Archaeal nitrification in the ocean

Cornelia Wuchter*, Ben Abbas*, Marco J. L. Coolen*[†], Lydie Herfort*, Judith van Bleijswijk*, Peer Timmers*, Marc Strous*, Eva Teira*[‡], Gerhard J. Herndl*, Jack J. Middelburg[¶], Stefan Schouten*, and Jaap S. Sinninghe Damsté*^{||}

*Departments of Marine Biogeochemistry and Toxicology and of Biological Oceanography, Royal Netherlands Institute for Sea Research (NIOZ), P.O. Box 59, 1790 AB, Den Burg, The Netherlands; [†]Department of Microbiology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands; and [‡]Centre for Estuarine and Marine Ecology, Netherlands Institute for Ecology (NIOO-KNAW), P.O. Box 140, 4400 AC, Yerseke, The Netherlands

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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 Crenarchaeota may play a role in the oceanic nitrogen cycle. We enriched a crenarchaeote from North Sea water and showed that its abundance, and not that of bacteria, correlates with ammonium oxidation to nitrite. A time series study in the North Sea revealed that the abundance of the gene encoding for the archaeal ammonia monooxygenase alpha subunit (*amoA*) is correlated with a decline in ammonium concentrations and with the abundance of Crenarchaeota. 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 1,000 m, where most of the ammonium regeneration and oxidation takes place, showed that crenarchaeotal *amoA* copy numbers are also 1–3 orders of magnitude higher than those of bacterial *amoA*. Our data thus suggest a major role for Archaea in oceanic nitrification.

Crenarchaeota | enrichment culture | North Sea | North Atlantic Ocean | nitrifying bacteria

Archaea constitute one of the three domains 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 nonextremophilic 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 comprise both Crenarchaeota and 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 $\approx 20\%$ of all prokaryotic cells in the global ocean (1). The metabolism of these planktonic Crenarchaeota is a subject of current debate. *In situ* labeling (8) and microautoradiography (7) experiments showed that marine Crenarchaeota can use 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 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 (*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 β - and γ -proteobacteria (16).

Here, we provide experimental evidence that Crenarchaeota present in seawater 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 ref. 17 for details). During this experiment, coastal North Sea water was incubated in the dark for 6 months at 25°C in an 850-liter 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 possibility, water from this mesocosm tank was incubated in the dark at 22°C and 25°C, and inorganic nutrients, including ammonium, were added while pH was kept constant at 8.2, the regular pH of surface seawater. The abundance of Crenarchaeota was monitored with catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH) (18) and revealed a substantial enrichment of crenarchaeotal cells at both temperatures (Fig. 1 *Lower*; see also Fig. 4 and Table 2, which are published as supporting information on the PNAS web site). After a lag phase, crenarchaeotal abundance increased with a doubling time of 2 days to $4\text{--}5 \times 10^6$ cells per ml in the incubation series and comprised at that time up to 40–70% of DAPI-stainable cells. Bacterial abundance increased during the first 3 days of incubation and then remained at <5% of DAPI-stainable cells during the whole incubation period (Fig. 1 *Lower*). Generally, 20–30% of the DAPI-stained cells are found to be composed of dead cell material (19), suggesting that our enrichment culture was dominated by Crenarchaeota at day 11 (Fig. 1 *Lower*). In fact, molecular analyses selective for Archaea revealed

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Abbreviations: AOB, ammonia-oxidizing bacteria; CARD-FISH, catalyzed reporter deposition–FISH; DGGE, denaturing gradient gel electrophoresis; QPCR, quantitative PCR.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ659389–DQ659410 (archaeal 16S rDNA) and DQ784527–DQ784538 (*amoA*)].

[†]Present address: Woods Hole Oceanographic Institution, Department of Chemistry and Geochemistry, 360 Woods Hole Road, Woods Hole, MA 02543.

[‡]Present address: Departamento Ecología e Biología Animal, Universidade de Vigo, Campus Lagoas-Marcosende, 36200 Vigo, Spain.

[¶]To whom correspondence should be addressed. E-mail: damste@nioz.nl.

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Fig. 2. Phylogenetic analyses of archaeal *amoA* recovered from the enrichment culture (red), the North Sea (blue), and the Atlantic Ocean (green). Neighbor-joining bootstrap tree of 594-bp long *amoA*-like nucleotide sequences published by Francis *et al.* (14) and Könneke *et al.* (10) to which we added our partial sequences (217 bp) using the ARB parsimony tool. The *amoA* gene recovered from the incubation experiment is closely related (91% nucleotide identity) to that of *Candidatus "N. maritimus"* (10) and of the Sargasso Sea environmental sequences (20) (up to 90% nucleotide identity). The *amoA* recovered from the North Sea time series is also closely related to that of *Candidatus "N. maritimus"* (91% nucleotide identity) and some Sargasso Sea environmental sequences (98% nucleotide identity).

chaetotal 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, because 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 $\approx 70 \mu\text{M}$ nitrate within ≈ 3 months (Fig. 3) and the crenarchaeotal abundance, an *in situ* archaeal nitrification rate of $\approx 7 \text{ fmol of NH}_3 \text{ cell}^{-1} \cdot \text{day}^{-1}$ 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, because sedimentary nitrification also might have contributed by up to 65% (24), and the presence of bacterial *amoA* suggests that bacterial nitrifiers could have contributed as well. 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 that 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 and dominate the prokaryotic community below the photic zone (1, 5). Nitrate depth profiles from the ocean typically show low concentrations in the upper ocean to levels varying from ≈ 20 to $40 \mu\text{M}$ in deeper waters of 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 inorganic nitrogen in the upper

Fig. 3. Crenarchaeotal abundance in the North Sea between August 2002 and July 2003 as a response to changing nutrient concentrations. (*Top*) Nutrient concentrations (micromolar). (*Middle*) Cell abundances (cells per milliliter) of Crenarchaeota as determined by CARD-FISH (18) and abundances of 16S rDNA copies of Crenarchaeota as determined by QPCR (see *Materials and Methods*). (*Bottom*) Abundances of archaeal and β -proteobacterial *amoA* copy numbers as determined by QPCR. 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 β -proteobacterial ammonia oxidizers was observed in this period ($\approx 1.0 \times 10^4$) compared with the rest of the year ($\approx 2.3 \times 10^3$).

ocean waters by primary producers, ammonium regeneration from decomposing descending particulate organic nitrogen, subsequent oxidation of ammonium to nitrite by members of the *Nitrosomonas/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), whereas marine Crenarchaeota do constitute ≈ 20 –30% of the total prokaryotic community (1).

Analysis of cell numbers of Crenarchaeota in the upper 1,000 m of the North Atlantic as determined by CARD-FISH (7) and the abundance of archaeal *amoA* (Table 1) revealed a ratio of 1.2 ± 1.3

copies per cell, which is in between the ratios 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 enrichment culture *amoA* sequence and that of *Candidatus* “*N. maritimus*” (Fig. 2). Quantification of bacterial *amoA* gene in the same set of water samples (Table 1) revealed that the *amoA* gene abundance of β -proteobacteria was lower by 1–3 orders of magnitude compared with archaeal *amoA*, whereas *amoA* gene abundance of γ -proteobacteria was below our 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 $\approx 3.3 \times 10^{14}$ mol·N·yr $^{-1}$, assuming Redfield stoichiometry (28). If all of the generated ammonium would be oxidized by Crenarchaeota fixing one carbon atom for every ≈ 10 nitrogen molecules oxidized (29), then one would expect an archaeal inorganic carbon fixation rate of $\approx 3.3 \times 10^{13}$ 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 $\approx 4.5 \times 10^{13}$ mol·C·yr $^{-1}$ may be taken up by Crenarchaeota (7), assuming that Euryarchaeota and Crenarchaeota are growing at equal rates. Archaeal nitrification thus may 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, together with the recently established importance of *Planctomycetes* in denitrification (30, 31) and unicellular cyanobacteria in dinitrogen fixation (32, 33), show the important role of hitherto unrecognized prokaryotes in the oceanic biogeochemical cycling of nitrogen.

Materials and Methods

Incubation Experiment Setup. Coastal North Sea water was kept in the dark at 25°C in an 850-liter mesocosm tank without addition of nutrients for 6 months (17). After these 6 months, batch cultures with aged mesocosm water were incubated in 20 liters of Clearboy tanks (Nalge Nunc, Rochester, NY) at 22° and 25°C in the dark. Nutrients were added before the incubation at concentrations of 150 μ M NaNO $_3$, 150 μ M NH $_4$ Cl, 25 μ M NaH $_2$ PO $_4$, and 2,666 μ M NaHCO $_3$ together with a sterile mix of 12.5 mg/liter yeast, 5 mg/liter 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-liter tanks were continuously stirred and left 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 biweekly from August 2002 to July 2003.

CARD-FISH Analyses. Fifteen-milliliter 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 0.2- μ m polycarbonate filters (25-mm filter diameter; Millipore, Billerica, MA) with 0.45- μ m cellulose nitrate filters (Millipore) as supporting filters and stored frozen at -20°C until further analysis. Total picoplankton were enumerated after DAPI staining (34), and 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), β - and γ -proteobacteria (23), respectively, and specific probes were applied for the marine Crenarchaeota Group I,

clearly differed from that in the Atlantic Ocean (data not shown).

DGGE fragments were excised from the gels and subsequently sequenced for phylogenetic comparison with reference sequences from GenBank (39) by using the ARB software package (40). Archaeal 16S rDNA and *amoA* sequences obtained in this study have been deposited in GenBank under accession nos. DQ659389–DQ659410 and DQ784527–DQ784538, respectively.

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