according to the following procedure: cultures were filtered onto 0.2 µm polycarbonate GTTP membranes (Millipore) and the filters subsequently cut with sterile scissors and placed into 15 ml Greiner tubes. Zirconium beads and 6 ml sodium dodecyl sulfate (SDS) extraction buffer (10 mM Tris-HCl pH 8, 25 mM Na_2EDTA pH 8, 100 mM NaCl, 1% v/v SDS) were added. The tubes were bead-beaten (10 min) and placed in a 70°C water bath (30 min). Three ml phenol (pH 7.9) was added and after centrifugation (at 10,000 *g* for 10 min), the watery phase was removed, placed into a new tube and 3 ml phenol-chloroform-isoamylalcohol (25:24:1) was added. This was followed by an additional purification step (same procedure) using chloroform. Subsequently, 2 volumes ethanol (-20°C, 100% v/v) and 0.02 volumes NaCl (5 M) were added, the solution incubated (-20°C, 2 h) and centrifuged for 45 min (4°C, 10,000 *g*). The pellet was washed with ethanol (70% v/v), centrifuged for 45 min (4°C, 10,000 *g*), dried and dissolved in 100 µl Tris-HCl (10 mM, pH 8). The extracted DNA was purified using the PowerClean Purification Kit (MO BIO Laboratories).

Quantification of bacterial 16S rRNA and archaeal *amo*A genes was performed using 10 µl total volume containing 5 µl LightCycler 480 SYBR Green I Master Mix (Roche), 3.6 µl PCR H_2O, 0.2 µl of each primer and 1 µl of sample. For bacterial 16S rRNA and archaeal *amo*A gene standards, a serial dilution of purified PCR products (PCR Extract, 5Prime) obtained from an ocean surface water DNA sample was used, and gene copies quantified using the 16S rRNA gene primers 1361F (5'-CGGTGAATACGTTCYCGG-3') and 1492R (5'-GGWTACCTTGTTACGACTT-3') and the *amo*A gene primers 296F (5'-CTGAYTGGGCYTGGACATC-3') and 551R (5'-TTCTTCTTTGTYGCCCARTA-3'), respectively. Amplifications were performed in a LightCycler 480 II (Roche). Melting curve analysis was performed at the end of all qPCR runs and the presence of only specific products confirmed via standard agarose gel electrophoresis.

Determination of optimal growth conditions and alternative substrates

Cultures were incubated in SCM media of different pH (6.8, 7.1, 7.3, 7.5, 8.0, adjusted with HEPES, T = 30°C) and temperatures (24°C, 28°C, 32°C, 37°C, pH adjusted to 7.2). When ammonium concentrations dropped below 300 µM, triplicates of cultures were inoculated for a second time under the same conditions to enable adaptation to the new parameters prior to analysis. Every second day, 1 ml of culture was fixed with glutaraldehyde (5%) for 10 min and subsequently stored at -80°C for further analysis. Samples were diluted in BSA buffer, stained with SYBRGreen I (final conc: 1:10,000) and cell abundances measured via flow cytometry (Accuri C6). Temperature and pH optima were determined by calculating the archaeal generation time, g, and division rate, v (number of generations d^{-1}):

$$N = N_0 \times 2^n$$

(N_0 = Number of cells at the beginning of exponential growth, N = number of cells at the end of exponential growth, n = number of generations)

$$g = t/n$$

(g = generation time (days), t = duration of exponential growth (days), n = number of generations)

$$v = 1/g$$

(v = division rate (d^{-1}))

For urea experiments, ammonium-depleted cultures were inoculated and consecutively transferred into medium supplemented with 0.25-1 mM urea. For taurine investigations, taurine was added in concentrations of 25 µM, 250 µM and 1 mM to medium without ammonium and 250 µM to medium containing 1 mM ammonium chloride. Cell abundances and nitrite production were measured over 14 d (see above).

Determination of ammonium uptake kinetics

Ammonium-depleted cultures in early-stationary phase were inoculated in 150 ml flasks (3-5x10^6 cells ml^{-1}) containing ammonium-free medium and incubated at 30°C overnight. Ammonium was subsequently added in concentrations of 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 600 nM, 800 nM, 1000 nM, 1500 nM and 2000 nM to triplicate cultures. Ammonium concentrations were determined utilizing the fluorometric OPA

method for low ammonium concentrations (Holmes *et al.* 1999), using 10 ml culture and 2 ml OPA reagent for the reaction. After 2-3 h of incubation, fluorescence was measured with a spectrofluorophotometer (RF-1501, Shimadzu) and ammonium concentrations were calculated from the time zero (T_0) standard curves.
The ammonium uptake rate per hour was calculated and kinetic constants were obtained with SigmaPlot, fitting the Michaelis-Menten equation to the obtained ammonium uptake rates:

$$V = (V_{max} \, x \, [S]) \, x \, (K_m + [S])^{-1}$$

(V = velocity, V_{max} = maximum velocity ($\mu M \, h^{-1}$), K_m = half-saturation constant for ammonium oxidation (μM), [S] = concentration of ammonium in μM).

DNA extraction and pyrosequencing

Highly enriched 1L batch cultures with cells in early stationary phase were used for genomic DNA extraction. Microbial biomass was obtained by centrifuging 4x250 ml culture (45 min, 14,000 *g*) in a high performance centrifuge (Avanti J-26 XP, Beckman Coulter). Each pellet was resuspended in 0.5 ml sodium dodecyl sulfate (SDS) extraction buffer (0.7 M NaCl, 0.1 M $Na_2(SO_3)$, 0.1 M Tris/HCl pH 7.5, 0.05 M EDTA pH 8, 1 v/v% SDS) and the mixture transferred into Lysing Matrix tubes.
Phenol/chloroform/isoamyl alcohol (0.5 ml in total, 25:24:1) was added and the tubes placed in a Fast prep machine (speed 4 for 30 s) to lyse the cells and separate genomic DNA from proteins. After cooling the tubes on ice (2 min) and centrifuging (10 min, 10,000 *g*), 0.5 ml chloroform/isoamyl alcohol (24:1) was added to the supernatant and after another centrifugation step (10 min, 10,000 *g*), 2× volume polyethylene glycole (PEG) solution (1.6 M NaCl, 30% PEG) was added and the mixture incubated overnight. Subsequently, the tubes were centrifuged for at least 30 min (4°C, 10,000 *g*), the pellet washed with cold ethanol (70% v/v), dried, eluted in 50 µl ultrapure H_2O and stored at -20°C. Extracted DNA was pyrosequenced using a 454/FLX-Titanium sequencer (Roche). The total number of reads was 114,359 and 80,867 and the average read length 622 and 623 bp, for the isolates D3C and NF5, respectively.

Genome assembly and annotation

The obtained reads were length- and quality-filtered (parameters: min. length = 150bp, min. average quality = 25) using the prinseq pipeline (Schmieder & Edwards 2011). Draft genomes were de-novo assembled with Newbler 2.6 (Margulies et al. 2005). The resulting contigs were blasted with Blastn (Altschul et al. 1997) and contigs affiliated with Bacteria removed from the draft genome (in case of NF5). Auto-annotations of the draft genomes were performed with the program ConsPred (Thomas Weinmaier, unpublished) and genomes compared via reciprocal Blastp. Pairwise alignments of the assemblies were compared to *N. maritimus* and visualized with the Promer program in the MUMmer 3.23 package (Kurtz et al. 2004). SignalP 4 (Petersen et al. 2011) was used for the prediction of signal peptides and SecretomeP (Bendtsen et al. 2005) used to predict non-classical secretion pathways for enzymes.

Phylogenetic analyses

Phylogenetic distance trees of sequenced and characterized Thaumarchaeota based on 16S rRNA (1411 positions), 23S rRNA (2951 positions) and *amo*A (600 positions) gene sequences were constructed in Geneious 7.1 (Kearse *et al.* 2012), using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering and Jukes-Cantor genetic distance corrections. Bootstrap values were calculated from 1000 re-samplings using neighbor joining.

Results

Enrichment of the two AOA isolates D3C and NF5

The two AOA isolates were obtained from coastal surface water of the Northern Adriatic Sea (approx. 0.5 m depth) off the coast of Piran (Slovenia) in November and December 2011. Enrichment in completely inorganic medium amended with antibiotics (Kanamycin, Streptomycin, Carbenicillin, Spectinomycin) over 2 years resulted in the two stable enrichments, NF5 and D3C. CARD-FISH and qPCR analyses showed enrichment of both isolates of ≥ 99% (± 1% error rate) (Fig. S1). The absence of betaproteobacterial *amo*A genes and nitrate production indicated that autotrophic bacterial contaminants, which would bias the ammonia oxidation measurements, were not present.

Cells of the two isolates were small, regular rods with a length of 0.5-1.8 µm and width of ~0.2 µm as revealed by scanning electron microscopy (Fig. 1). After a lag phase of 2-5 d, both enrichment cultures stoichiometrically converted ammonia into nitrite (Fig. 2) when grown in chemically defined inorganic medium supplemented with 1 mM NH_4Cl and 2 mM sodium bicarbonate. After 7-12 d of incubation, all ammonium was consumed and cultures reached cell abundances of up to 10^8 cells ml^{-1}. The generation times of both enrichments were on average 27-58 h. The high nitrite concentrations (up to 1 mM) resulting from the oxidation of ammonia were not found to affect cell growth.

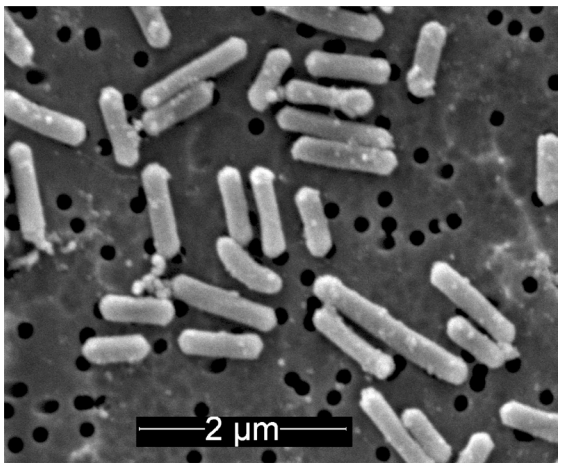


Figure 1. SEM picture of the NF5 enrichment culture (by Jean-Marie Volland)

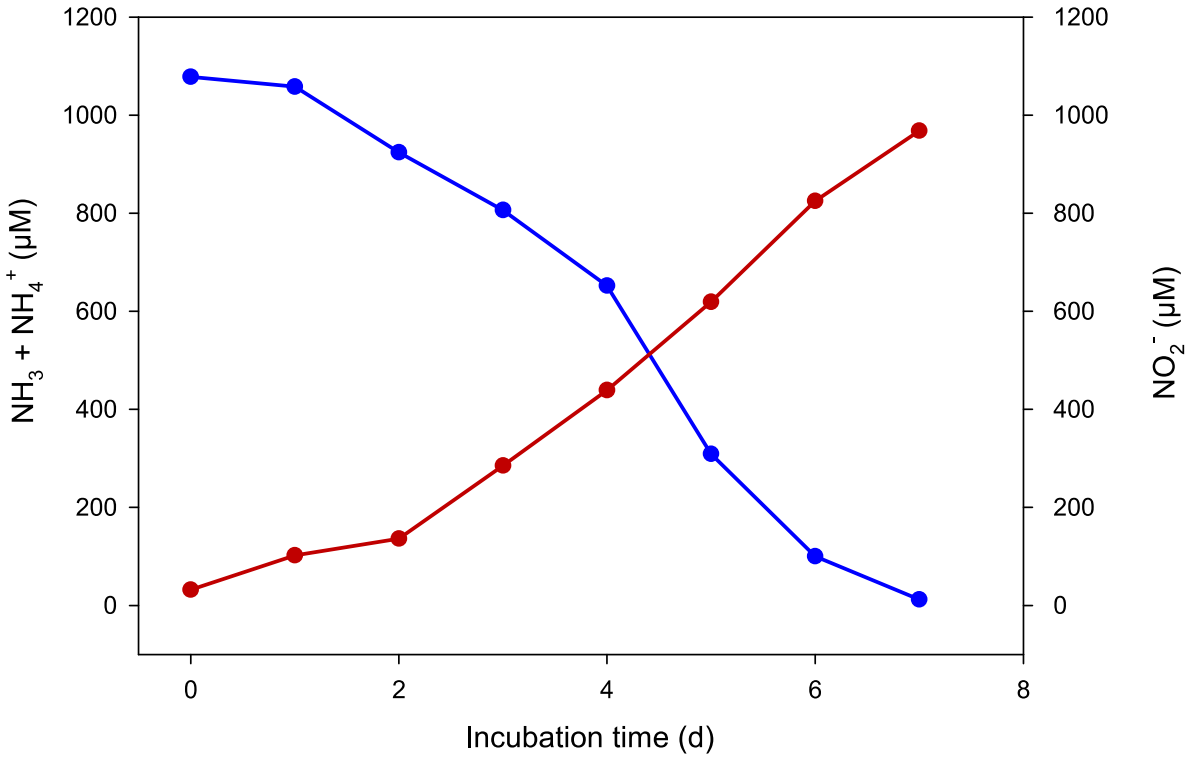


Figure 2. Typical growth curve of NF5 with near-stoichiometric conversion of ammonium (blue line) into nitrite (red line)

Physiological characterization

Physical parameters

Both enrichment cultures were tested for growth at a temperature range of 24-37°C and a pH range of 6.8-8.0 with growth optima determined via ammonium and cell abundance measurements. All incubations were carried out with ammonium concentrations of 1 mM. To determine the temperature optimum for growth, the pH was adjusted to 7.1-7.3 with HEPES buffer, while the cultures were incubated at 30°C for determination of the pH optimum.

The temperature optimum of NF5 ranged from 28°C to 30°C (Fig. 3A-B), whereas D3C exhibited highest growth rates at 30-32°C (Fig. 4A-B). Generally, no growth was observed at 37°C and cell abundances were found to decrease. The optimal pH ranged from 7.2-7.3 and 7.1-7.3 for NF5 and D3C (Fig. 5A-B, Fig. 6A-B), respectively. Cultures did not grow at pH 8, however, cell numbers were not found to decrease and remained constant over 30 days.

Collectively, the growth optima of the two archaeal cultures differed slightly in terms of temperature and pH.

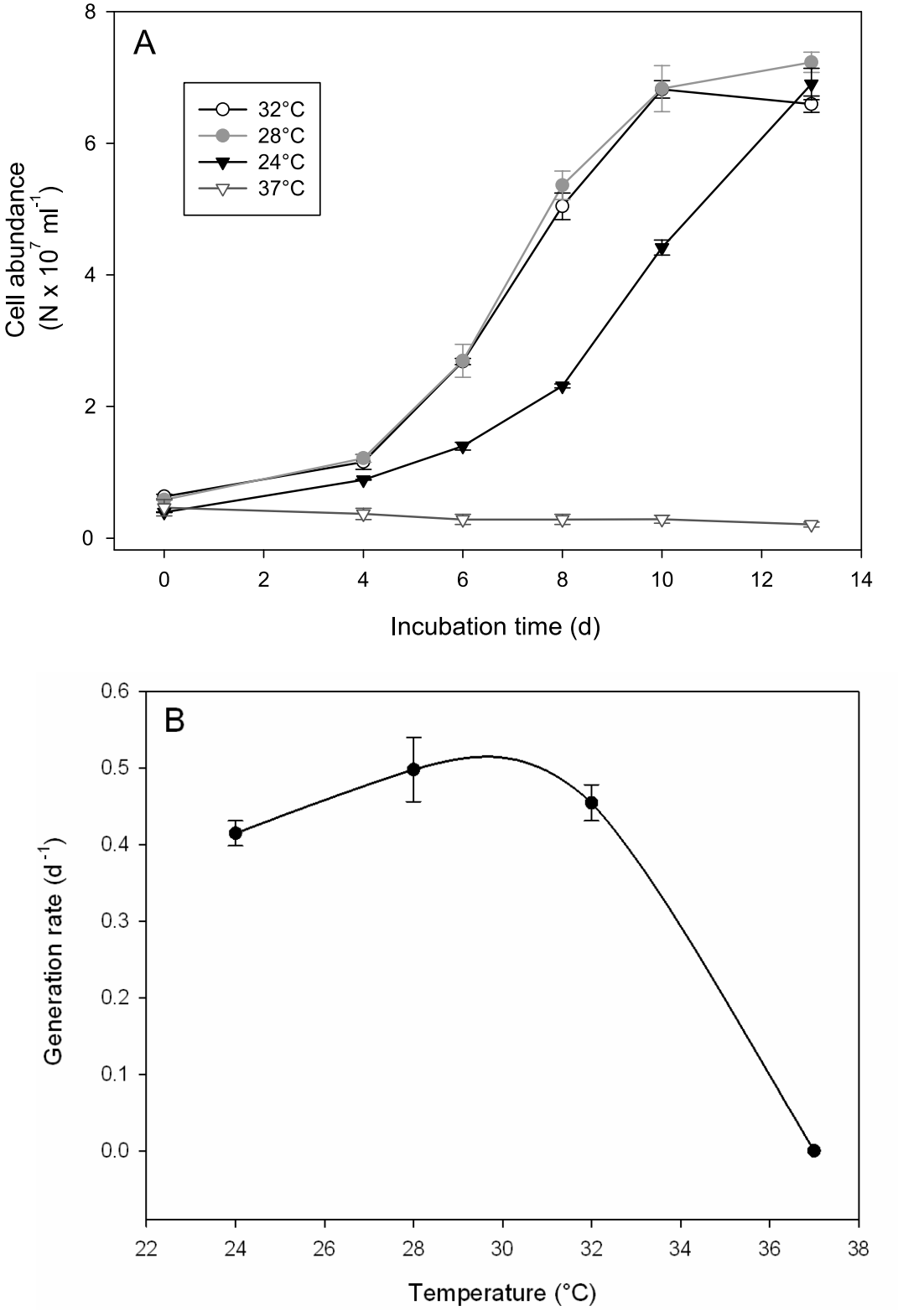


Figure 3. Cell abundances (A) and generation rates (B) of NF5 when incubated at different temperatures. Plotted data represents means of measurements from triplicate incubations. Error bars represent SD.

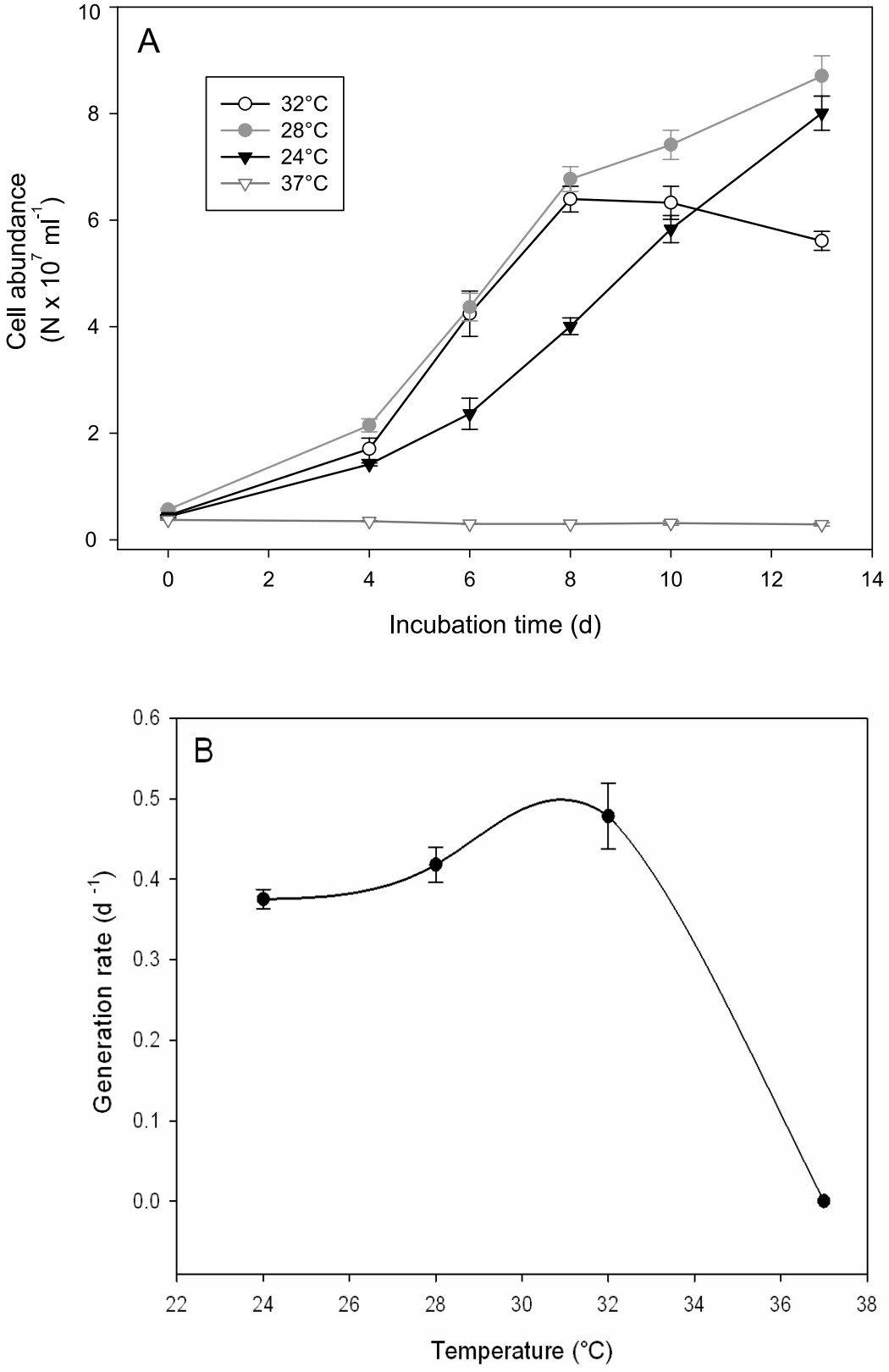


Figure 4. Cell abundances (A) and generation rates (B) of D3C when incubated at different temperatures. Plotted data represents means of measurements from triplicate incubations. Error bars represent SD.

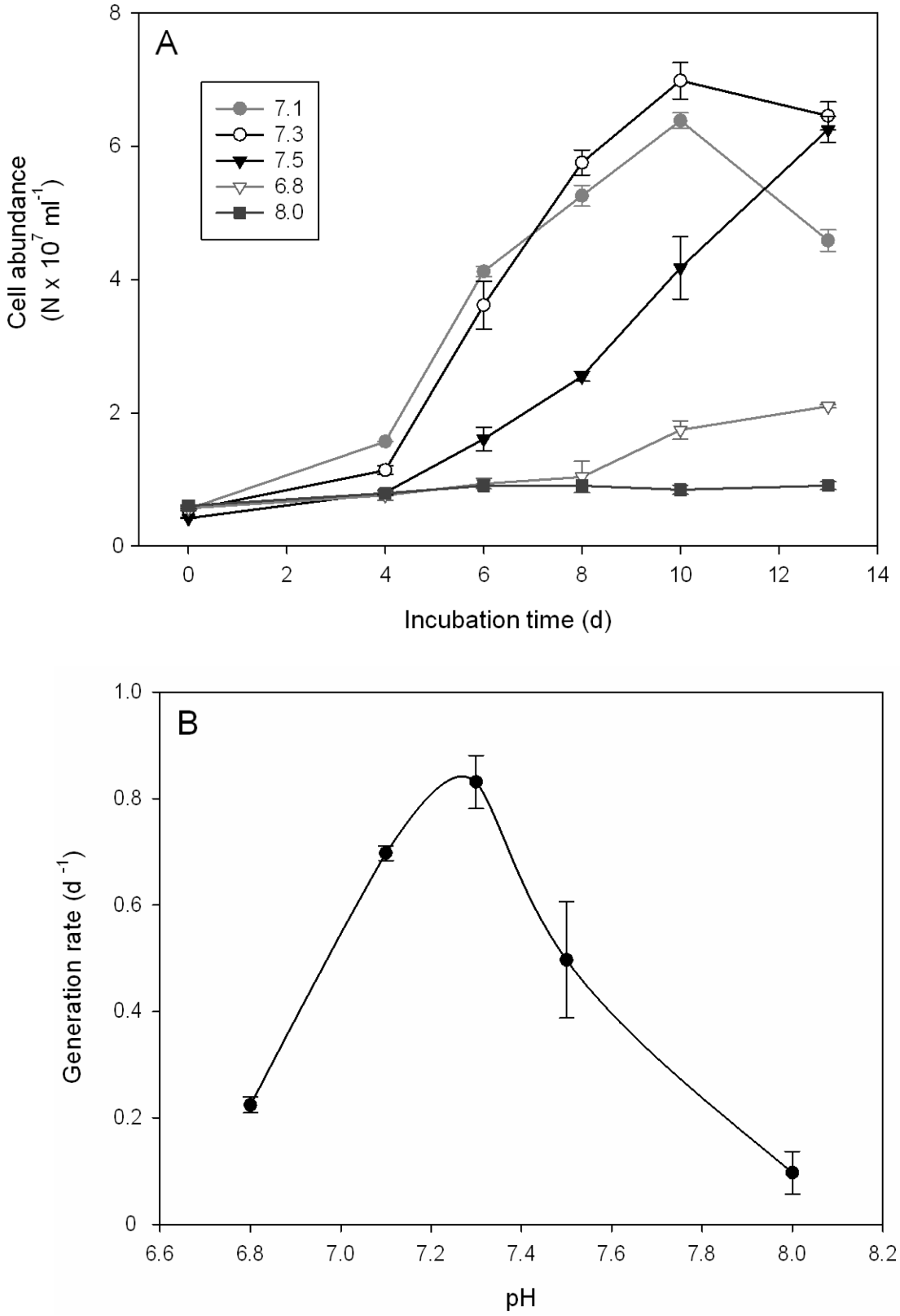


Figure 5. Cell abundances (A) and generation rates (B) of NF5 when incubated at different pH. Plotted data represents means of measurements from triplicate incubations. Error bars represent SD.

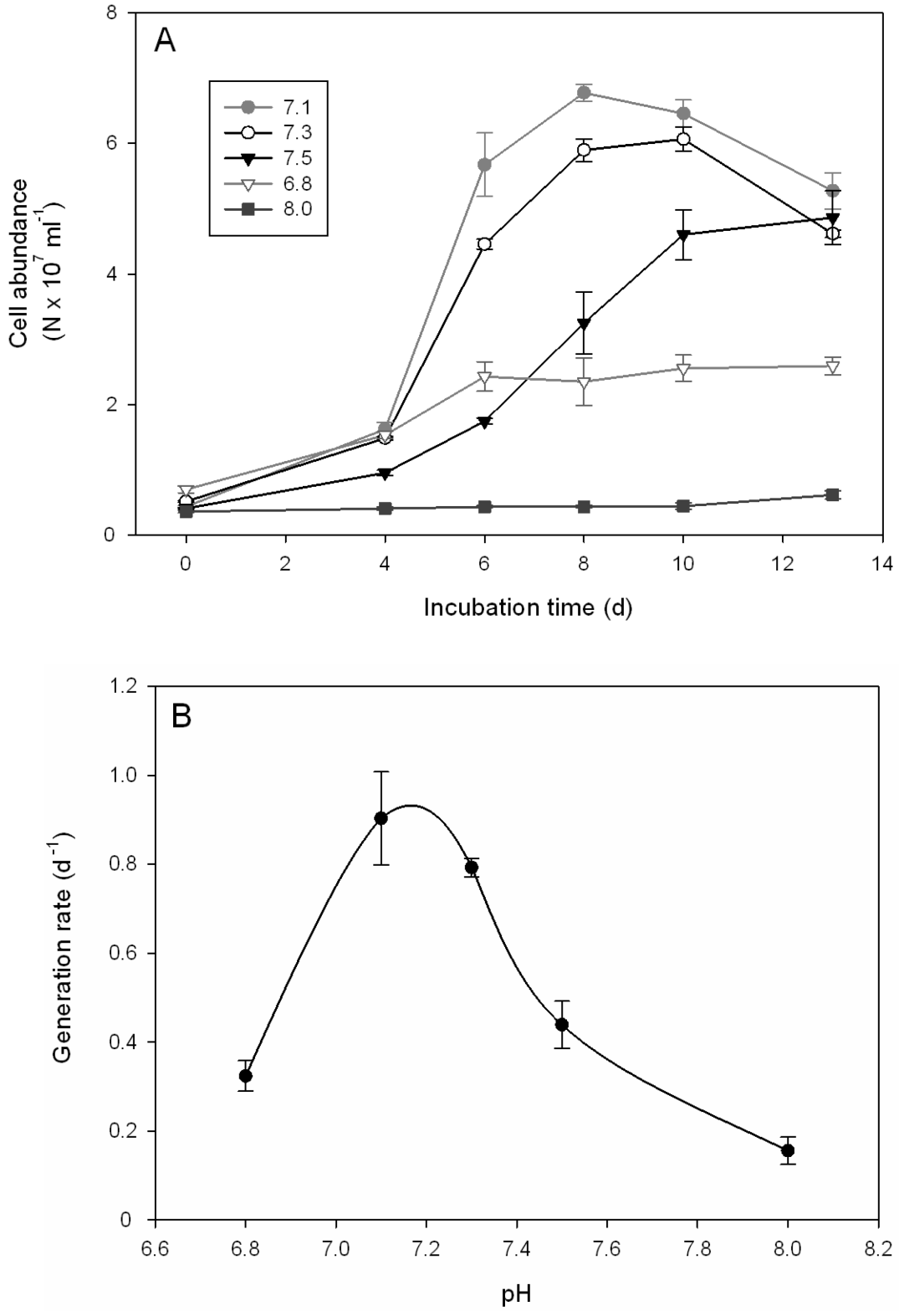


Figure 6. Cell abundances (A) and generation rates (B) of D3C when incubated at different pH. Plotted data represents means of measurements from triplicate incubations. Error bars represent SD.

Utilization of alternative substrates

The capability of both enrichment cultures to utilize taurine and urea as alternative substrates was investigated. In both isolates, addition of taurine did not yield an increase in cell abundance as compared to the control treatments (data not shown). When the cultures were incubated in medium supplemented with 1 mM urea (as sole energy source), the cell abundance of NF5 decreased (Fig. 7A), whereas D3C grew to cell abundances comparable to growth in medium containing ammonium. This indicated that D3C was able to utilize urea as an alternative substrate for ammonia oxidation (Fig. 7B). Additionally, growth of D3C under several urea concentrations (1 mM, 250 μM) and on different pH (7.1 and 7.5) was investigated. When grown at pH 7.1, the addition of 1 mM and 250 μM urea resulted in the production of 800 μM and 500 μM nitrite, respectively (Fig. 8A). At pH 7.5, however, addition of 1 mM and 250 μM urea, yielded the production of 350-400 μM nitrite for both treatments (Fig. 8B). A stoichiometric conversion of urea-bound ammonia into nitrite could only be observed at pH 7.0-7.3 for urea concentrations < 500 μM, indicating concentration and pH dependence of the reaction (data not shown). After 3 d of incubation, ammonium could not be detected (<10 μM), however, after 5 d of incubation, ammonium was detected in concentrations of 100-160 μM in the medium, indicating that either urea was cleaved outside the cell or that excess ammonia was released from the cell again. The pH remained constant throughout the experiments.

The addition of urea (250 μM, 500 μM, 1 mM) to medium containing ammonium (1 mM) resulted in a continuous ammonium decrease independent from the amount of urea added, suggesting that freely available ammonium is taken up preferentially (data not shown).

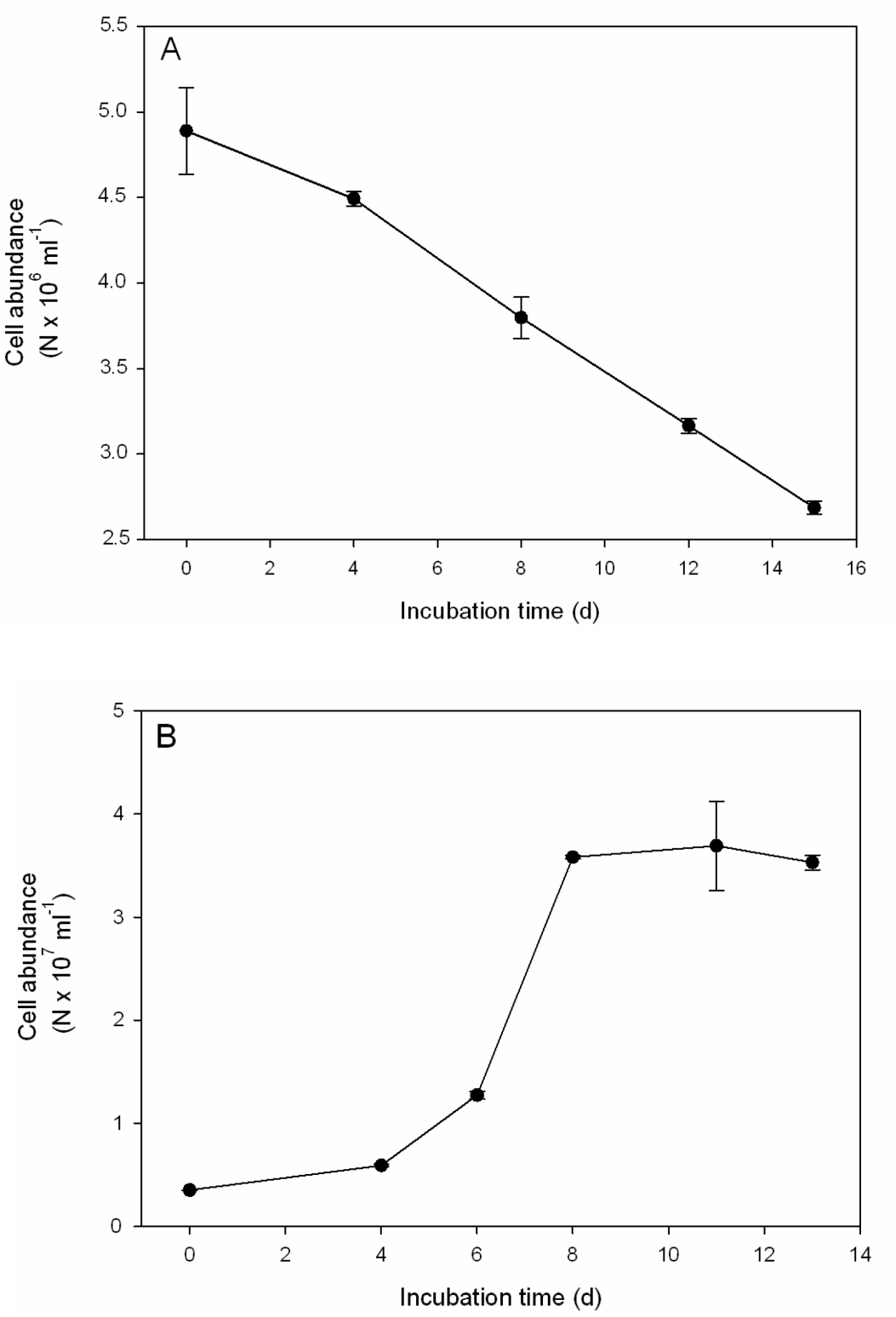


Figure 7. Growth of NF5 (A) and D3C (B) in media containing urea (1 mM) as sole energy source

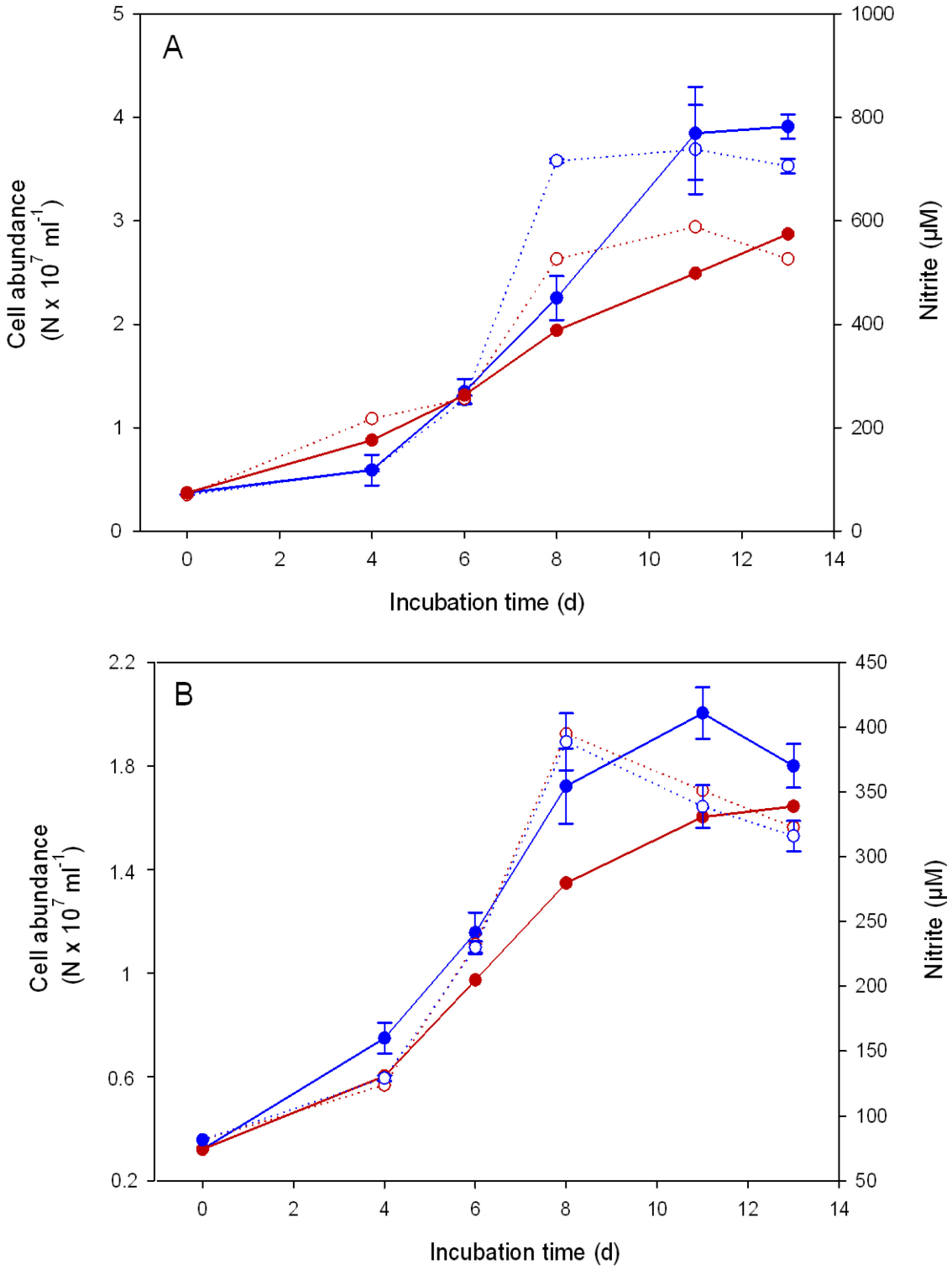


Figure 8. Urea utilization of D3C at pH 7.1 (A) and pH 7.5 (B) for 1 mM urea (blue) and 250 µM urea (red), solid line: nitrite production, dotted line: number of cells ml $^{-1}$

Ammonium uptake kinetics

Addition of ammonium in concentrations between 50 nM and 1.5 µM resulted in ammonium uptake rates of 90-150 nM h^{-1} (Fig. 9). Generally, uptake rates decreased for ammonium concentrations of 1.5 µM to 0.3 µM as expected for Michaelis-Menten-type kinetics, however, rates increased again for low ammonium concentrations (0.05-0.2 µM).

When the data were fitted to a Michaelis-Menten-like curve, the mean apparent half-saturation constants (K_m) for ammonium uptake were 33.6 nM and 54 nM total NH_3+NH_4 and maximum reaction velocities (V_{max}) reached 133 and 141 nmol h^{-1} for NF5 and D3C, respectively. However, actual half-saturation constants for both enrichments may even be lower than those reported, due to possible luxury uptake of ammonium after ammonium starvation and the methodological limitations in measuring very low ammonium concentrations.

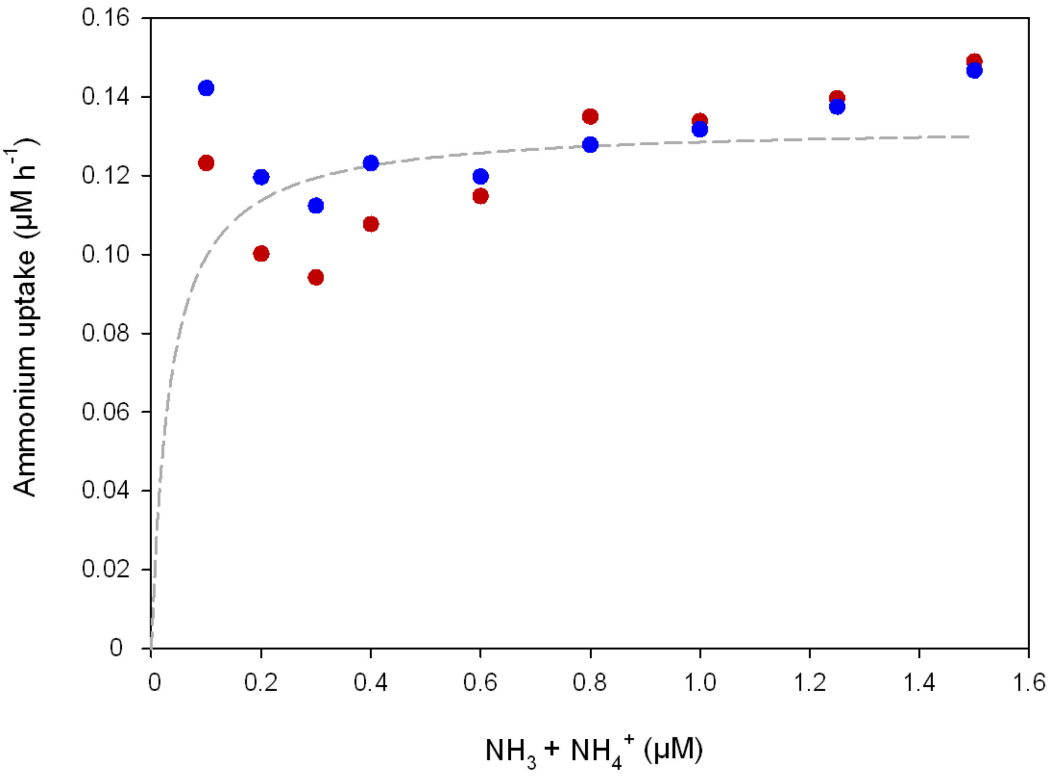


Figure 9. Ammonium uptake kinetics of NF5 (red dots) and D3C (blue dots)

Genomic characterization

Genome assembly

The draft genome assembly of the NF5 enrichment culture resulted in 23 contigs (11 contigs > 30,000 bp, 2 contigs < 10,000 bp > 3,000 bp, 10 contigs < 3,000 bp) containing 63,618 reads. For the D3C enrichment, 89,704 reads were assembled to 3 contigs (1 contig > 1,600,000 bp, 1 contig > 90,000 bp, 1 contig < 3,000 bp). A Blastn search of the contigs against a non-redundant bacterial database (NCBI) showed that all three contigs of D3C and the 13 larger contigs of NF5 were most closely related to *Nitrosopumilus maritimus* SCM1, *Nitrosopumilus koreensis* AR1 and *Nitrosopumilus sediminis* AR2. Ten short contigs (500-1,500 bp) of NF5 were affiliated with the Bacteroidetes orders Flavobacteriales and Sphingobacteriales, and the alphaproteobacterial orders Rhodobacter, Rhodospirillales and Rhizobiales, possibly representing contaminants. Thus, these contigs were excluded from the draft genome sequence and further analyses.

The draft genome sequence of the D3C enrichment culture was found to be ~1.71 Mb in length, containing 2,212 predicted CDS (Coding DNA Sequences), and had an average G+C content of 33.8%. The NF5 enrichment had an approximate genome size of 1.80 Mb, 2,300 predicted CDS and an average G+C content of 33.4% (Table 1). Across the entire length of the genome, D3C showed average coverage of 70% and average nucleotide identity of 88% of overlapping regions to the genomes *of N. maritimus* SCM1 and *N. korrensis* AR1, and a lower coverage and nucleotide identity of 59% and 82% to the genome of *N. sediminis* AR2. NF5 had an average coverage and nucleotide identity of 61% and 84%, respectively, to the aforementioned *Nitrosopumilus* genomes.

Table 1. Comparison of the genomic features of characterized Thaumarchaeota isolates

	Isolate D3C	**Isolate NF5**	*Nitrosopumilus maritimus* SCM1	*Nitrosopumilus sediminis* AR2	*Nitrosopumilus koreensis* AR1	*Nitrosoarchaeum limnia* SFB1	*Cenarchaeum symbiosum* A	*Nitrososphaera gargensis*
Affiliation	Nitrosopumilus cluster, group I.1a	Nitrosopumilus cluster, group I.1a	Nitrosopumilus cluster, group I.1a	Nitrosopumilus cluster, group I.1a	Nitrosopumilus cluster, group I.1a	Nitrosopumilus cluster, group I.1a	Nitrosopumilus cluster, group I.1a	Nitrososphaera cluster, group I.1b
Habitat	Marine surface water	Marine surface water	Tropical fishtank	Marine arctic sediment	Marine arctic sediment	Estuarine sediment	Sponge symbiont	Microbial mat (Garga spring)
Genome size (Mb)	1.71	1.8	1.65	1.69	1.64	1.77	2.05	2.83
Average G+C content (%)	33.8	33.4	34.2	33.6	34.2	32.4	57.7	48.3
Number of predicted genes	2,212	2,300	1,997	2,162	1,986	2,171	2,063	3,602
Protein coding density (%)	89.5	90.1	91.9	N/A	N/A	83	91.2	N/A

Genome annotation

Energy and carbon metabolism

Both genomes encode proteins involved in ammonia oxidation. One *amo* gene cluster, containing the putative ammonia monooxygenase genes *amo*ABC could be identified in both isolates. Interestingly, genes encoding a putative second *amo*B and one *amo*A fragment, distantly located from the *amo* cluster, showing only <60% amino acid identity to thaumarchaeal ammonia monooxygenases, were found in the D3C genome. Additionally, genes for several blue-copper domain-containing proteins and multicopper oxidases, and genes for putative copper-containing nitrite reductases (*nir*K) (one in D3C and two in NF5), as well as two putative ammonium transporters were identified in both genomes. In addition to the conventional ammonia oxidation pathway, the D3C genome contained an *ure* gene cluster, consisting of three urease encoding subunits (*ure*ABC), four accessory proteins (*ure*DEFG) and two urea transporters (DUR3), which appeared to be closely related to *Nitrosopumilus sediminis* AR2 (> 80% nucleotide identity). SignalP did not detect any signal peptides in the *ure* subunits of D3C, however, evidence for a non-classical secretion pathway was found on the *ure*AB subunits via SecretomeP, suggesting secretion of the urease enzyme out of the cell.

As for all Thaumarchaeota which have been characterized thus far, a gene set encoding all required enzymes of a modified 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) pathway for inorganic carbon fixation were present in both enrichment cultures. Additionally, both genomes contained a number of genes coding for enzymes of the tricarboxylic acid (TCA) cycle, excluding citrate-cleaving enzymes. NF5 possessed a gene encoding NAD(P)-dependent malic enzyme, which catalyzes the conversion of malate to pyruvate. A NAD(P)-dependent malic enzyme was found in the genomes of *Nitrosoarchaeum limnia*, *Nitrosopumilus salaria* and *Nitrososphaera gargensis,* but seems to be absent in other Thaumarchaeota genomes (Spang *et al.* 2012).

In addition to ammonium transporters and urea transporters (in case of D3C), both genomes contained transporters for di- and oligopeptides, amino acids, sulfonates and phosphonates, as well as the phosphate transport system pstSCAB.

Motility

In the genome of NF5, a high number of genes associated with motility were identified, which were absent in the D3C genome. NF5 harbored multiple flagellin genes, the putative flagellar accessory genes *fla*FGHIJ, one gene encoding the preflagellin peptidase *fla*K and two genes coding Flp pilus assembly proteins (*tad*C). Additionally, a full gene set of chemotaxis proteins *che*ABCRWY and two methyl-accepting chemotaxis proteins were identified, located adjacent to the *fla* gene cluster. Most of the motility-related genes were in close proximity to each other on the same contig, however, *tad*C genes were located in one cluster on another contig and genes for putative *che*Y-like response regulators as well as histidine kinase-like proteins were found throughout the entire genome.

Generally, motility-associated proteins of NF5 showed high amino acid similarity to that of *N. limnia*, whereas chemotaxis related proteins seemed to be less conserved among these two species (average amino acid identity = 58%).

CRISPR defense system and integrated elements

One putative CRISPR (clustered regularly interspaced palindromic repeat) viral defense system within the NF5 genome was identified via CRISPR Finder, consisting of 24 bp repeat regions and three spacers (33 bp in length). Additionally, two CRISPR-associated *cas*1 genes were identified (50% amino acid similarity to each other), however, as the *cas* genes were not directly associated with the putative CRISPR regions and the genome was lacking additional *cas* genes, the functioning as viral defense system appears to be unlikely.

In both genomes, integrase-family proteins (two in NF5 and four in D3C), located in regions with mainly hypothetical proteins were found, suggesting the presence of putative proviruses or integrated plasmids. The two integrase proteins of NF5 and one of D3C were located next to t-RNAs, which represent typical insertion sites.

Protection from osmotic shock and ultraviolet radiation

One putative mechanosensitive ion channel (MscS) was detected in NF5, possibly involved in osmotic homeostasis. However, genes for ectoine biosynthesis, as found in *N. maritimus*, could not be identified in either of the two genomes. Both genomes contained a gene encoding deoxyribodipyrimidine photolyase, also present in *Nitrosoarchaeum*

limnia (59% and 55% amino acid identity to NF5 and D3C, respectively) but absent in other characterized Thaumarchaeota genomes.

Table 2 shows a comparison of the metabolic and functional gene diversity of different characterized Thaumarchaeota species including the isolates D3C and NF5.

Table 2. Metabolic and functional gene diversity of characterized Thaumarchaeota

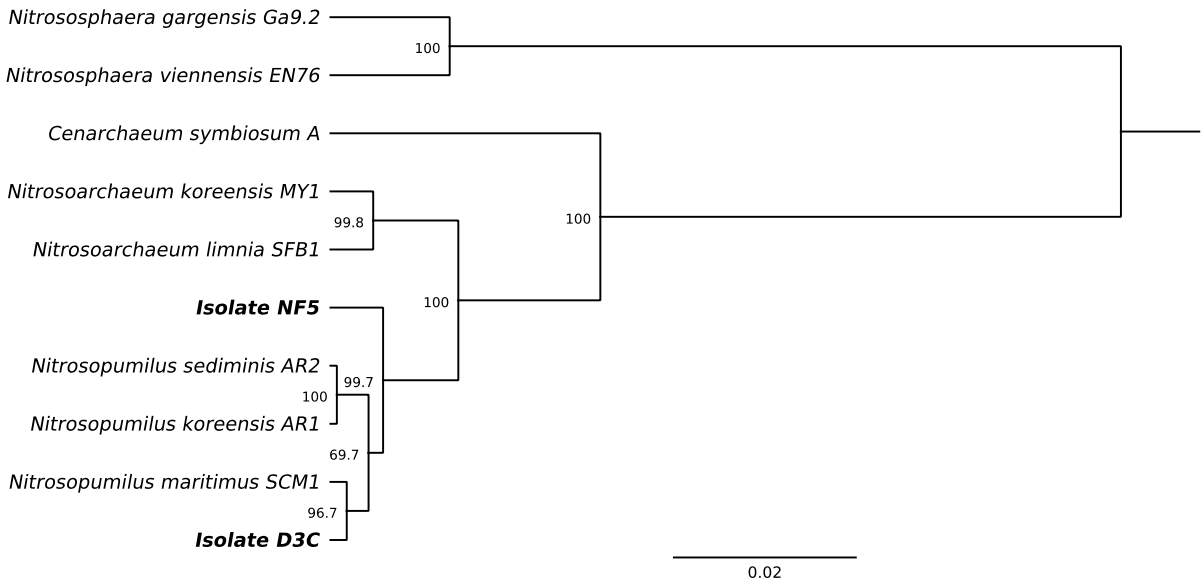
	Isolate D3C	**Isolate NF5**	*Nitrosopumilus maritimus* SCM1	*Nitrosopumilus sediminis* AR2	*Nitrosopumilus koreensis* AR1	*Nitrosoarchaeum limnia*	*Cenarchaeum symbiosum* A	*Nitrososphaera gargensis*
Ammonia oxidation								
Urea degradation and transport								
Inorganic carbon fixation								
Transporter for organic substrates								
Motility								
Photolyase								

Phylogenetic analyses

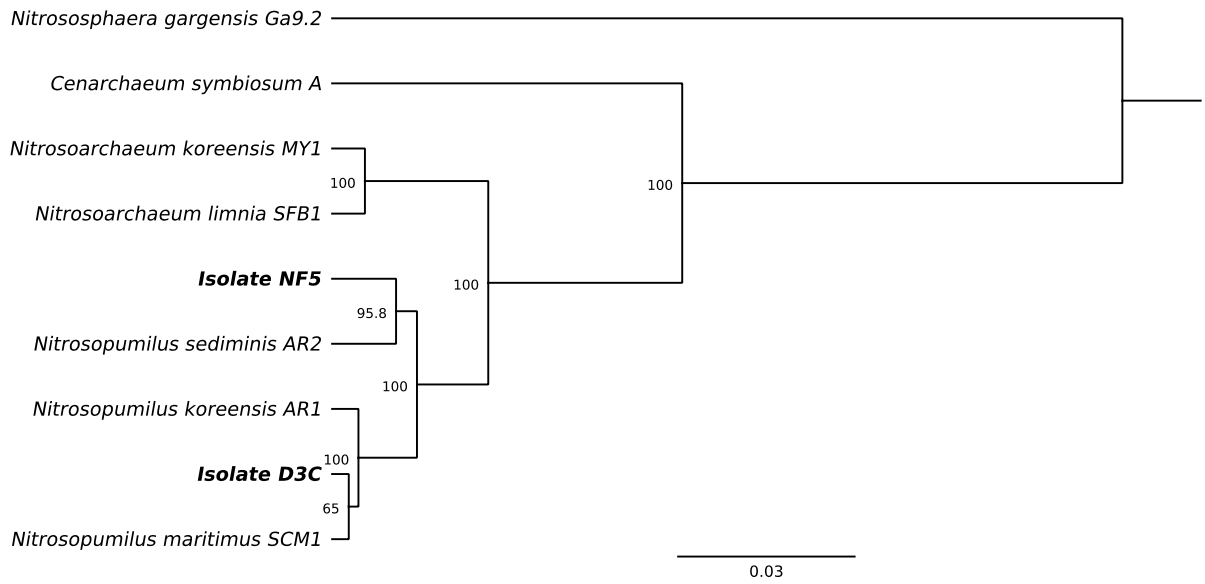
Phylogenetic analyses were carried out based on 16S and 23S rRNA, and ammonia monooxygenase subunit A (*amo*A) gene sequences. The 16S rRNA, 23S rRNA and *amo*A gene sequences of NF5 and D3C differed by 14, 70 and 34 point mutations, respectively, and both strains were affiliated to group 1.1a AOA. Phylogenetic distance trees showed that the *Nitrososphaera* group 1.1b formed a separate cluster and *Cenarchaeum symbiosum* branched off earlier within the group 1.1a. However, within the *Nitrosopumilus* genus, the relation of different species to each other appeared to be less clear. Comparing 16S rRNA gene sequences, the NF5 enrichment seemed to be more distantly related to other Nitrosopumilus species (Fig. 10A), however, distance trees based on 23S rRNA and *amo*A gene sequences grouped NF5 and *Nitrosopumilus sediminis* AR2 together.

The D3C enrichment culture seemed to be closely related to *Nitrosopumilus maritimus* SCM1 and *Nitrosopumilus koreensis* AR1, indicated by a low bootstrap support of this particular branch for both trees (65 and 67.9% for 23S rRNA and *amo*A tree, respectively) (Fig. 10B-C).

A



B



C

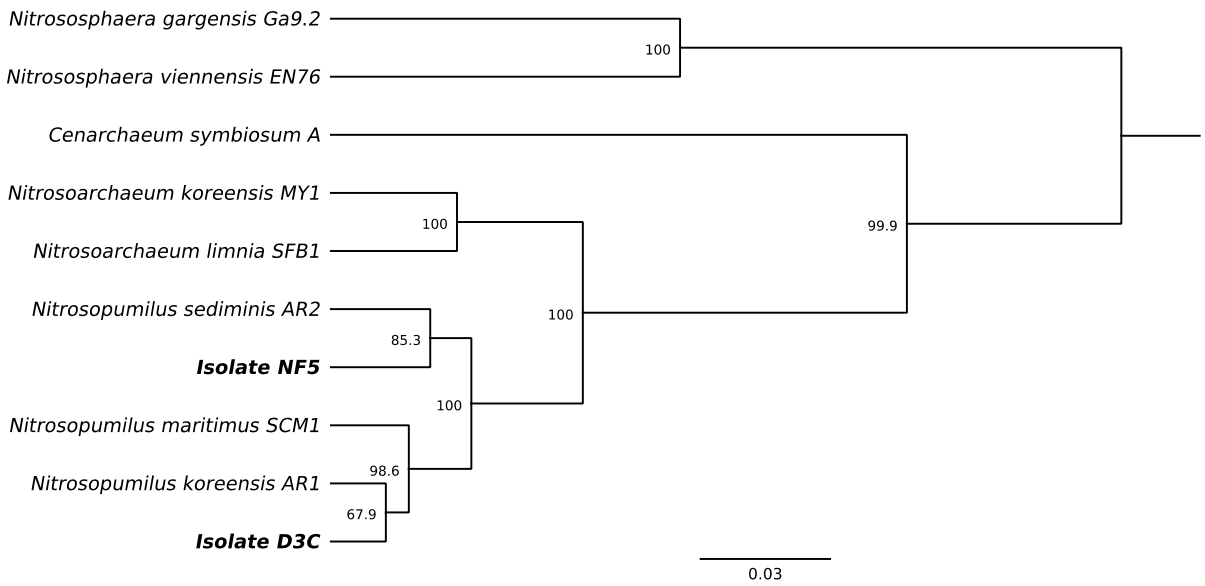


Figure 10. Phylogenetic distance trees based on 16S rRNA (A), 23S rRNA (B) and *amo*A (C) gene sequences of characterized Thaumarchaeota, using UPGMA clustering and Jukes-Cantor distance corrections. Bootstrap values (> 50% support) are shown at the respective nodes.

Discussion

Within group 1.1a AOA, 16S rRNA genes appear to be especially conserved among the different members, making it difficult to assess phylogenetic relationships between closely related species (Table S1A-B). Although metabolic genes are often targets of horizontal gene transfer (Koonin *et al.* 2001), the *amo*A gene is widely used as a phylogenetic marker for ammonia oxidation (Rotthauwe *et al.* 1997). Consequently its phylogeny may not accurately reflect the evolution of AOA, however, constructed distance trees based on 23S rRNA gene sequences of cultivated *Nitrosopumilus* representatives are highly similar to that based on *amo*A gene sequences and thus likely reflect actual phylogenetic relationships between these members.

Cultivation of marine Thaumarchaea often followed the culturing conditions established for *Nitrosopumilus maritimus* (e.g. in this study) (Koenneke *et al.* 2005). The relatively low pH (7.0-7.6), high ammonium concentrations (0.25-1 mM) and/or high temperatures (20-30°C) exert a specific selective pressure on the organisms of interest, potentially leading to cultivation of species closely related to *N. maritimus*. Thus, currently utilized culturing approaches may not lead to isolates resembling the actual diversity of AOA in the ocean.

Our results demonstrate, however, that besides their high 16S rRNA and *amo*A gene similarities, even closely related Thaumarchaota seem to be more diverse than previously assumed. The D3C and NF5 enrichments were isolated from the same location in two consecutive months, but exhibit quite different metabolic strategies and environmental adaptations, including the capability to use the alternative substrate urea (Table 2).

The presence of urease genes in marine Thaumarchaeota has only been reported for marine polar waters (Alonso-Saez *et al.* 2012), in the psychrophilic sponge symbiont *Cenarchaeum symbiosum* (Hallam *et al.* 2006) and in *Nitrosopumilus sediminis,* an isolate from Arctic sediments (Park *et al.* 2012), potentially indicating a dominance of urea metabolism in cold regions. However, the D3C enrichment culture, isolated from Adriatic surface waters, contains urease genes as well as urea transporters and growth on urea as sole energy source was experimentally confirmed for this isolate. There is evidence from genomic analysis that the urease enzyme of D3C is secreted and urea might thus be cleaved extracellularly. This is supported by the fact that ammonium was detected in low concentrations in the medium after urea addition, which was also

demonstrated for the *Nitrososphaera* group 1.1b (Michaela Stieglmeier pers. comm.). However, two urea transporters were found in the genome sequence of D3C, possibly also allowing direct uptake of urea. The secretion of urease, the efficiency of the enzyme and/or the uptake of urea appear to be pH dependent as indicated by low cell abundances and low nitrite production at pH 7.5 as compared to pH 7.1. The exact mechanisms of the thaumarchaeal urea metabolism remain unknown and although easily accessible ammonium seems to be taken up preferentially, urea could serve as a viable alternative energy source when ammonium is short in supply.

The finding of a second putative *amo*B and an amoA fragment in the D3C genome, which exhibit low amino acid identities (< 60%) to thaumarchaeal ammonia monooxygenases, could possibly indicate the potential of this isolate to oxidize different substrates. Copper-containing membrane-bound monooxygenases (CuMMO) have a wide substrate range, including methane and alkanes (Semrau *et al.* 2010; Sayavedra-Soto *et al.* 2011), which could serve as potential energy and/or carbon sources.

As already demonstrated for *N. maritimus*, cultures grow exponentially until ammonium is depleted below 10 nM (limit of detection). Remarkably, the NF5 and D3C enrichments seem to exhibit even lower half-saturation constants (33.6 nM and 54 nM total NH_3+NH_4 for NF5 and D3C, respectively) for ammonium uptake than *N. maritimus* (133 nM NH_3+NH_4, (Martens-Habbena *et al.* 2009)). This even higher substrate affinities are consistent with the apparent high abundances of Thaumarchaeota in marine environments. Although ammonium concentrations are beyond the detection limit of 10 nM in most oligotrophic deep water layers of the ocean, ammonia could still be the energy source driving thaumarchaeal chemoautotrophy since the supply rates are generally more important determinants for the importance of a given substrate as energy source than their concentrations (Fuhrman & Ferguson 1986). As shown in Fig. 9, ammonium uptake rates increased for very low ammonium concentrations (0.05-0.2 μM), resulting in an unusual pattern of two potential uptake rate maxima. In addition to the possible luxury uptake after ammonium starvation and the methodological limitations which have already been mentioned, another possibility would be the functioning of each of the two ammonium transporters at specific substrate concentration range (Nakagawa & Stahl 2013), which could result in two ammonium uptake rate maxima.

The apparent adaptation of NF5 and D3C to low nutrient environments is also reflected by the large number of genes for metabolism and transport of nutrients, as previously reported for thaumarchaeal genomes (Walker *et al.* 2010; Blainey *et al.* 2011;

Spang *et al.* 2012). In addition to the genomic repertoire supporting autotrophy, the D3C and NF5 genomes contain transporters for organic substances including di- and oligopeptides, amino acids, sulfonates and phosphonates, indicating a potential for mixotrophy. Both genomes contain a number of genes coding for enzymes of the tricarboxylic acid (TCA) cycle, however, the absence of citrate-cleaving enzymes excludes the reverse functioning of the pathway. Rather, it suggests that these enzymes are utilized for biosynthetic purposes or a complete oxidative TCA cycle, as already proposed for *N. maritimus* (Walker *et al.* 2010). NF5 seems to be more flexible in the conversion of pyruvate than most characterized Thaumarchaeota, possessing a gene encoding NAD(P)-dependent malic enzyme, which could possibly be involved in gluconeogenesis and anaplerosis (Spang *et al.* 2012).

The presence of motility-associated genes of marine Thaumarchaeota has only been reported for *Nitrosoarchaeum limnia* and was assumed to be crucial for survival and fitness in estuarine environments (Mosier *et al.* 2012). The presence of genes for chemotaxis and flagella assembly in the genome of NF5 indicates that this isolate might actively seek appropriate microenvironments in marine surface waters, possibly being able to attach to particles.

The genomes of D3C and NF5 both contain a gene encoding photolyase, an enzyme involved in the repair of DNA damage induced by ultraviolet light (Heelis *et al.* 1993; Ozer *et al.* 1995), indicating adaption to high ultraviolet (UV) radiation. For *N. maritimus*, which was isolated from an aquarium, as well as for *N. koreensis* and *N. sediminis* from arctic sediments and the sponge symbiont *C. symbiosum*, protection from UV-induced DNA damage may not represent an essential trait consequently, as they lack the enzyme. The presence of a photolyase encoding gene in these two isolates corroborates with their presence in the surface waters of the Northern Adriatic Sea where biologically effective UV radiation reaches about 10 m depth (Herndl *et al.* 1993).

Taken together, our results provide insight into the functional diversity and metabolic versatility of marine Thaumarchaeota. The finding of phylogenetically close but functionally distinctive members of the Nitrosopumilus genus in the Northern Adriatic Sea points towards a possible niche differentiation among the different closely related species and indicates genetic and physiological differences between closely related AOA populations in the marine environment.

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Supplementary Information

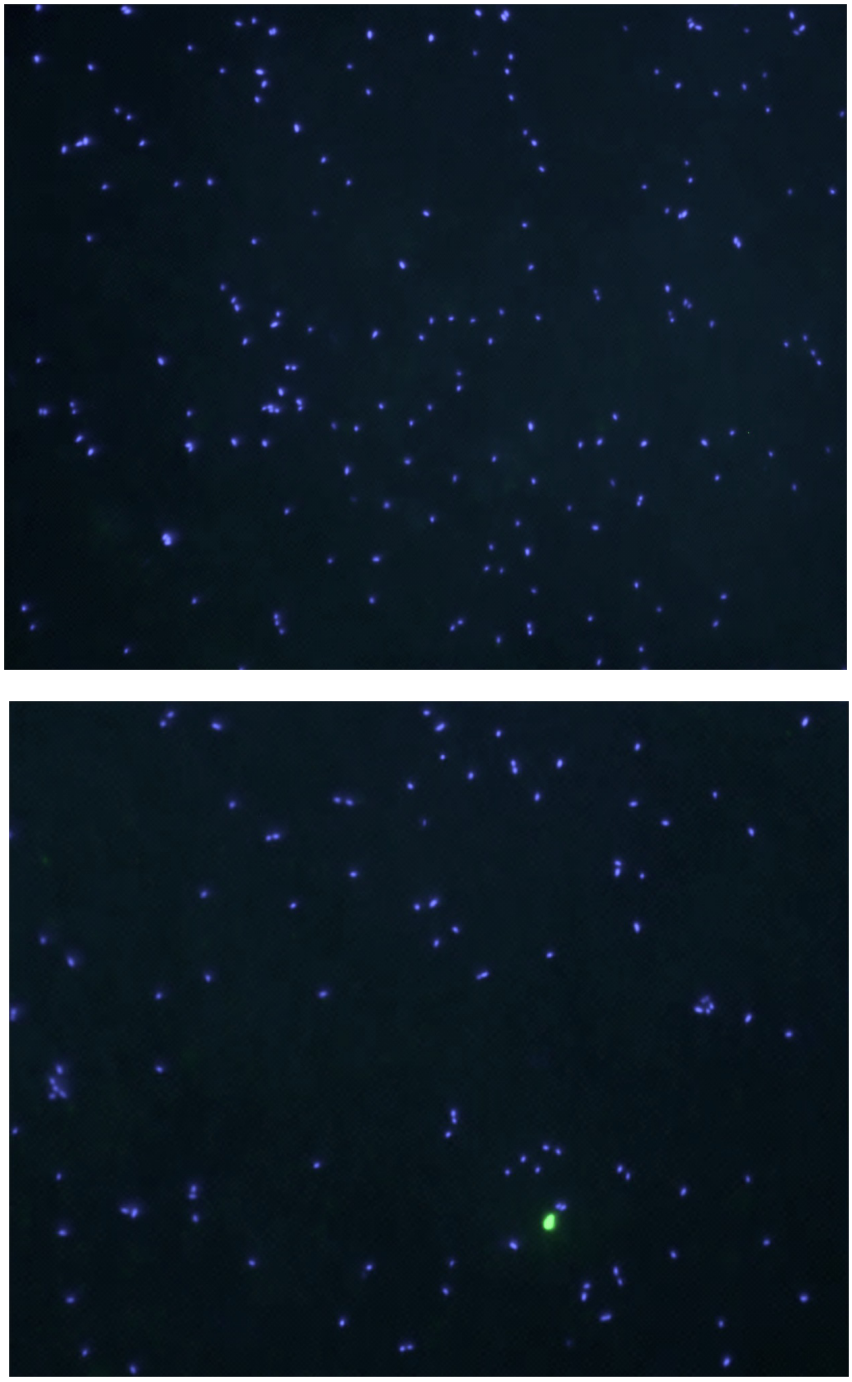

Figure S1. CARD-FISH picture of the D3C (top) and the NF5 (below) enrichment culture using EUBI-III probes.

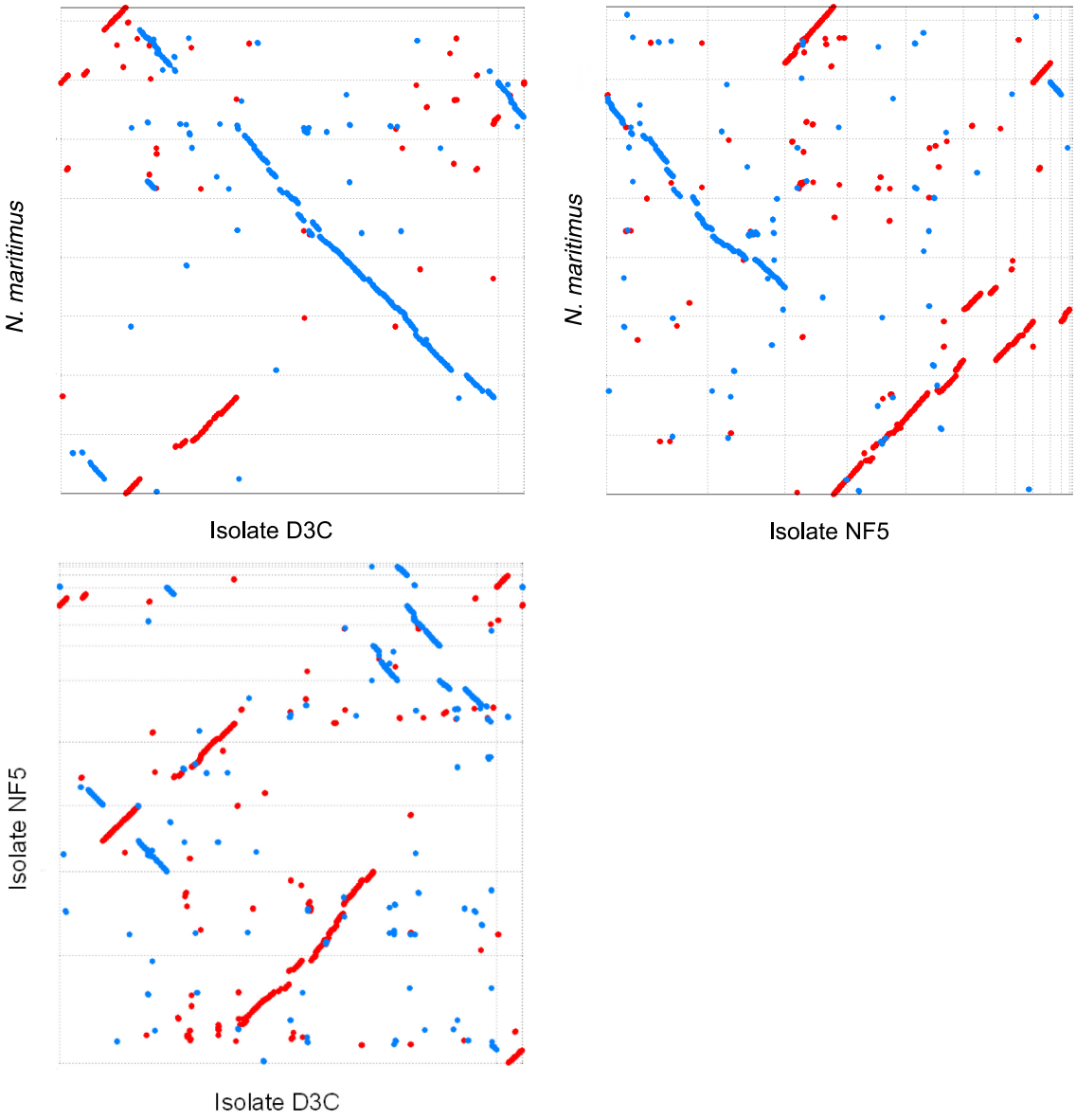

Figure S2. Dot plot representation of the pairwise alignments of theNF5, D3C and *N. maritimus* genomes using the Promer program (MUMmer 3.23 package). In all plots, a dot indicates a match (of at least six AA) between the two genome sequences being compared, with forward matches coloured in red and reverse matches coloured in blue.

Table S1. Nucleotide identities (%) of phylogenetic marker genes of characterized Thaumarchaeota. (* 88-95% coverage, ** 49-50% coverage)

A

NF5	D3C	*N. maritimus*	*N. koreensis*	*N. sediminis*	*C. symbiosum*	*N. gargensis*
16S rRNA	99	99	99	99	94	84
23S rRNA	98	97	97	98	89	78
amoA	95	94	94	96	80*	72
amoB	94	94	94	92	73*	67**
amoC	94	93	98**	92	80	77

B

D3C	NF5	*N. maritimus*	*N. koreensis*	*N. sediminis*	*C. symbiosum*	*N. gargensis*
16S rRNA	99	99	99	99	94	85
23S rRNA	98	99	99	97	89	80
amoA	95	97	97	95	79	73
amoB	94	96	97	91	74*	69**
amoC	94	97	98**	91	82	78

Table S2. Metabolic and functional genes present in D3C and NF5

		D3C	NF5
Energy metabolism	Ammonia monooxygenase subunit A		
	Ammonia monooxygenase subunit B		
	Ammonia monooxygenase subunit C		
	Urea amidohydrolase (urease), subunit alpha		
	Urea amidohydrolase (urease), subunit beta		
	Urea amidohydrolase (urease), subunit gamma		
	Blue copper domain-containing proteins		
	Multicopper oxidases		
	Putative copper-containig nitrite reductase		
3-Hydroxypropionate/4-Hydroxybutyrate Cycle	Acetyl-CoA/ propionyl-CoA carboxylase		
	Aspartate-semialdehyde dehydrogenase		
	Hydroxyacyl-CoA dehydrogenase		
	Acetate-coA ligase		
	3-hydroxybutyryl-CoA dehydrogenase		
	Methylmalonyl-CoA epimerase/mutase		
	Succinate-semialdehyde dehydrogenase		
	Acetyl-CoA acetyltransferase		
	NAD-dependent alcohol dehydrogenase		
Tricarboxylic acid (TCA) cycle	2-oxoglutarate ferredoxin oxidoreductase		
	Aconitate hydratase		
	Isocitrate/isopropylmalate dehydrogenase		
	Succinyl-CoA synthetase		
	Succinate dehydrogenase/fumarate reductase		
	Malate dehydrogenase		
	Succinyl-CoA synthetase		
	Citrate synthase		
	Phosphoenolpyruvate carboxykinase		
Transporters	Ammonium transporters		
	Urea transporters		
	Phosphate transport system (pstSCAB)		
	Phosphonate transporter		
	Sulfonate transporter		
	Glycerol transporter		
	Amino acid transporter		
	Dipeptides/oligopeptides transporter		
Motility	Flp pilus assembly protein		
	Flagellar assembly protein		
	ATPase involved in flagellar biogenesis		
	Preflagellin peptidase		
	Archaeal flagellins		
	Type IV secretory pathway protein (flagella biosynthesis)		
	Chemotaxis related histidine kinase		
	MCP methylation inhibitor protein		
	Chemotaxis response regulator protein		
	Chemotaxis signal transduction protein		
	CheY-like receiver		
	Methyl-accepting chemotaxis protein		
	Methylase of chemotaxis methyl-accepting proteins		

Zusammenfassung

Die Entdeckung der in aquatischen und terrestrischen Lebensräumen ubiquitär vorkommenden Ammoniak oxidierenden Thaumarchaeota hat zu einem vermehrten Interesse geführt, deren Physiologie und ökologische Funktion besser zu verstehen.

Die aerobe Oxidation von Ammoniak zu Nitrit ist der erste, geschwindigkeitsbestimmende Schritt der Nitrifikation. Es wurde lange Zeit angenommen, dass dieser essentielle Prozess, welcher reduzierte anorganische Stickstoffverbindungen in ihre oxidierte Form umwandelt, einzig und allein von Ammoniak oxidierenden Bakterien (AOB) durchgeführt wird. Die Abundanz von Archaeen assoziierten Markergenen für Ammoniakoxidation, Ammoniakmonooxygenasen (*amo*), in der gesamten Wassersäule, sowie deren numerische Dominanz über die der Bakterien, führten jedoch zu der Ansicht, dass Ammoniak oxidierende Archaeen (AOA) wesentlich am Nitrifikationsprozess beteiligt sind. Daraus folgt, dass AOA zu den am häufigsten vertretenen Organismen in marinen Ökosystemen zu zählen scheinen und eventuell eine wichtige Rolle in globalen Stickstoff-und Kohlenstoffkreisläufen spielen.

Im Rahmen dieser Arbeit wurden die physiologische und genomische Charakterisierung von zwei AOA Spezies der Gattung *Nitrosopumilus* beschrieben, welche aus den Oberflächengewässern des nördlichen Adriatischen Meeres isoliert wurden. Die Isolate D3C und NF5 sind in der Lage, in vollständig anorganischem Medium zu wachsen, und experimentell ermittelte Halbsättigungskonstanten der Ammoniakaufnahme beider Isolate (K_m = 54 nM und 34 nM $NH_3 + NH_4^+$ für D3C und NF5) indizieren die Anpassung an noch nährstoffärmere Bedingungen, als sie für *Nitrosopumilus maritimus* (Martens-Habbena *et al.* 2009) beschrieben wurden. Die Nutzung von alternativen Substraten neben Ammoniak wurde für beide Isolate experimentell ermittelt und mit Genomdaten ergänzt.

Die Anreicherungskultur D3C besitzt neben den Genen für Ammoniakoxidation auch Gene für den Abbau und Transport von Harnstoff. Die Nutzung von Harnstoff als alternatives Primärsubstrat für Ammoniakoxidation wurde durch Kultivierungsversuche bestätigt. Des Weiteren wurden im Genom der Anreicherungskultur NF5 eine große Anzahl an Genen, welche mit Motilität und Chemotaxis assoziiert sind, identifiziert, und die im Genom der Anreicherungskultur D3C fehlen. Beide Genome enthalten ein Gen welches Photolyase kodiert, ein Enzym das in der Reparatur von UV induzierten DNA

Schäden involviert ist und eine mögliche Anpassung an das Leben im Oberflächenwasser darstellen könnte.

Die Entdeckung von phylogenetisch nah verwandten, jedoch funktionell deutlich unterschiedlichen Vertretern der Gattung *Nitrosopumilus* in der Nordadria, gibt einen Einblick in die Funktionsvielfalt der marinen Thaumarchaeota und weist auf eine mögliche Nischendifferenzierung zwischen den verschiedenen eng verwandten Arten hin.

Curriculum Vitae

Personal Information

Name: Barbara Bayer

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Professional Background and Education

2012 – 2014	MSc in Ecology with main focus on Marine Biology
	University of Vienna, Austria
2008 – 2012	BSc in Biology with main focus on Microbiology and Genetics
	University of Vienna, Austria
2007	EF High School Semester
	Gardner Edgerton High School, Gardner, Kansas (US)
2000 – 2008	High School, Bernoulligymnasium, Vienna, Austria

Research Activities

2013 – 2014	Master Thesis at the Department of Marine Biology
	University of Vienna, Austria
	Title: *Characterization of Thaumarchaeota isolated from the Northern Adriatic Sea* (Supervisor: Prof. Dr. Gerhard J. Herndl)
2013	Special Course in Marine Ecology
	Marine Biology Station Piran, Slovenia
	Project: *The symbiosis of Zoothamnium niveum: host settlement and symbiont acquisition* (Supervisor: Dr. Andrea Nussbaumer)
2012	Bachelor Thesis at the Center of Marine Sciences (CCMar)
	University of Algarve, Portugal
	Title: *Molecular Analyses of Bacterial Symbionts in the Sponge Sarcotragus spinosulus* (Supervisor: Rodrigo Costa, PhD)

Work Experience

2011	Diving Center „Delphinus Divers“, Armação de Pêra, Portugal
2009	Bar-Restaurant „Las Tres Marias“, Vienna, Austria
2007	Veterinary Medicine Ambulance Dr. Gerhard König, Vienna, Austria

Research Interests

Marine Microbial Ecology, Energy and carbon sources in the meso- and bathypelagic water layers, Cultivation of microorganisms, Microbial metabolic pathways, Archaea Biology and Phylogeny, Genome Annotation

Publications and Conferences

17th Ocean Sciences Meeting, Honolulu, Hawaii

Poster: Characterization of Thaumarchaeota isolated from the Northern Adriatic Sea. ***Bayer B.**, J. Vojvoda, P. Offre, D. De Corte, C. Schleper, and G. J. Herndl*

*Mooshammer, M., M. Stieglmeier, **B. Bayer**, L. Jochum, M. Melcher M, W. Wanek*. Nitrogen isotope fractionation during archaeal ammonia oxidation: Coupled estimates from $\delta^{15}NH_4^+$ and $\delta^{15}NO_2^-$ measurements (submitted)