Archaeal nitrification in the ocean

Cornelia Wuchter[1], Ben Abbas[1], Marco J.L. Coolen[1,*], Lydie Herfort[1], Judith van Bleijswijk[1], Peer Timmers[2], Marc Strous[2], Eva Teira[1], Gerhard J. Herndl[1], Jack J. Middelburg[3], Stefan Schouten[1], Jaap S. Sinninghe Damsté[1]

[1] *Royal Netherlands Institute for Sea Research (NIOZ), Departments of Marine Biogeochemistry and Toxicology and of Biological Oceanography, PO Box 59, 1790 AB Den Burg, The Netherlands*

[2] *Department of Microbiology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands*

[3] *Netherlands Institute for Ecology (NIOO-KNAW), Centre for Estuarine and Marine Ecology, PO Box 140, 4400 AC Yerseke, The Netherlands*

** Present address: Woods Hole Oceanographic Institution, Department of Chemistry and Geochemistry, 360 Woods Hole Rd., Woods Hole, MA 02543, US*

Correspondence and requests for materials should be addressed to J.S. Sinninghe Damsté (Tel: +31 222 368550; Fax: +31 222 319674; E-mail: damste@nioz.nl).

Category: Physical Sciences, Environmental Sciences

Manuscript information: 19 text pages, 4 figures, 1 table, supplementary information

Word count abstract: 192

Character count: 51687

Abstract

Marine Crenarchaeota are the most abundant single group of prokaryotes in the ocean but their physiology and role in marine biogeochemical cycles are unknown. Recently, a member of this clade was isolated from a sea aquarium and shown to be capable of nitrification, tentatively suggesting that they may play a role in the oceanic nitrogen cycle. We enriched a crenarchaeote from North Sea water and show that it oxidizes ammonium to nitrite. A time series study in the North Sea revealed that the abundance of the gene encoding for the archaeal ammonia monooxygenase alfa subunit (*amoA*) is correlated with the decline in ammonium concentrations and with the abundance of Crenarcheota. Remarkably, the archaeal *amoA* abundance was 1-2 orders of magnitude higher than those of bacterial nitrifiers which are commonly thought to mediate the oxidation of ammonium to nitrite in marine environments. Analysis of Atlantic waters of the upper 1000 m, where most of the ammonium regeneration and oxidation takes place, showed that crenarchaeotal *amoA* copy numbers are also one to three orders of magnitude higher than those of bacterial *amoA*. Our data thus suggest a major role for Archaea in oceanic nitrification.

Introduction

Archaea constitute the third domain of life next to the Eukaryotes and Bacteria. Until a decade ago, Archaea were thought to mainly consist of organisms thriving in extreme environments such as sulfidic hot springs, salt brines and anoxic environments. With the advent of molecular biological techniques it became clear that Archaea are actually widespread and occur in diverse environments such as oceans, lakes and soils (1-4). However, these non-extremophilic Archaea are not closely related to cultured relatives and thus not much is known about their physiology and role in biogeochemical cycling.

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 (1, 5). Marine Crenarchaeota are typically relatively more abundant in deep neritic waters and in the meso- and bathypelagic zones of the ocean (1, 5-7) and are thought to account for ca. 20% of all prokaryotic cells in the global ocean (1). The metabolism of these planktonic Crenarchaeota is subject of current debate. In-situ labelling (8) and microautoradiography (7) experiments showed that marine Crenarchaeota can utilize dissolved inorganic carbon as carbon source but are also able to take up amino acids (7, 9), suggesting a heterotrophic lifestyle. Recently, a crenarchaeote, *Candidatus* "Nitrosopumilus maritimus", was isolated from a sea aquarium and shown to be autotrophic and able to oxidize ammonium to nitrite (10). Positive correlations between the abundance of Crenarchaeota and nitrite were observed in the Arabian Sea (11) and in the Santa Barbara Channel time series (12), and with particulate organic nitrogen in Arctic waters (13). Furthermore, a diverse set of putative archaeal ammonia monooxygenase encoding genes (e.g. *amoA*) were recently reported from shallow (<300 m) marine waters and sediments (14) and was

found in fosmid sequences of *Cenarchaeum symbiosum* (15). These findings hint that Crenarchaeota may be involved in the marine nitrogen cycle, possibly as nitrifiers, but the relevance of crenarchaeotal nitrification for the marine nitrogen cycle is unknown. Until now it has been assumed that marine nitrification is mainly performed by two different groups of bacteria belonging to the Beta- and Gammaproteobacteria (16).

Here, we provide experimental evidence that Crenarchaeota present in sea water are capable of aerobic ammonium oxidation (nitrification) by enrichment of a nitrifying crenarchaeote from coastal waters. We also show by quantitative analysis of both archaeal and bacterial *amoA* in coastal and open ocean waters that marine Crenarchaeota are likely important players of the present day marine nitrogen cycle.

Results and Discussion

Enrichment culture of a nitrifying Crenarchaeote

We enriched a member of the Crenarchaeota from North Sea waters. The experimental setup was originally designed to examine the temperature adaptation of marine crenarchaeotal membrane lipids [see (17) for details]. During this experiment, coastal North Sea water was incubated in the dark for 6 months at 25 ºC in an 850 l mesocosm tank without addition of nutrients. A substantial increase in archaeal membrane lipids was observed which coincided with an almost complete consumption of ammonium (17). This initial experiment tentatively suggested a link between North Sea Crenarchaeota and the oxidation of ammonium. To further investigate this, water from this mesocosm tank was incubated in the dark at 22 and 25ºC and inorganic nutrients, including ammonium, were added whilst pH was kept constant at 8.2, the regular pH of surface sea water. The abundance of Crenarchaeota was monitored with Catalytic Reporter Deposition-Fluorescence In Situ Hybridisation

(CARD-FISH) (18) and revealed a substantial enrichment of crenarchaeotal cells at both temperatures (Fig. 1b; Table S1 in supplementary information). 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 at that time up to 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 (19), suggesting that our enrichment culture was dominated by Crenarchaeota at day 11 (Fig. 1b). In fact, molecular analyses selective for Archaea revealed that the incubated waters were dominated by a single member of the crenarchaeotal phylogenetic cluster I.1a (6) (Fig. 2) with 99% sequence similarity over the nearly complete 16S rRNA gene to the nitrifying crenarchaeote, *Candidatus* "N. maritimus" (10). When ammonium levels dropped, nitrite concentrations increased concomitantly with the increase in crenarchaeotal abundance (Fig. 1a), similar to what was previously observed in the large mesocosm tank (see above) (17). Importantly, the abundance of Beta- and Gammaproteobacteria, which include the known nitrifying bacteria, remained low (<1% of DAPI-stained cells) throughout the incubation. If all the detected Proteobacteria would have been nitrifiers and all the nitrification activity would have been mediated by these nitrifiers, the activity per cell would have been >100 fmol NH_3 $cell^{-1}$ day^{-1}, substantially more than ever reported before. However, if Crenarchaeota were responsible for nitrification, the ammonium conversion rates were between 2 and 4 fmol NH_3 $cell^{-1}$ day^{-1} at 22 and 25°C, respectively. These figures are well within the ranges reported previously for bacterial aerobic ammonium oxidation (16) and compare very well with the ~4 fmol NH_3 $cell^{-1}day^{-1}$ for *Candidatus* "N. maritimus" [estimated from Fig. 3 in (10)].

Further evidence for ammonia oxidation by the enriched marine crenarchaeote was provided by the identification of a single *amoA*. Its sequence is closely related (92% nucleotide sequence similarity; 97% amino acid sequence similarity) to the *amoA* obtained from the archaeon *Candidatus* "N. maritimus" (10) and to archaeal *amoA* recovered from the Sargasso Sea (4, 20) (91% nucleotide sequence similarity; 95% amino acid sequence similarity; Fig. 3) and only distantly related to known bacterial *amoA* sequences. Quantification of the abundance of the *amoA*- and marine crenarchaeotal 16S rDNA at day 7 in the enrichment culture yielded a relative ratio of 0.9:1 suggesting that the enriched crenarchaeote possesses a singly copy for *amoA*. Thus, the distribution of the coinciding single phylotypes of *amoA*- and marine crenarchaeotal 16S rDNA in relation to the observed changes in nutrient concentrations strongly suggests that the enriched marine crenarchaeote was involved in nitrification. Our result is in agreement with the recent findings of an ammonium oxidizing crenarchaeote isolated from an aquarium (10) and the presence and expression of *amoA* in soil Crenarchaeota (21). Importantly, our findings suggest that this metabolism may be widespread among Crenarchaeota thriving in marine waters.

Importance of archaeal nitrification in an ocean margin system

The ecological significance of the observed crenarchaeotal nitrification was investigated in the coastal waters of the North Sea from which the marine crenarchaeote was enriched. PCR amplification of archaeal 16S rDNA using a general archaeal primer followed by phylogenetic analysis of sequenced denaturing gradient gel electrophoresis (DGGE) fragments revealed that Crenarchaeota dominated the archaeal community from late fall to early spring. The recovered 16S rDNA crenarchaeotal sequences from the North Sea during the crenarchaeotal winter bloom

were closely related to each other and to the enriched crenarchaeote with sequence similarities >96% and all belonged to the Group I.1a (6) of the Crenarchaeota (Fig. 2). The abundance of Crenarchaeota varied coincidentally with inorganic nitrogen species in North Sea waters: when ammonium levels dropped from 12.7 to 8.5 μM and nitrite levels were rising from 0.8 to 2.5 μM between November and December, crenarchaeotal abundance increased by 2 orders of magnitude (Fig. 4b). In December abundances of Crenarchaeota dropped considerably, for reasons presently unclear, whilst ammonium levels remained relatively constant at ~9 μM. Then in early January crenarchaeotal abundance again increased substantially by one order of magnitude whilst ammonia levels dropped from 10 to 3 μM. In contrast to the crenarchaotal cell abundance, cell abundances of Beta- and Gammaproteobacteria as determined by CARD-FISH remained high but invariant throughout December to March with $4\pm1x10^5$ cells ml^{-1} and did not show a distinct elevation in abundance when ammonium concentrations dropped.

Functional gene analyses of the North Sea time series yielded one dominant archaeal *amoA*. This gene was closely related to that of *Candidatus* "N. maritimus" (92% nucleotide and 97% amino acid similarity) (Fig. 3). Importantly, quantification of the archaeal *amoA* copies by Q-PCR showed a strong positive linear correlation with both crenarchaeotal cell counts by CARD-FISH (r^2=0.81, n=9) and crenarchaeotal 16S rDNA gene abundance (r^2=0.94, n=39). The slopes of these correlation lines are 2.5 and 2.8, respectively suggesting that each crenarchaeotal cell possessed 2-3 copies of *amoA*. This is significantly more than the singly copy for *amoA* per cell in the enriched crenarchaeote but it has been shown previously that number of *amoA* copies per cell in ammonia oxidizing bacteria can vary from 1 for Gammaproteobacteria to 2 or 3 for Betaproteobacteria (22). Obviously, there is

variability in *amoA* copies within crenarchaeota as well. Compared to the archaeal *amoA*, betaproteobacterial *amoA* were present in equal or slightly higher numbers only during late spring and summer when the nitrification activity was low, whilst *amoA* of Gammaproteobacteria could not be detected at all with our primer set. However, from late fall to early spring during times of high nitrification activity bacterial *amoA* was 1-2 orders of magnitude less abundant than *amoA* derived from Crenarchaeota (Fig. 4c). A recent study of ammonium-oxidizing Betaproteobacteria in the North Sea indicated the same seasonal abundance pattern (23). Thus, our results strongly suggest that the dominant crenarchaeotal species in North Sea water were predominantly responsible for nitrification during winter.

It should be noted that a perfect correlation between ammonium concentrations and cell abundance or *amoA* copy numbers is not to be expected as not only ammonia oxidation will influence ammonium concentrations but also ammonium regeneration, input from rivers, advection from the Atlantic Ocean, and release of ammonium from sediments (24). To roughly estimate nitrification rates we used the amount of nitrate formed during the period of ammonium oxidation. Based on the regeneration of ~70 μM nitrate within ~3 months (Fig. 4) and the crenarchaeotal abundance, an *in situ* archaeal nitrification rate of ca. 7 fmol NH_3 $cell^{-1}$ day^{-1} was calculated, which is ~2 times higher than in our enrichment experiments and in cultures of *Candidatus* "N. maritimus" (10). This *in situ* archaeal nitrification rate is an upper estimate as sedimentary nitrification might also have contributed by up to 65% (24) and the presence of bacterial *amoA* suggests that bacterial nitrifiers could also have contributed. CARD-FISH of Crenarchaeota indicated that the cells were often associated with particles, an important source of ammonium in the marine water column. Our data suggest that the Crenarchaeota present in this ocean margin

system are indeed involved in nitrification and may contribute more to nitrification than the known bacterial nitrifiers which were formerly held responsible for this process.

Importance of archaeal nitrification in the open 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 (1, 5). 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 (1, 5). Nitrate depth profiles from the ocean typically show low concentrations in the upper ocean to levels varying from ca. 20 to 40 μM for deeper waters in the Atlantic and Pacific Ocean (25). These profiles are thought to be the result of four different processes taking place within the water column: the uptake of nitrogen in the upper ocean waters by primary producers, ammonium regeneration from decomposing descending particulate organic nitrogen, subsequent oxidation of ammonium to nitrite by members of the *Nitrosonomas/Nitrosospira* and *Nitrosococcus* groups, and oxidation of nitrite to nitrate by nitrite oxidizers such as *Nitrobacter* sp. (26). However, no molecular ecological study has so far revealed large numbers of known nitrifying bacteria in marine waters (16), while marine Crenarchaeota do constitute ca. 20-30% of the total prokaryotic community (1).

Analysis of cell numbers of Crenarchaeota in the upper 1000 m of the North Atlantic as determined by CARD-FISH (7) and the abundance of archaeal *amoA* (Table 1) revealed a ratio of 1.9±1.4 copies per cell, in between the ratio's observed in the North Sea time series and the enriched crenarchaeote. The recovered *amoA*

sequences fall into two phylogenetic clusters including the one containing the North Sea and enrichment culture *amoA* sequences and that of *Candidatus* "N. maritimus" (Fig. 3). Quantification of bacterial *amoA* in the same set of water samples (Table 1) revealed that *amoA* of Betaproteobacteria is lower by one to three orders of magnitude compared to archaeal *amoA,* whilst *amoA* derived from Gammaproteobacteria were below detection limit. These combined results suggest that Crenarchaeota in the mesopelagic layer of the open ocean are also involved in nitrification and may play a more important role than bacterial nitrifiers.

Our data can be combined with literature data to give a rough estimate of the global importance of archaeal nitrification. Mineralization in the meso- and bathypelagic zones of the ocean [2.2×10^{15} mol C yr^{-1} (27)] releases ca. 3.3×10^{14} mol N yr^{-1} assuming Redfield stoichiometry (28). If all the generated ammonium would be oxidized by Crenarchaeota fixing one carbon atom for every ca. ten nitrogen molecules oxidized (29), then one would expect an archaeal inorganic carbon fixation rate of ca. 3.3×10^{13} mol C yr^{-1}. This estimate is consistent with the estimated rate of global inorganic carbon fixation in the dark ocean by Archaea of 6.6×10^{13} mol C yr^{-1} of which ca. 4.5×10^{13} mol C yr^{-1} may be taken up by Crenarchaeota (7), 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 although it remains uncertain whether all pelagic Crenarchaeota are nitrifiers. These data show, together with the recently established importance of Planctomycetes in denitrification (30, 31) and unicellular cyanobacteria in dinitrogen fixation (32, 33), the important role of hitherto unrecognized prokaryotes in the oceanic biogeochemical cycling of nitrogen.

Material and Methods

Incubation experiment setup. Coastal North Sea water was kept in the dark for 6 months at 25 ºC in an 850 l mesocosm tank without addition of nutrients (17). After these 6 months 20 l batch cultures with aged mesocosm water were incubated in 20 l Nalgene Clearboy tanks at 22 and 25 °C in the dark. Nutrients were added before the incubation at concentrations of 150 µM $NaNO_3$, 150 µM NH_4Cl, 25 µM NaH_2PO_4 and 2666 µM $NaHCO_3$ together with a sterile mix of 12.5 mg l^{-1} yeast, 5 mg l^{-1} peptone extract, vitamins and trace elements. The pH was regularly adjusted to 8.2 by adding sterile 0.1 M NaOH or HCl and salinity was maintained at 27 by addition of distilled water. The 20 l tanks were continuously stirred and open throughout the experiment allowing constant gas exchange with the air. Samples for nutrient analysis, CARD-FISH and DNA were taken every 3-4 days.

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 CARD-FISH were taken on a bi-weekly schedule from August 2002 to July 2003.

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 0.45 µm cellulose nitrate filters (Millipore) as supporting filters and stored frozen at -20ºC until further analysis. Total picoplankton were enumerated after DAPI staining (34), while Bacteria and Archaea were enumerated by CARD-FISH (18) under the epifluorescence microscope. The oligonucleotide probes Eub338, BET42 and GAM42 were used for enumeration of Bacteria (35), Beta- and Gamma-proteobacteria (23), respectively, and specific probes were applied for the marine Crenarchaeota Group I,

Cren537 (5'-TGACCACTTGAGGTGCTG-3') (17). All probes were tested for their specificity prior to the study. Cell walls were permeabilized with lysozyme (Sigma; 10 mg ml^{-1} in 0.05 M EDTA, 0.1 Tris-HCl [pH 8]) for Eub338 (17) 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}. Hybridization 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 Gammaproteobacteria 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 Gammaproteobacteria 28%. The larger errors are usually associated with low cell numbers where slides contained substantially less than 200 cells (e.g. Beta- and Gammaproteobacteria in the incubation experiments).

QPCR. The numbers of archaeal 16S rDNA-, archaeal *amoA*- as well as bacterial *amoA* copies in all samples were determined in duplicate using an iCycler system (Biorad). A total of 40 cycles were run with PCR conditions and reagents as described previously (36) but with annealing temperatures and primer combinations as listed in Table S2. Fluorescently measured (Picogreen, Molecular Probes) exact volumes and known concentrations (10 ng) of template DNA was added to the reaction mixtures. Accumulation of newly amplified double stranded gene products was followed online as the increase in fluorescence due to the binding of the fluorescent dye SYBRgreen (Molecular Probes). Calibration of the samples was performed with known copies (between 10^{-2} and 10^{7}) of *Sulfolobus acidocaldarius* DSM 639 (archaeal 16S rDNA), enriched marine Crenarchaeote from the North Sea (archaeal *amoA*), *Nitrosomonas*

europea (*amoA* of beta-AOB), and *Nitrosococcus oceanus* (*amoA* of gamma-AOB) which were generated during PCR with the same primers as used for the amplification of the environmental genes (Table S2). As a control of the specificity of the QPCR, the runs were repeated with only 32 cycles so that most amplicons reached the threshold cycle. In addition, one µl of the first reaction with 32 cycles was added to a fresh mixture of PCR ingredients and run for 12 to 15 cycles but this time with primers including the 40-bp-long GC clamp to allow subsequent DGGE analysis (36). Aliquots of these QPCR products were run on an agarose gel in order to identify unspecific PCR products such as primer dimers or fragments with unexpected fragment lengths (Table S2). Sequence analysis of the excised DGGE fragments (see methods below) revealed the diversity of the amplicons generated by QPCR and therefore was the ultimate proof that the QPCR reactions were in fact specific.

Total DNA extraction. For the QPCR and phylogenetic 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 total DNA was extracted as described previously (17). The cell lyses efficiency of this method was ca. 90% as determined by counting the percentage of DAPI-stained cells which remained in suspension or were still attached to the filter or zirconium beads after the lyses steps during our extraction procedure. This whole procedure was performed three times.

Phylogeny of sequenced DGGE fragments. Archaeal 16S rDNA amplicons were analyzed by DGGE as described previously (37). Archaeal *amoA* amplicons were analyzed by DGGE using a similar protocol with the exception that the DGGE was run for 3 h and the gradient of denaturants was 10 to 50%. To check the specificity of QPCR of Betaproteobacterial AOB, *amoA* amplicons from Betaproteobacterial AOB were analyzed by DGGE as described recently (38) and sequencing of DGGE

fragments revealed the specificity of the QPCRs despite the low copy numbers The community structure of beta-AOB in the North Sea clearly differed from the Atlantic Ocean (data not shown).

DGGE-fragments were sliced from the gels and subsequently sequenced for phylogenetic comparison with reference sequences from the NCBI database (39) using the ARB software package (40). Archaeal *amoA* sequences obtained in this study have been deposited in the NCBI sequence database under accession numbers xxx to xxx *(in progress)*

Acknowledgements

Analytical assistance was provided by Bouwe Kuipers, Govert van Noort, Harry Witte and Karel Bakker (Royal NIOZ). Dr. Karen Casciotti (WHOI) is thanked for supplying the *Nitrobacter* and *Nitrosomonas* cultures. This study was partially supported by grants to JSSD and MJLC (VENI) from the Netherlands Organisation for Scientific Research (NWO).

References

1. Karner, M. B., DeLong, E. F. & Karl, D. M. (2001) *Nature* **409**, 507-510.
2. MacGregor, B. J., Moser, D. P., Alm, E. W., Nealson, K. H. & Stahl, D. A. (1997) *Appl. Environ. Microbiol.* **63**, 1178-1181.
3. Hershberger, K. L., Barns, S. M., Reysenbach, A.-L., Dawson, S. C. & Pace, N. R. (1996) *Science* **384**, 420.
4. Schleper, C., Jurgens, G. & Jonuscheit, M. (2005) *Nature Rev. Microbiol.* **3**, 479-488.
5. Fuhrman, J. A., McCallum, K. & Davis, A. A. (1992) *Nature* **356**, 148-149.

6. Massana, R., DeLong, E. F. & Pedrós-Alió, C. (2000) *Appl. Environ. Microbiol.* **66**, 1777-1787.

7. Herndl, G. J., Reinthaler, T., Teira, E., van Aken, H., Veth, C., Pernthaler, A. & Pernthaler, J. (2005) *Appl. Environ. Microbiol.* **71**, 2303-2309.

8. Wuchter, C., Schouten, S., Boschker, H. T. S. & Sinninghe Damsté, J. S. (2003) *FEMS Microbiol. Lett.* **219**, 203-207.

9. Ouverney, C. C. & Fuhrman, J. A. (2000) *Appl. Environ. Microbiol.* **66**, 4829-4833.

10. Könneke, M., Bernhard, A. E., de, l. T., Jr., Walker, C. B., Waterbury, J. B. & Stahl, D. A. (2005) *Nature* **437**, 543-546.

11. Sinninghe Damsté, J. S., Rijpstra, W. I. C., Hopmans, E. C., Prahl, F. G., Wakeham, S. G. & Schouten, S. (2002) *Appl. Environ. Microbiol.* **68**, 2997-3002.

12. Murray, A. E., Blakis, A., Massana, R., Strawzewski, S., Passow, U., Alldredge, A. & DeLong, E. F. (1999) *Aquat. Microb. Ecol.* **20**, 129-145.

13. Wells, L. E. & Deming, J. W. (2003) *Aquat. Microb. Ecol.* **31**, 19-31.

14. Francis, C. A., Roberts, K. J., Beman, J. M., Santoro, A. E. & Oakley, B. B. (2005) *Proc. Natl. Acad. Sci. USA* **102**, 14683-14688.

15. Hallam, S. J., Mincer, T. J., Schleper, C., Preston, C. M., Roberts K & et al. (2006). *PLoS Biol* **4**: e95.

16. Ward, B. B. (2002) in *Encyclopedia of Environmental Microbiology*, ed. Capone D.A. (Wiley, New York), pp. 2144-2167.

17. Wuchter, C., Schouten, S., Coolen, M. J. L. & Sinninghe Damsté, J. S. (2004) *Paleoceanography* **19**, PA4028, doi:10.1029/2004PA001041, 2004-.

18. Teira, E., Reinthaler, T., Pernthaler, A., Pernthaler, J. & Herndl, G. J. (2004) *Appl. Environ. Microbiol.* **70**, 4411-4414.

19. Heissenberger, A., Leppard, G. G. & Herndl, G. J. (1996) *Appl. Environ. Microbiol.* **62**, 4521-4528.

20. Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., Wu, D. Y., Paulsen, I., Nelson, K. E., Nelson, W. *et al.* (2004) *Science* **304**, 66-74.

21. Treusch, A. H., Leininger, S., Kletzin, A., Schuster, S. C., Klenk, H. P. & Schleper, C. (2005) *Environ. Microbiol.* **7**, 1985-1995.

22. Norton, J. M., Alzerreca, J. J., Suwa, Y. & Klotz, M. G. (2002) *Arch. Microbiol.* **177**, 139-149.

23. Sekar, R., Fuchs, B. M., Amann, R. & Pernthaler, J. (2004) *Appl. Environ. Microbiol.* **70**, 6210-6219.

24. Middelburg, J. J. & Soetaert, K. (2005) in *The Sea, Volume 13*, eds. Robinson, A. R. & McCarthy, J. R. B. J. (Harvard University Press, Cambridge), p. 353.

25. Arrigo, K. R. (2005) *Nature* **437**, 349-355.

26. Zehr, J. P. & Ward, B. B. (2002) *Appl. Environ. Microbiol.* **68**, 1015-1024.

27. del Giorgio, P. A. & Duarte, C. M. (2002) *Nature* **420**, 379-384.

28. Lenton, T. M. & Watson, A. J. (2000) *Glob. Biogeochem. Cycl.* **14**, 225-248.

29. Tijhuis, L., van Loosdrecht, M. C. M. & Heijnen, J. J. (1993) *Biotechnol. Bioeng.* **42**, 509-519.

30. Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jørgensen, B. B., Kuenen, J. G., Sinninghe Damsté, J. S., Strous, M. & Jetten, M. S. M. (2003) *Nature* **422**, 608-611.

31. Kuypers, M. M. M., Lavik, G., Woebken, D., Schmid, M., Fuchs, B. M., Amann, R., Jørgensen, B. B. & Jetten, M. S. M. (2005) *P. Natl. Acad. Sci. USA* **102**, 6478-6483.

32. Zehr, J. P., Waterbury, j. B., Turner, P. J., Montoya, J. P., Omoregie, E., Steward, G. F., Hansen, A. & Karl, D. M. (2001) *Nature* **412**, 635-638.

33. Montoya, J. P., Holl, C. M., Zehr, J. P., Hansen, A., Villareal, T. A. & Capone, D. G. (2004) *Nature* **430**, 1027-1031.

34. Porter, K. G. & Feig, Y. S. (1980) *Limnol. Oceanogr.* **25**, 943-948.

35. Amann, R. I. (1995) *Mol. Microbiol. Ecol. Manual* **3.3.6**, 1-15.

36. Coolen, M. J. L., Boere, A., Baas, M., Wakeham, S. G. & Sinninghe Damsté, J. S. (2006) *Paleoceanography* **21**, PA1005-doi:10.1029/2005PA001188.

37. Coolen, M. J. L., Hopmans, E. C., Rijpstra, W. I. C., Muyzer, G., Schouten, S., Volkman, J. K. & Sinninghe Damsté, J. S. (2004) *Org. Geochem.* **35**, 1151-1167.

38. Hornek R., Pommerening-Röser A., Koops H.P, Farnleitner A.H., Kreuzinger N., Kirschner A. & Mach R.L. (2006) *J. Microbiol. Meth.*, in press.

39. Nicolaisen, M. H. & Ramsing, N. B. (2002) *J. Microbiol. Meth.* **50**, 189-203.

40. Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G. *et al.* (2004) *Nucl. Acids Res.* **32**, 1363-1371.

41. Holmes, A. J., Costello, A., Lidstrom, M. E. & Murrell, J. C. (1995) *FEMS Microbiol. Lett.* **132**, 203-208.

Figure legends

Figure 1. Archaeal nitrification in an enrichment culture of a crenarchaeote from the North Sea. (**A**) Nutrient concentrations (μM) in the course of the experiment. (**B**) crenarchaeotal, eubacterial, betaproteobacterial and gammaproteobacterial cell numbers (cells ml^{-1}) as determined by CARD-FISH (18). Aged sea water from a large mesocosm experiment (see text and Materials and Methods) with added inorganic nutrients was incubated at two different temperatures in the dark. The data shown are those obtained at 25ºC. The crenarchaeotal population in our enrichment culture was shown to consist of a single species, phylogenetically closely related to Crenarchaeota in the North Sea (Figs. 2 and 3).

Figure 2. Phylogenetic analyses of crenarchaeotal 16S rRNA genes recovered from the enrichment culture and the North Sea time series. Neighbour joining tree of Archaea showing the affiliation of partial crenarchaeotal 16S rDNA gene sequences recovered from the North Sea waters time series 2002/2003 (blue colors) and the almost complete 16S rDNA sequence of the Crenarchaeote in the enrichment culture (*E. coli* positions between 20 and 1406) (red colour) with reference sequences obtained from the NCBI database. Classification of clades according to Massana et al. (6).

Figure 3. Phylogenetic analyses of archaeal *amoA* recovered from the enrichment culture (red colour), the North Sea (blue colour) and the Atlantic Ocean (green colour). Neighbour joining tree of 256 bp long *amoA*-like nucleotide sequence according to Francis et al. (14). The *amo*A- gene recovered from the incubation experiment is closely related (92% sequence similarity) to that of *Candidatus* "N. maritimus" (10) and of the Sargasso Sea environmental sequences (20) (up to 91% sequence similarity). The *amoA* recovered from the North Sea time series is also closely related to that of *Candidatus* "N. maritimus" (92% sequence similarity) and of some Sargasso Sea environmental sequences.

Figure 4. Crenarchaeotal abundance in the North Sea between August 2002 and July 2003 as a response to changing nutrient concentrations. (**A**) Nutrient concentrations (μM). (**B**) Cell abundances (cells ml^{-1}) of Crenarchaeota as determined by CARD-FISH (18) and abundances of 16S rDNA copies of Crenarchaeota as determined by quantitative-PCR (see Methods) and (**C**) abundances of archaeal, betaproteobacterial and gammaproteobacterial *amoA* copy numbers as determined by quantitative-PCR. The sharp increases in crenarchaeotal cell numbers and archaeal *amoA* copy numbers in November and January co-occur with the transformation of ammonia to nitrate. In contrast, a far less pronounced increase in *amoA* copy numbers of betaproteobacterial ammonia oxidizers was observed in this period ($1.0x10^4$) compared to the rest of the year (2.3×10^3).

Table 1. Crenarchaeotal cell abundance as determined by CARD-FISH and archaeal and bacterial *amoA* copy numbers as determined by Q-PCR in water samples from the Atlantic Ocean. Samples were taken during the TRANSAT-1 and 2 cruises (7).

Sample code	***Latitude (°N)***	***Longitude (°E)***	***Depth (m)***	***Cren-archaeota (cells ml^{-1})***	***Archaeal amoA (copies ml^{-1})***	***Beta-Proteobac-terial amoA (copies ml^{-1})***	***Gamma-Proteobacterial amoA (copies ml^{-1})***
T1S23	61.683	-16.750	100	1.8E+04	4.4E+04	1.4E+03	n.d.
T1S32	61.633	-20.187	1016	1.5E+04	1.2E+04	5.3E+01	n.d.
T1S34	61.633	-20.187	100	2.8E+04	1.9E+04	8.4E+02	n.d.
T1S50	60.183	-25.700	150	1.7E+04	3.6E+04	8.5E+02	n.d.
T1S61	57.453	-27.919	100	3.9E+04	4.3E+04	1.5E+03	n.d.
T1S71	55.314	-30.432	400	2.7E+04	0.5E+04	6.7E+00.	n.d.
T1S72	55.314	-30.432	150	3.1E+04	1.9E+04	3.6E+02	n.d.
T1S82	52.667	-34.167	400	2.2E+04	0.8E+04	1.7E+01	n.d.
T1S83	52.667	-34.167	100	2.3E+04	1.0E+04	2.2E+02	n.d.
T1S120	49.734	-26.134	600	8.0E+03	3.0E+04	2.0E+02	n.d.
T1S121	49.734	-26.134	148	3.8E+04	3.1E+04	1.2E+03	n.d.
T1S154	41.600	-26.533	150	6.9E+03	3.6E+04	1.7E+02	n.d.
T2S14	40.104	-66.498	95	8.4E+04	2.4E+04	1.7E+00	n.d.
T2S25	41.149	-62.433	95	2.7E+04	0.9E+04	2.5E+00	n.d.
T2S36	43.383	-58.083	99	2.9E+04	3.7E+04	9.8E+02	n.d.
T2S58	40.317	-49.250	100	9.4E+04	2.0E+04	5.6E+02	n.d.
T2S73	45.567	-45.067	243	2.9E+04	1.6E+04	1.4E+02	n.d.
T2S74	45.567	-45.067	100	5.2E+04	3.2E+04	3.9E+02	n.d.
T2S83	47.300	-42.217	266	1.7E+04	1.0E+04	2.3E+02	n.d.
T2S84	47.300	-42.217	103	1.0E+05	5.5E+04	1.7E+03	n.d.

n.d = not detected, i.e. no specific products were formed upon Q-PCR or they were below the detection limit of ~2.5e+02 copy ml^{-1} as determined for gammaproteobacterial *amoA*.

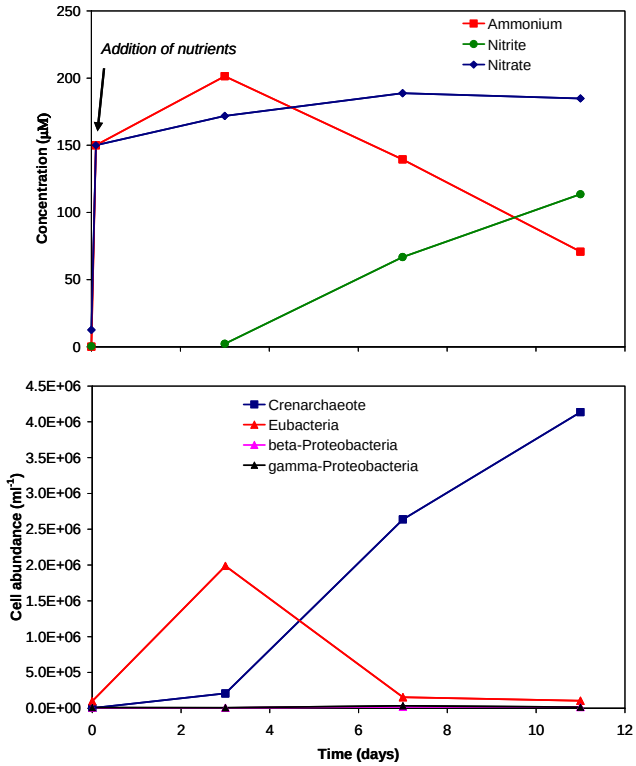
Addition of nutrients
Ammonium
Nitrite
Nitrate
Concentration (µM)
250
200
150
100
50
0
Crenarchaeote
Eubacteria
beta-Proteobacteria
gamma-Proteobacteria
Cell abundance (ml-1)
4.5E+06
4.0E+06
3.5E+06
3.0E+06
2.5E+06
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
0
2
4
6
8
10
12
Time (days)

Figure 1

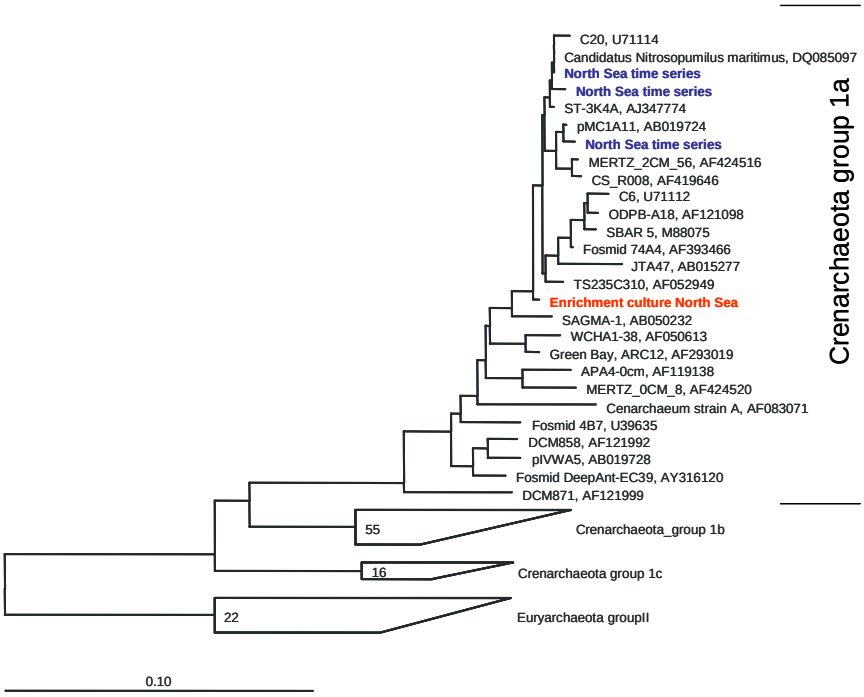
C20, U71114
Candidatus Nitrosopumilus maritimus, DQ085097
North Sea time series
North Sea time series
ST-3K4A, AJ347774
pMC1A11, AB019724
North Sea time series
MERTZ_2CM_56, AF424516
CS_R008, AF419646
C6, U71112
ODPB-A18, AF121098
SBAR 5, M88075
Fosmid 74A4, AF393466
JTA47, AB015277
TS235C310, AF052949
Enrichment culture North Sea
SAGMA-1, AB050232
WCHA1-38, AF050613
Green Bay, ARC12, AF293019
APA4-0cm, AF119138
MERTZ_0CM_8, AF424520
Cenarchaeum strain A, AF083071
Fosmid 4B7, U39635
DCM858, AF121992
pIVWA5, AB019728
Fosmid DeepAnt-EC39, AY316120
DCM871, AF121999
Crenarchaeota group 1a
55
Crenarchaeota_group 1b
16
Crenarchaeota group 1c
22
Euryarchaeota groupII
0.10

Figure 2

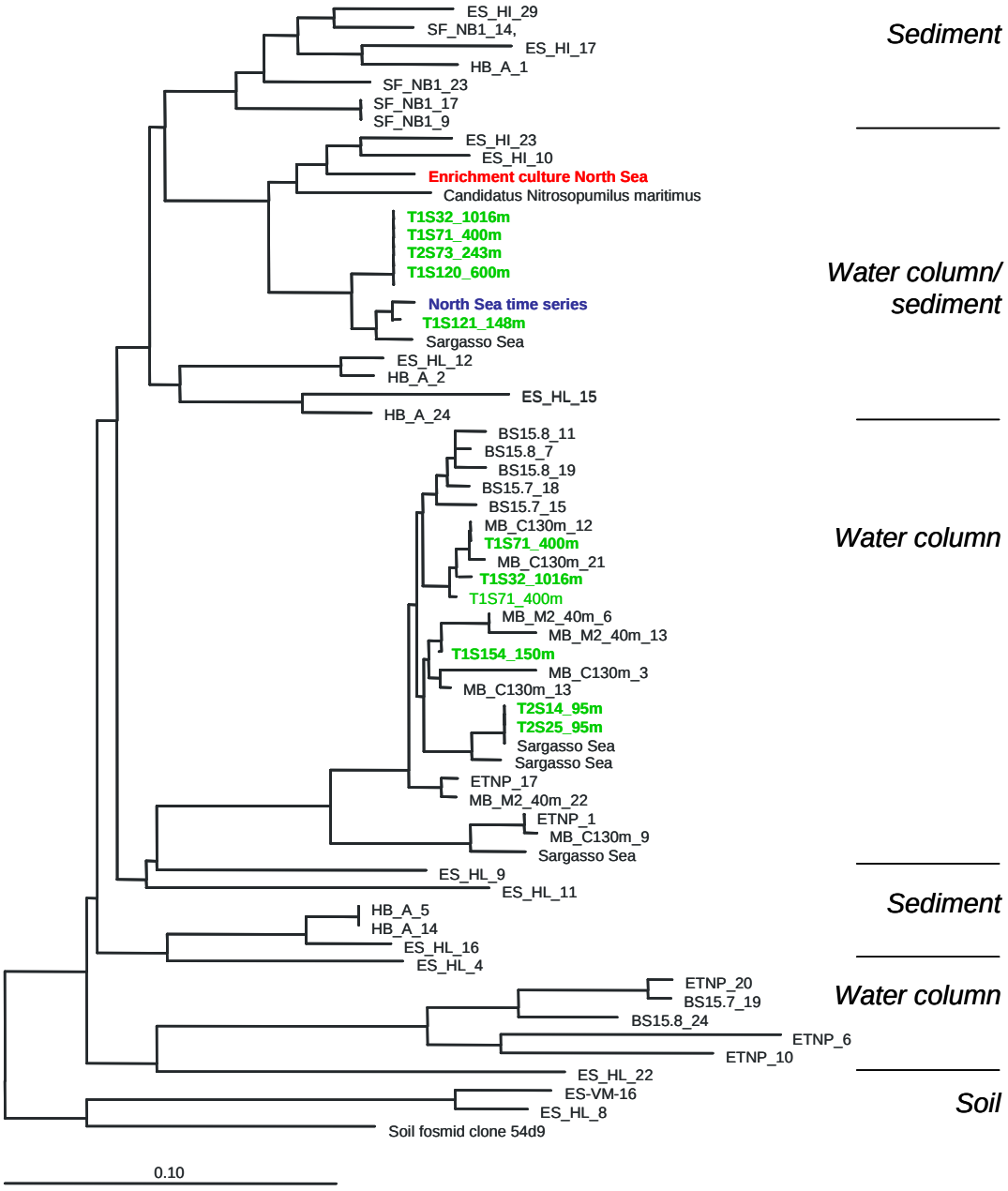
ES_HI_29
SF_NB1_14,
ES_HI_17
HB_A_1
SF_NB1_23
SF_NB1_17
SF_NB1_9
ES_HI_23
ES_HI_10
Enrichment culture North Sea
Candidatus Nitrosopumilus maritimus
T1S32_1016m
T1S71_400m
T2S73_243m
T1S120_600m
North Sea time series
T1S121_148m
Sargasso Sea
ES_HL_12
HB_A_2
ES_HL_15
HB_A_24
BS15.8_11
BS15.8_7
BS15.8_19
BS15.7_18
BS15.7_15
MB_C130m_12
T1S71_400m
MB_C130m_21
T1S32_1016m
T1S71_400m
MB_M2_40m_6
MB_M2_40m_13
T1S154_150m
MB_C130m_3
MB_C130m_13
T2S14_95m
T2S25_95m
Sargasso Sea
Sargasso Sea
ETNP_17
MB_M2_40m_22
ETNP_1
MB_C130m_9
Sargasso Sea
ES_HL_9
ES_HL_11
HB_A_5
HB_A_14
ES_HL_16
ES_HL_4
ETNP_20
BS15.7_19
BS15.8_24
ETNP_6
ETNP_10
ES_HL_22
ES-VM-16
ES_HL_8
Soil fosmid clone 54d9
0.10
Sediment
Water column/ sediment
Water column
Sediment
Water column
Soil

Figure 3

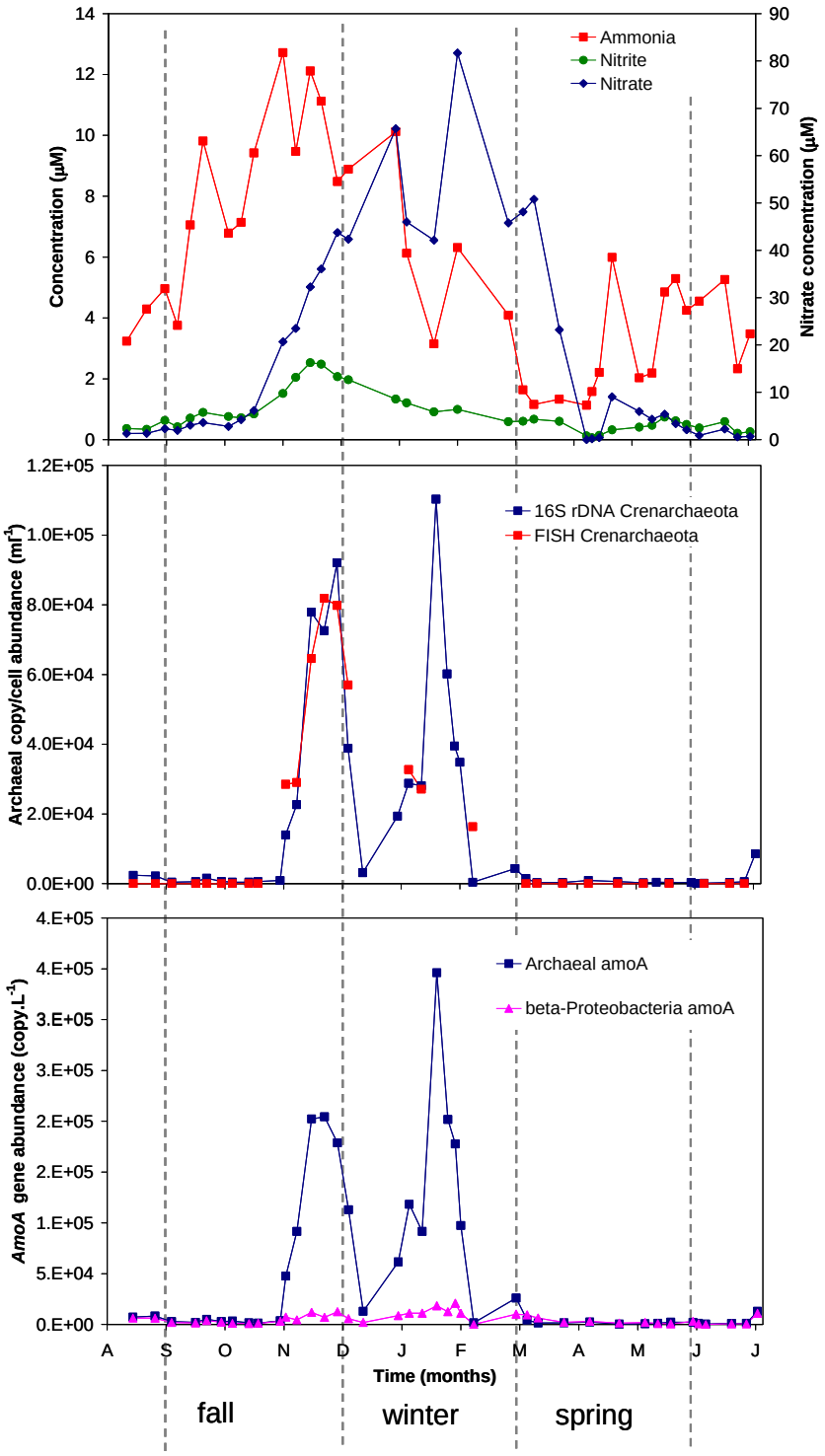
Concentration (µM)
Nitrate concentration (µM)
Ammonia
Nitrite
Nitrate
Archaeal copy/cell abundance (ml-1)
16S rDNA Crenarchaeota
FISH Crenarchaeota
AmoA gene abundance (copy.L-1)
Archaeal amoA
beta-Proteobacteria amoA
A S O N D J F M A M J J
Time (months)
fall
winter
spring

Figure 4

Supplementary information.

Table S1. Nutrient concentrations, DAPI counts and crenarchaeotal, bacterial, Betaproteobacterial and Gammaproteobacterial cell abundances in aged mesocosm water incubated in 20 l tanks at 22 and 25°C in the dark. Nutrients were directly added after 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
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

Table S2. Primers used for detection and quantification of archaea and bacterial ammonium oxidizers.

Target	**Gene**	**Primer pair**	**Fragment (bp)**	**T_{an} (°C) (Q)PCR**	**Ref.**
Archaea	16S rDNA	Parch 519f / ARC915r	396	63.0	37
Archaea	*amoA*	Arch-*amoA*-for / Arch-*amoA*-rev	256	58.5	This work
Betaproteobacterial ammonia oxidizers	*amoA*	*amoA*-1F / *amoA*r-new	490	61.5	38
Gammaproteobacterial ammonia oxidizers	*amoA*	A189-for / A682-rev	525	56.0	41

Primers developed during this work: Arch-*amoA*-for (5'-CTG AYT GGG CYT GGA CAT C-3'); Arch-*amoA*-rev (5'-TTC TTC TTT GTT GCC CAG TA-3'). To prevent complete melting of amplicons during DGGE, a 40-bp-long GC-clamp was attached to the 5'end of primers ARC915r, Arch-*amoA*-for, and *amoA*-2R-TC. Universal primers for Archaea were used to determine the almost complete 16S rDNA (*E. coli* positions 20 to 1406) of the North Sea marine crenarchaeotal enrichment culture.