Chapter 3

Ammonia oxidation by a marine Crenarchaeote

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Abstract.

Nitrogen cycling plays a crucial role in sustaining oceanic primary production [*Tyrrell* 1999]. Decomposition of particulate nitrogen releases ammonia that is subsequently re-oxidized by prokaryotes. Nitrification is performed in two steps, i.e. the oxidation to nitrite and then to nitrate [*Ward* 2002; *Kowalchuk and Stephen* 2001]. Two different groups of bacteria belonging to the beta- and gamma-Proteobacteria are involved in the first nitrification step [*Zehr and Ward* 2002]. Here we show, however, that an enrichment culture of a member of the pelagic Crenarchaeota, one of the most abundant groups of prokaryotes in the ocean [*Karner et al.,* 2001; *Herndl et al.,* 2005], is able to oxidize ammonia to nitrite. Evidence for this was provided by the detection of an archaeal ammonia monooxygenase gene in the enrichment culture the concentration of which was positively correlated with Crenarchaeotal cell abundance and nitrite concentrations. Field studies in the coastal North Sea indicated that Crenarchaeota may play an important role in marine nitrification. The participation of Archaea in nitrification indicates, together with the recently established importance of planctomycetes in denitrification [*Kuypers et al.,* 2003] and unicellular cyanobacteria in dinitrogen fixation [*Zehr et al.,* 2001; *Montoya et al.,* 2004], the important role of hitherto unrecognized prokaryotes in the oceanic biogeochemical cycling of nitrogen.

3.1 Introduction

Planktonic Archaea in the ocean consist of two major groups, the Crenarchaeota and the Euryarchaeota of which the former appears to be the most abundant [*Fuhrman et al.,* 1992]. Marine Crenarchaeota typically occur in higher relative abundance in deep neritic waters and in the meso-and bathypelagic zones of the ocean [*Fuhrman et al.,* 1992; *Massana et al.,* 2000; *Karner et al.,* 2001; *Herndl et al.,* 2005] and are thought to account for ca. 20% of all prokaryotic cells in the global ocean [*Karner et al.,* 2001]. The metabolism of these planktonic Crenarchaeota is still enigmatic, primarily because none of its members is currently available in culture. In-situ labelling [*Wuchter et al.,* 2003] and microautoradiography experiments [*Herndl et al.,* 2005] showed that marine Crenarchaeota can utilize dissolved inorganic carbon as carbon source and are also able to take up amino acids [*Herndl et al.,* 2005; Ouverney and Fuhrman 2000]. Essentially nothing is known about the energy source of marine Crenarchaeota. Positive correlations between marine crenarchaeotal abundance and nitrite were observed in the Arabian Sea [*Sinninghe Damsté et al.,* 2002] and in the Santa Barbara Channel time series [*Murray et al.,* 1999], and with particulate organic nitrogen in Arctic waters [*Wells and Deming* 2003]. These findings suggest that marine Crenarchaeota may be involved in the marine nitrogen cycle, possibly as nitrifiers.

In this study we provide experimental evidence that marine Crenarchaeota are capable to oxidise ammonia and are actively involved in the marine nitrogen cycle.

3.2 Material and Methods

3.2.1 Incubation experiment setup: Coastal North Sea water was kept in the dark for 6 months at 25 ºC in an 850l mesocosm tank without addition of nutrients [*Wuchter et al.,* 2004]. After these 6 months 20l batches of the aged mesocosm water were taken and incubated in 20l Nalgene Clearboy tanks at 22 and 25 °C in the dark. Nutrients were added before the incubation and initial nutrient concentrations were: 150 µM $NaNO_3$, 150 µM NH_4Cl, 25 µM NaH_2PO_4 and 2666 µM $NaHCO_3$. A sterile mix of 12.5 mg l^{-1} yeast, 5 mg l^{-1} peptone extract, vitamins and trace elements were added as well. Temperature, pH and salinity were monitored and kept constant. The pH was regularly adjusted to 8.2 by adding

sterile 0.1M NaOH or HCl and salinity was maintained at 27 PSU by addition of de-mineralized water. The 20l tank was continuously stirred and open throughout the experiment allowing constant gas exchange with the air. Nutrients, CARD-FISH samples and DNA samples were taken every 3-4 days for 25 days.

3.2.2 Coastal North Sea time series: The sampling site is situated at the western entrance of the North Sea into the Wadden Sea at the Island Texel (53°00'25"N, 4°78'27"E). Water samples for DNA and fluorescence in situ hybridization (FISH), samples were taken on a bi-weekly schedule from August 2002 to July 2003.

3.2.3 CARD-FISH analyses: 15 ml water samples were fixed with formaldehyde (final concentration 4%) and stored at 4°C for at least 4 h. Thereafter, the samples were filtered onto 0.2µm polycarbonate filters (Millipore, 25 mm filter diameter) with 0.45µm cellulose nitrate filters (Millipore) as supporting filters and stored frozen at -20ºC until further analysis. Total picoplankton were enumerated after DAPI staining [*Porter and Feig* 1980], while Bacteria and Archaea were enumerated by catalysed reporter deposition fluorescence in situ hybridisation (CARD-FISH) [*Teira et al.,* 2004] under the epifluorescence microscope. The oligonucleotide probes Eub338, BET42 and GAM42 were used for enumeration of Bacteria [*Amann* 1995], beta- and gamma-Proteobacteria [*Sekar et al*., 2004], respectively, and specific probes were applied for the marine Crenarchaeota Group I, Cren537 (5'-TGACCACTTGAGGTGCTG-3') [*Teira et al.,* 2004]. All probes were tested for their specificity prior to the study. The archaeal and general bacterial probes target the same cells as the polynucleotide probes used in previous studies [*Karner et al.,* 2001]. Cell walls were permeabilised with lysozyme (Sigma; 10 mg ml^{-1} in 0.05 M EDTA, 0.1 Tris-HCl [pH 8]) for Eub338 [*Teira et al.,* 2004] or with proteinase-K for Cren537 ([1844 U mg^{-1}, 10.9 mg mL^{-1}, Sigma]; 0.2 µl ml^{-1} in 0.05 EDTA, 0.1 Tris-HCl [pH 8]) at 37ºC for 1 h. Probe working solution (50 ng μl^{-1}) was added at a final concentration of 2.5 ng μl^{-1}. Hybridisation was done at 35ºC for 8-12 h. Negative control counts (hybridization with HRP-Non338) averaged 1.5 %. The average counting error in cell abundances for DAPI staining was 29%, for Crenarchaeota 29%, for bacteria 40% and for beta-and gamma-Proteobacteria 92%. For the North Sea time series the average counting error in cell abundance for DAPI staining was 18%, for Crenarchaeota 42% and for beta- and gamma-Proteobacteria 28%.The larger errors

are usually associated with low cell numbers where slides contained substantially less than 200 cells (e.g. beta- and gamma-Proteobacteria for the incubation experiments).

3.2.4 DNA analysis: 1 l of coastal North Sea water or water from the incubation experiments was filtered through a 0.2 µm pore size polycarbonate filter and stored at -80ºC until further analyses. For details about DNA extraction and analyses see *Wuchter et al.,* [2004]. The almost complete archaeal 16S rDNA sequence of the archaeal enrichment culture (*E. coli* positions between 14 and 1406) was recovered by using primer combination of Arch20f and Univ1406r.

3.2.5 Functional gene analysis: Partial, 258-bp-long *amo*A genes in the archaeal enrichment cultures were amplified by PCR using our newly developed primers *amo*A-for (5'-CTGAYTGGGCYTGGACATC-3') and *amo*A-rev (5'-TACTGGGCAACAAAGAAGAA -3'). The obtained sequence was compared to reference sequences from the GenBank database using the ARB software package –software [*Ludwig et al.,* 2004]. The *amo*A-like gene was quantified by quantitative-PCR analyses: 1ml water samples were shock frozen in liquid nitrogen every 3-4 days for 25 days during the course of the experiment and stored at -80ºC until further analyses.The water samples were defrosted and centrifuged at 10000 x g for 20 minutes and the cells were re-suspended in 50µl 10mM Tris-HCl. Cells were lysed by heating the cell suspension in a PCR Cycler (30 min at 95ºC). An aliquote of the lysed cells was used to quantify the number of *amo*A copies in each sample by Q-PCR using an iCycler system (BioRad). For the calibration of the samples, known *amo*A gene copy numbers of an *amo*A PCR product were subjected to the Q-PCR along with the samples. It should be noted that the archaeal cell counts are in general higher than the *amo*A-like gene copy numbers likely due to the low DNA extraction efficiency for Q-PCR analyses as only a heat treatment was applied for cell lyses and PCR efficiency was 80%.

3.3 Results and Discussion

The experimental setup was originally designed to examine the temperature adaptation of marine crenarchaeotal membrane lipids [see *Wuchter et al.,* 2004 for details]. During this experiment coastal North Sea water was kept in the dark for 6 months at 25 ºC in an 850l mesocosm tank without addition of nutrients. A substantial increase in the archaeal membrane lipids was observed which coincided with a drop of ammonium levels to almost zero

[*Wuchter et al.,* 2004]. This initial experiment tentatively suggested a link between Crenarchaeota and the oxidation of ammonia. To further investigate this, water from this mesocosm tank was incubated in the dark at 22ºC and 25ºC and nutrients, including ammonia, were added. The abundance of marine Crenarchaeota was determined using CARD-FISH [*Teira et al.,* 2004] and revealed at both temperatures a substantial enrichment of crenarchaeotal cells (Fig. 1b; Table S1). After a lag phase, crenarchaeotal abundance increased with a doubling time of 2 days to 4-5x10^6 cells ml^{-1} in the incubation series and comprised between 40-70% of DAPI-stainable cells. Bacterial abundance increased during the first three days of incubation and then remained <5% of DAPI-stainable cells during the whole incubation time (Fig. 1b). Generally 20-30% of the DAPI-stained cells are composed of dead cell material [*Heissenberger et al.,* 1996] indicating that our enrichment culture was dominated by marine Crenarchaeota (Fig. 1b). In fact, molecular analyses using two independent PCR approaches selective for Archaea revealed that the incubated waters was dominated by a single archaeal species, closely related (>96% sequence similarity) to the marine Crenarchaeotal phylogenetic cluster 1α [*Massana et al*., 2000] (Fig S1).When ammonium levels dropped, nitrite concentrations increased concomitantly with the increase in crenarchaeotal abundance (Fig. 1a), similar to what was observed in the large mesocosm tank [*Wuchter et al.,* 2004]. Finally, crenarchaeotal cell abundances decreased at the end of the experiment possibly due to lyses by viruses, grazing of flagellates or the toxic influence of the high nitrite concentrations.

The kinetics and stoichiometry of chemolithoautotrophic aerobic ammonium oxidation are well known and fixed by thermodynamic and biochemical constraints [*Tijhuis et al.,* 1993]. In our enrichment culture, a nitrifying population of ca. 5x10^6 cells ml^{-1} is required to explain the observed rate of ammonium oxidation assuming maximum rates for nitrifying bacteria of 10 fmol NH_3 $cell^{-1}$ day^{-1} [*Zart and Bock,* 1998]. Surprisingly, the enriched Crenarchaeote constituted the only prokaryotic species which approached these numbers (4x10^6 cells ml^{-1}; Fig. 1b). If the Crenarchaeote was responsible for ammonium oxidation then ammonium conversion rates were 2 and 4 fmol NH_3 $cell^{-1}$ day^{-1} at 22 and 25°C, respectively. These figures are well in the ranges reported previously for bacterial aerobic ammonium oxidation [*Ward* 2002]. Oxidation of ammonia to nitrite and nitrate in aquatic systems is generally thought to be performed by different groups of Bacteria. First, ammonium is

oxidized to nitrite by members of the *Nitrosonomas/Nitrosospira* (beta-Proteobacteria) and *Nitrosococcus* (gamma-Proteobacteria) groups and then nitrite is converted to nitrate by phylogenetically diverse nitrite oxidizers such as *Nitrobacter* sp. (alfa-Proteobacteria) [*Ward* 2002; *Zehr and Ward* 2002]. The beta- and gamma-proteobacterial groups, which include but likely are not exclusively comprised of nitrifiers, remained below $6x10^4$ cells ml^{-1} during the entire period of ammonium oxidation and crenarchaeotal cell growth, almost two orders of magnitude lower in abundance than the Crenarchaeote (Table S1). Considering the thermodynamic and biochemical constraints bacterial nitrifiers can not be responsible for the high nitrification rates observed in our crenarchaeotal enrichment culture.

More direct evidence for ammonia oxidation by the enriched marine Crenarchaeote was provided by molecular analyses of ammonia monooxygenase (*amo*A) genes in the incubation experiments. Application of specific amplifiers resulted in the detection of one *amo*A-gene (Figure S2). Its nucleotide and amino acid sequence is closely related (83-91% sequence homology) to the *amo*A-like genes obtained from the Sargasso Sea [*Schleper et al.,* 2005] (Fig. 2) and only distantly related to known bacterial *amo*A-genes. The *amo*A-like genes from the Sargasso Sea were obtained by an environmental genome shotgun cloning approach which resulted in the identification of an *amo*A-gene on a genome fragment that, based on the co-occurrence of the 16S rDNA on that genome fragment, is of presumed archaeal origin [*Venter et al.,* 2004]. Thus, the identification of this single archaeal *amo*A-like gene in our incubation experiments strongly suggests that the enriched marine Crenarchaeote contained and expressed the necessary functional gene for ammonia oxidation. Further evidence was obtained by quantification of the archaeal *amo*A-like gene during the incubation experiment using quantitative PCR. This revealed a strong correlation ($r = 0.99$) between the crenarchaeotal cell abundances and the archaeal *amo*A-like gene copy number abundance during the course of the experiment (Fig. 1c) confirming the link between the Crenarchaeote and the functional gene. In addition, the archaeal *amo*A-like gene copy number abundance correlate well with the decline in ammonia and increase in nitrite showing that the marine Crenarchaeote enriched in our incubation experiments was responsible for the oxidation of ammonia to nitrite. Thus, we have for the first time enriched a mesophilic crenarchaeotal species, phylogenetically closely related (>96% sequence homology) to the species

comprising 20% of the marine prokaryotes, and demonstrated that it is involved in nitrification.

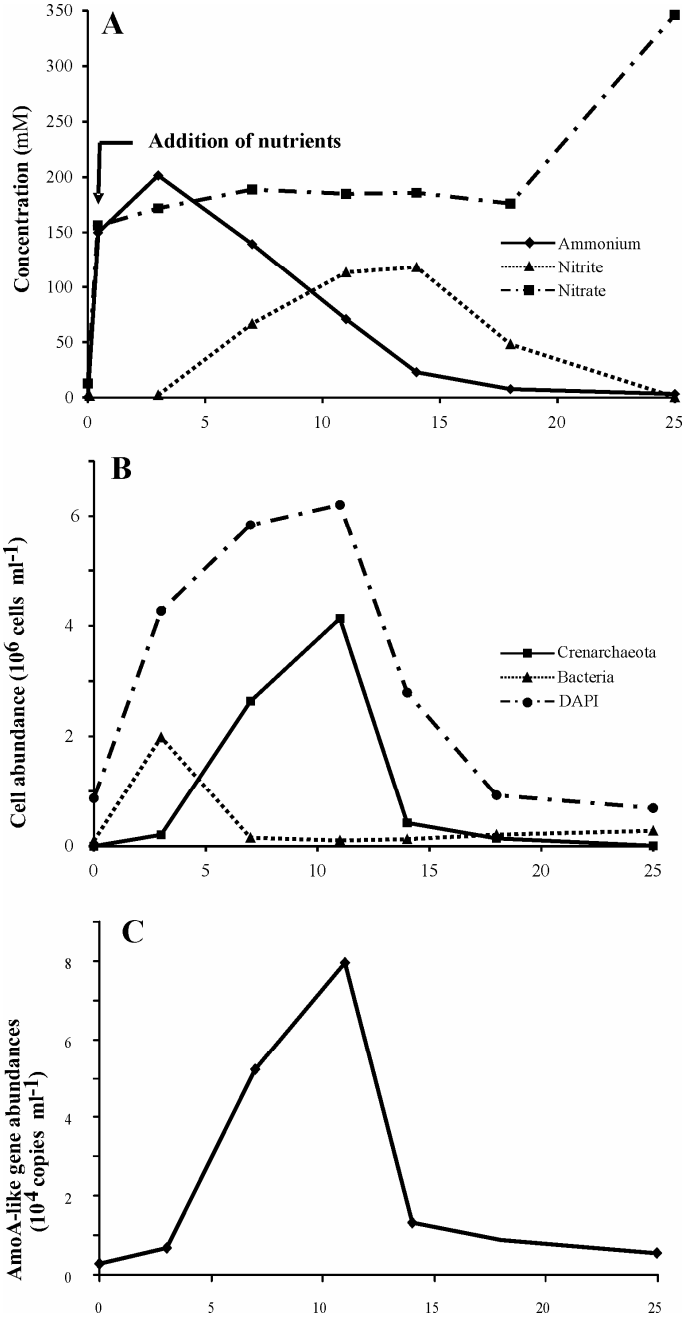

Figure 1. Archaeal nitrification in an enrichment culture. (**A**) Nutrient concentrations (μM) in the course of the experiment. (**B**) DAPI counts and archaeal and bacterial cell numbers (cells ml^{-1}) as determined by CARD-FISH (**C**) *amo*A-like gene copy numbers as determined by Q-PCR. Batches of pre-aged water from the large mesocosm experiment (see text and Methods) with added nutrients were incubated at two different temperatures in the dark. The data shown are those obtained at 25ºC. The Crenarchaeote in our enrichment culture was shown to be comprised of a single species, phylogenetically closely related to Crenarchaeota in the North Sea (Fig. S1).

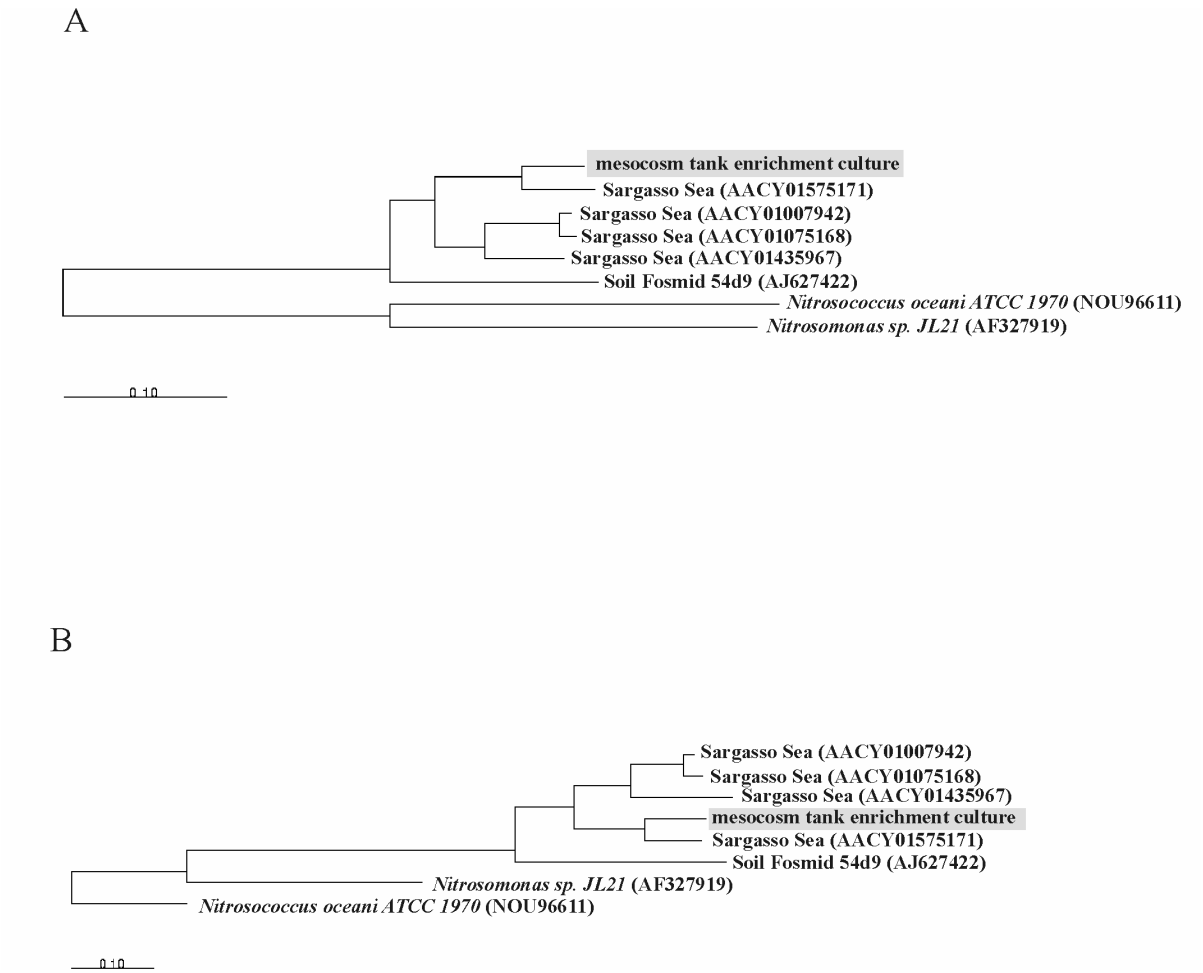

Figure 2. Phylogenetic analyses of *amo*A-like gene recovered from the enrichment culture. (**A**) Neighbour joining tree of 256 bp long *amo*A-like nucleotide sequence according to *Schleper at al.,* [2005]. The recovered *amo*A- like gene recovered from the incubation experiment is closely related to the Saragasso Sea environmental sequences. (**B**) Protein molphy tree of amino acids sequences (86aa) of *amo*A-like proteins showing the affiliation of the *amo*A-like protein recovered from the mesocosm experiment with closely related *amo*A-like proteins from the Genbank database. Sequence is submitted to GenBank.

The involvement of Crenarchaeota in nitrification was further investigated in the coastal waters of the North Sea. Marine Crenarchaeota dominated the archaeal community from late autumn to early spring (Fig. 3b) while marine Euryarchaeota were the dominant archaeal group in summer and early autumn [*Wuchter et al.,* unpublished results]. The recovered crenarchaeotal sequences from the North Sea during the crenarchaeotal winter bloom were closely related to each other with sequence similarities >96% and all belonged to the 1α group

[*Massana et al.,* 2000] of the marine Crenarchaeota (Fig. S1). The abundance of marine Crenarchaeota correlate strongly with inorganic nitrogen species, particularly nitrite ($r = 0.82$, $p<0.001$; Table S2). In contrast, cell abundances of beta- and gamma-Proteobacteria, which include the known nitrifying bacteria, remained invariant throughout this period with $4\pm1x10^5$ cells ml^{-1} (Fig. 2b) and do not show these pronounced correlation pattern with inorganic nutrients compared to the marine Crenarchaeota (Table S2). CARD-FISH of marine Crenarchaeota indicated that the cells were often associated with particles, an important source of ammonium in the marine water column. These data suggest that the marine Crenarchaeota present in this coastal environment are indeed involved in the nitrification process, with probably substantial consequences for the biogeochemical cycling of nitrogen in the ocean.

The ability of marine Crenarchaeota to perform chemolithoautotrophic nitrification would explain their distribution in the open ocean. These prokaryotes occur over a large depth range. Their absolute cell numbers are highest in the photic zone but decrease only moderately with depth, resulting in the dominance of Crenarchaeota below the photic zone [*Karner et al.,* 2001; *Herndl et al.,* 2005]. Nitrate depth profiles from the ocean typically show low concentrations in the upper ocean to levels varying from ca. 20 to 40 μM for deep waters in the Atlantic and Pacific Ocean, respectively [*Lenton and Watson.,* 2000]. These profiles are thought to be the result of four different processes occurring within the water column: the uptake of nitrate in the upper ocean waters by primary producers, ammonium regeneration from decomposing descending particulate organic nitrogen, subsequent oxidation of ammonia to nitrite by members of the *Nitrosonomas/Nitrosospira* and *Nitrosococcus* groups, and oxidation of nitrite to nitrate by nitrite oxidizers such as *Nitrobacter* sp. [*Zehr and Ward* 2002]. However, no molecular ecological studies have so far revealed large numbers of nitrifying bacteria in marine waters [*Ward* 2002], while marine Crenarchaeota do constitute ca. 20-30% of the total prokaryotic community [*Karner et al.,* 2001]. Mineralization in the meso- and bathypelagic zones of the ocean ($2.2x10^{15}$ mol C yr^{-1}) [*del Giorgio and Duarte* 2002] releases ca. $3.3x10^{14}$ mol N yr^{-1} assuming Redfield stoichiometry. If all the generated ammonium would be oxidised by Crenarchaeota fixing one carbon atom for every ca. ten nitrogen molecules oxidized [*Tijhuis et al.,* 1993], then one would expect an archaeal inorganic carbon fixation rate of ca. $3.3x10^{13}$ mol C yr^{-1}. This estimate is consistent with the

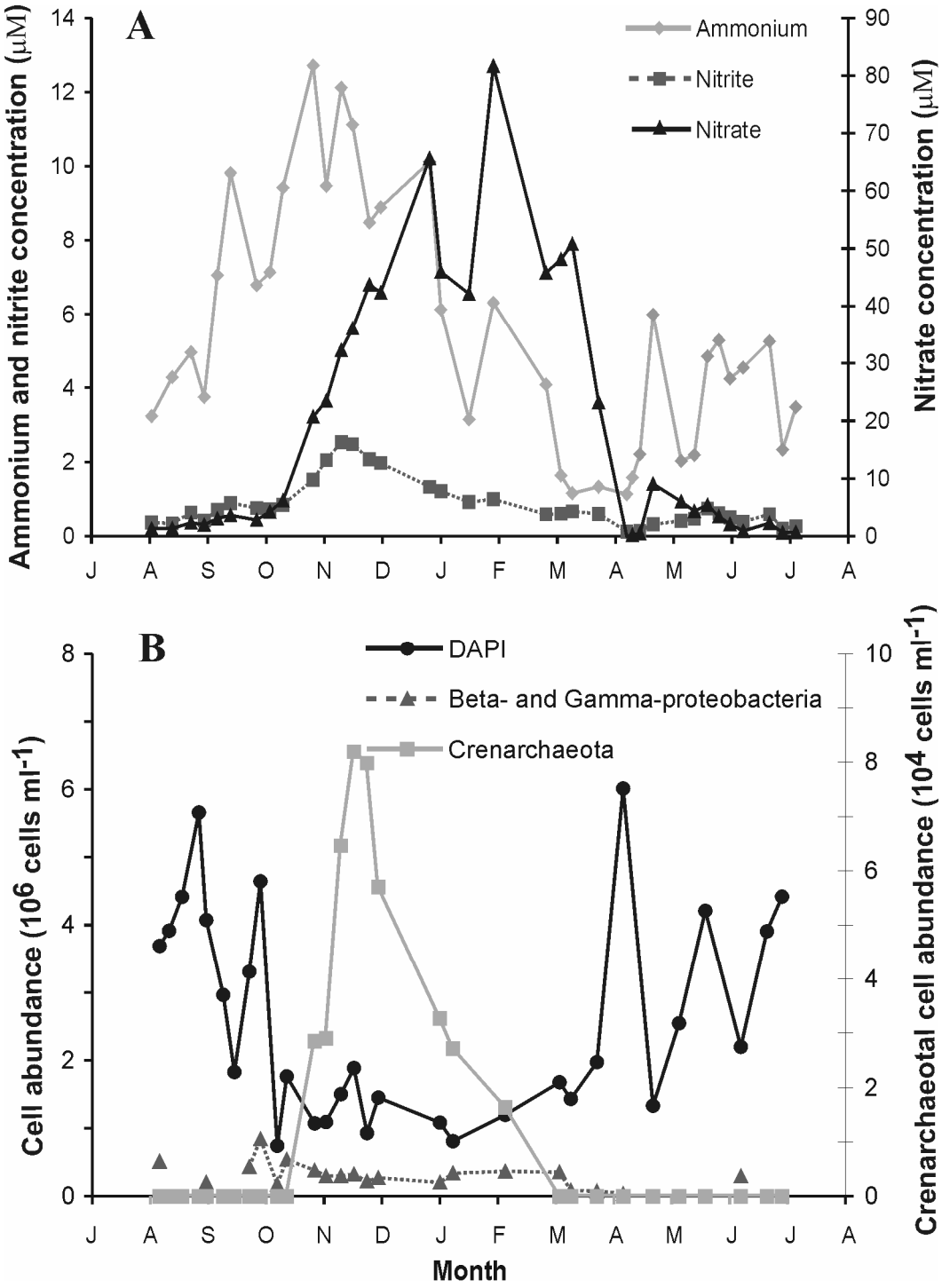

Figure 3. Crenarchaeotal abundance in the North Sea during 2002/2003 as a response to changing nutrient concentrations. (**A**) Nutrient concentrations (µM). (**B**) Cell abundances (cells ml^{-1}) of DAPI-stained cells and for Crenarchaeota and beta- and gamma-Proteobacteria as determined by CARD-FISH using selective molecular probes. The sharp increase in marine crenarchaeotal cell numbers co-occurs with the transformation of ammonium to nitrate.

estimated rate of global inorganic carbon fixation in the dark ocean by Archaea of $6.6x10^{13}$ mol C yr^{-1} of which ca. $4.5x10^{13}$ mol C yr^{-1} is taken up by Crenarchaeota [*Herndl et al.,* 2005], assuming that Eury- and Crenarchaeota are growing at equal rates. Archaeal nitrification may thus be an important process in the biogeochemical cycling of nitrogen in the ocean. These data show, together with the recently established importance of planctomycetes in denitrification [*Kuypers et al.,* 2003] and unicellular cyanobacteria in dinitrogen fixation [*Zehr et al.,* 2001; *Montoya et al.,* 2004], the possibly important role of hitherto unrecognized prokaryotes in the oceanic biogeochemical cycling of nitrogen.

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3.5 Supplementary material

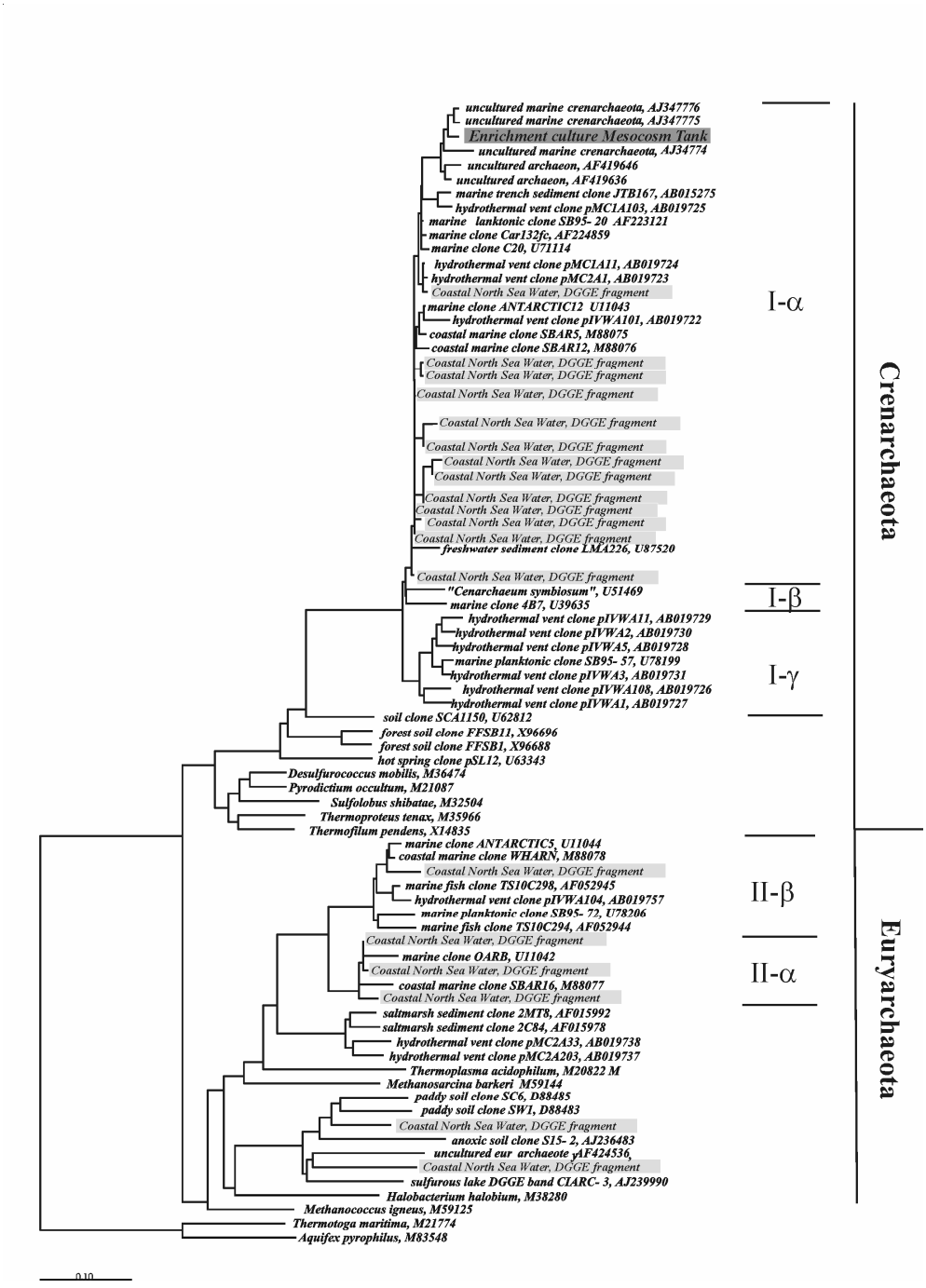

Figure S1. Partial phylogenetic tree of the Archaea showing the affiliation of the 15 partial crenarchaeal and 6 euryarchaeotal 16S rDNA gene sequences recovered from the North Sea waters time series 2002/2003 and the almost complete sequence of the Crenarchaeote in the enrichment culture (*E. coli* positions between 14 and 1406) with reference sequences obtained from the GenBank database. Sequences are submitted to GenBank. Classification of clades according to *Massana et al.* [2000]

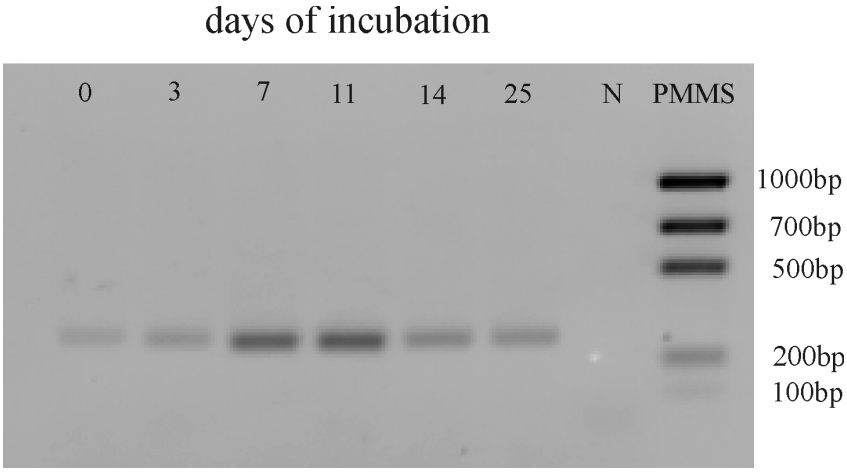

Figure S2. Agarose gel (2.5 wt%) with the Q-PCR product of the *amo*A like gene (256 bp) indicating the specificity of the Q-PCR reaction. Q-PCR was stopped after 39 cycles. N = negative control, PMMS = Precision Molecular Mass Ladder.

Table S1. Nutrient concentrations, DAPI counts and crenarchaeotal, bacterial, beta-proteobacterial and gamma-proteobacterial cell abundances in aged mesocosm water incubated at 22 and 25°C in the dark. Nutrients were directly added after the sampling for the initial nutrient concentrations and cell abundances

Time ***(day)***	***NH_4^+*** ***(µM)***	***NO_2^-*** ***(µM)***	***NO_3^-*** ***(µM)***	***DAPI counts (cells ml^{-1})***	***Cren-archaeota (cells ml^{-1})***	***Bacteria (cells ml^{-1})***	***Beta-Proteobacteria (cells ml^{-1})***	Gamma-Proteobacteria (cells ml^{-1})
22°C								
0	0.2	0.2	18	7E+05	0E+00	1E+05	2E+04	2E+05
3	175	2.0	154	2E+06	2E+05	1E+06	1E+04	4E+04
7	158	30	163	5E+06	2E+06	7E+05	1E+04	1E+04
11	91	31	101	8E+06	5E+06	5E+05	1E+04	4E+04
14	78	91	183	5E+06	2E+06	5E+05	2E+04	4E+04
18	21	40	89	3E+06	7E+05	9E+05	1E+04	6E+04
25	2.1	0.3	360	9E+05	2E+05	5E+05	1E+04	7E+04
25°C								
0	0.2	0.4	13	9E+05	1E+03	1E+05	4E+03	1E+04
3	201	2.2	172	4E+06	2E+05	2E+06	5E+03	7E+03
7	140	67	189	6E+06	3E+06	2E+05	2E+04	3E+04
11	71	114	185	6E+06	4E+06	1E+05	2E+04	2E+04
14	23	118	186	3E+06	4E+05	1E+05	2E+04	3E+04
18	7.5	48	176	9E+05	1E+05	2E+05	1E+04	2E+04
25	3.1	0.3	346	7E+05	8E+03	3E+05	2E+04	3E+04

Table S2. Pearson correlation matrix of nutrients and cells abundance of marine Crenarchaeota and beta- and gamma- Proteobacteria in North Sea time series. Most have p values > 0.1 which is not significant except for r-values denoted with * (p = 0.1-0.01) and ** (p < 0.001).

Nutrient	***Crenarchaeota***	***Beta-Proteobacteria***	Gamma-Proteobacteria
NH_4^+	0.58*	0.45	0.39
NO_2^-	0.82**	0.37	0.24
NO_3^-	0.62*	-0.06	-0.10

3.6 References

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