Chapter 4

Seasonal dynamics of marine Archaea in coastal North Sea waters determined by different molecular approaches

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Abstract.

The abundance and distribution of marine Archaea in coastal North Sea waters were followed by bi-weekly sampling over 1.5 years. Three independent quantification methods, catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH), quantitative PCR (Q-PCR) and glycerol dibiphytanyl glycerol tetraether (GDGT) lipid analyses were used to determine the abundance of marine Archaea and to compare the relative robustness of the different methods. The richness of the archaeal community was determined by denaturing gradient gel electrophoresis (DGGE) and sequencing. CARD-FISH and the Q-PCR method revealed similar abundance patterns and indicated that one archaeal cell contains on average 0.9 (±0.6) copy numbers of 16S rRNA genes. Crenarchaeotal abundance determined by CARD-FISH was closely related to GDGT concentrations, indicating that marine Crenarchaeota are indeed the source organisms for GDGT membrane lipids in marine oxygenated waters and that one marine crenarchaeotal cell contains on average 3 (± 0.5) pg GDGTs. CARD-FISH, DGGE and GDGT analyses revealed a distinct seasonal distribution pattern of pelagic marine Archaea in coastal North Sea waters. In the winter season, the marine Crenarchaeota Group I dominates the archaeal community whereas the marine Euryarchaeota Group II is more abundant during the summer and fall. This succession from Cren- to Euryarchaeota might point towards different metabolic requirements of these archaeal groups. At least some marine pelagic Crenarchaeota exhibit probably a chemoautotrophic mode of life while some marine pelagic Euryarchaeota are probably heterotrophic organisms.

4.1 Introduction

Until a decade ago, Archaea were thought to be restricted to anoxic, hypersaline, extremely warm and acidic environments. This view has changed dramatically, however, when phylogenetic analysis of environmental 16S rDNA sequences showed that Archaea are far more widespread than previously thought. Small-subunit rRNA genes of Archaea and their characteristic membrane lipids were retrieved from the pelagic realm of temperate and polar seas [*Fuhrman et al.,* 1992; *DeLong* 1992; *DeLong et al.,* 1994; *Hoefs et al.,* 1997; *DeLong et al.,* 1998; *Sinninghe Damsté et al.,* 2002a], marine sediments [*Vetriani et al.,* 1999; *Schouten et al.,* 2000], lake waters and sediments [*MacGregor et al.,* 1997; *Keough et al.,* 2003; *Powers et al.,* 2004], terrestrial soils [*Buckley et al.,* 1998; *Pesaro and Widmer* 2002; *Ochsenreiter et al.,* 2003] and peat bogs [Weijers et al., 2004].

Marine planktonic Archaea consist of two major groups, the Crenarchaeota and Euryarchaeota. Marine Crenarchaeota are typically more abundant than Euryarchaeota in deeper layers of neritic waters and in the meso- and bathypelagic zones of the ocean [*DeLong et al.,* 1999; *Massana et al.,* 2000; *Karner et al.,* 2001; *Church et al.,* 2003; *Herndl et al.,* 2005] and might account for about 20% of all prokaryotic cells in the global ocean [*Karner et al.,* 2001]. While the relative contribution of Crenarchaeota to total prokaryotic biomass increases with depth, the abundance of marine Euryarchaeota in the deep oceanic waters remains more invariant and relatively low throughout the water column compared to marine Crenarchaeota [*Karner et al.,* 2001; *Herndl et al.,* 2005]. In contrast, marine Euryarchaeota are relatively more abundant than Crenarchaeota in surface waters of open ocean and coastal systems [*Murray et al.,* 1999, *Massana et al.,* 2000, *Pernthaler* 2002].

Several molecular techniques exist to quantify the abundance of marine Archaea. Fluorescence in situ hybridization (FISH) has been developed to directly quantify individual components of complex prokaryotic communities [*DeLong et al.,* 1989, *Amann et al.,* 1990. The sensitivity of FISH was improved by the catalyzed reporter deposition (CARD-FISH) approach [*Pernthaler et al.,* 2002]. Recently, a CARD-FISH protocol with an improved cell permeabilization step for marine Archaea was introduced [*Teira et al.,* 2004]. The quantitative real-time PCR (Q-PCR) assay has also been used in marine sediments [*Coolen et al.,* 2002, *Inagaki et al.,* 2004] to assess the abundance and distribution of Archaea. Q-PCR offers a

rapid determination of the relative copy numbers of ribosomal genes from a specific group of microorganisms in mixed communities. The abundance of specific archaeal membrane lipids was determined in the Arabian Sea [*Sinninghe Damsté et al.,* 2002a]. Marine crenarchaeotal membrane lipids consist of glycerol dibiphytanyl glycerol tetraethers (GDGTs) of which one contains four cyclopentane rings and one cyclohexane ring, coined crenarchaeol [*Sinninghe Damsté et al.,* 2002b] [Fig. 1]. This crenarchaeol is exclusively attributed to Crenarchaeota, however, the concentration of crenarchaeol has not yet been directly related to the abundance of Crenarchaeota in any environment.

Although a number of tools exist to quantify the abundance of marine Archaea, they have not been used simultaneously to allow comparison of these methods. In this study, we determined the abundance and distribution of marine Cren- and Euryarchaeota in coastal North Sea surface waters over a seasonal cycle. We applied denaturing gradient gel electrophoresis (DGGE) to assess the diversity of the marine Archaea and used three independent methods, CARD-FISH, Q-PCR and GDGT analyses to determine the seasonal dynamics in the abundance of marine Archaea. From the distinct seasonal patterns in the abundance of Cren- and Euryarchaeota, we deduce that they might have different metabolic requirements and thereby occupy different ecological niches.

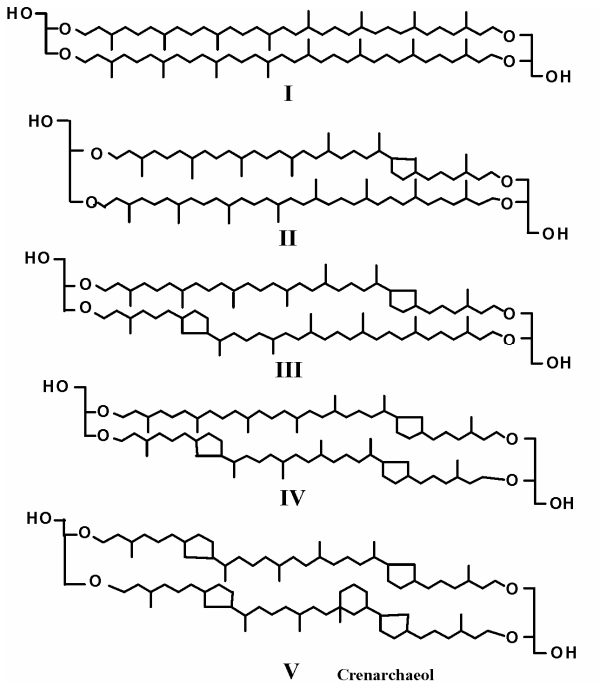

Figure 1. Structures of core tetraether membrane lipids of marine crenarchaeota.

4.2 Material and Methods

4.2.1 Study site and sampling. The sampling site is situated at the western entrance of the North Sea into the Wadden Sea at the southern tip of the Island Texel (53°00'25"N, 4°78'27"E). With each incoming tide, water from the coastal North Sea moves as far as 25 km into the Wadden Sea [*Postma,* 1954]. At high tide, water collected at the NIOZ jetty represents Dutch coastal waters since the estuarine influence is minimal. Strong tidal currents assure that the water is vertically mixed. Therefore, surface water samples taken during high tide are representative for the whole water column.

Water samples were taken for DNA and lipid analyses from February 2002 to July 2003 at bi-weekly intervals. For catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH), samples were taken bi-weekly from August 2002 to July 2003. For DNA analysis, 1 l of water was filtered through a 0.2 µm pore size polycarbonate filter (Schleicher & Schuell, 142 mm filter diameter) and stored at -80ºC until extraction. For CARD-FISH analyses, 50 ml water samples were fixed with formaldehyde (final concentration 4%) and stored at 4°C in the dark for at least 4 h. Thereafter, the samples were filtered onto 0.2 µm polycarbonate filters (Millipore, 25 mm filter diameter) with 0.45µm cellulose nitrate filters (Millipore) as supporting filters and stored frozen at -20ºC until further processing. For lipid analyses, 20 l of water was filtered sequentially through 3 µm and 0.7 µm combusted glass fibers (GF, Pall, 142 mm filter diameter), and finally through a 0.2 µm cellulose acetate (C/A) filter (Sartorius). The GF and C/A filters were stored at -20ºC until extraction.

4.2.2 Environmental parameters at the sampling site. Temperature, salinity, inorganic nutrients and Chlorophyll-*a* (Chl-*a*) are measured weekly at the sampling site in the frame of the long-term monitoring program of the NIOZ. Inorganic nutrient analyses are done on a TRAACS 800+ auto-analyzer and Chl-*a* following the standard protocol and spectrophotometric determination [*Parsons et al.,* 1984].

4.2.3 DNA analyses

4.2.3.1 Extraction of total DNA. Six ml of extraction buffer (10 mM Tris HCl, 25 mM EDTA, 1 vol% SDS, 100 mM NaCl and 0.1 ml zirconium beats) was added to each filter and total DNA extracted with phenol, phenol/chloroform/isoamyl-alcohol and chloroform, and precipitated with ice-cold ethanol according to *Sambrook et al.* [1989]. The DNA-pellet was

re-dissolved in ultra-pure DNA and Dnase-free sterile water (Sigma, St. Louis, MO, USA) and concentrated using the Microcon centrifugal filter system (Millipore). Salts and low molecular weight impurities such as humic acids were removed from the DNA extract during two centrifugal wash steps using 500 µl of ultra pure water. From each of the total DNA extracts, a subsample was subjected to agarose gel electrophoresis to determine the quality of the extracted DNA.

4.2.3.2 Amplification of archaeal 16S rDNA. Partial archaeal 16S rDNA genes were amplified by polymerase chain reaction (PCR). The PCR protocol and the archaeal primers were applied as described by *Coolen et al.,* [2004]. The primers Parch 519f (complementary reverse sequence of Parch 519r from *Øvreas et al.,* [1997] and GCArch 915r [*Stahl and Amann,* 1991], including a 40bp long GC clamp [*Muyzer et al.,* 1993] were used. The archaeal fragment length of the amplification products including the GC-clamp was 436 bp. The PCR reaction mix for each amplification reaction contained 20 µg of bovine serum albumin (BSA), 5 µl of 10x PCR buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$ (Amersham-Bio-Sciences)], 1 U of Taq polymerase (Amersham), each primer with a final concentration of 0.5 µM (Thermo-Electron, Ulm, Germany) and 250 µM of each dNTP (Amersham-Biosciences, Piscataway, NJ, USA). A final volume of 50 µl for each PCR reaction was prepared with DNase-free sterile water (Sigma). For most of the samples, the PCR reaction was initially inhibited by impurities in the DNA extracts, however, a 20-fold dilution of the DNA extracts eliminated this PCR-inhibition.

4.2.3.3 Denaturing gradient gel electrophoresis (DGGE). The amplified 16S rDNA products were separated by DGGE [*Muyzer et al.,* 1993]. The DGGE conditions were carried out according to *Coolen et al.,* [2004]. The archaeal PCR products were applied onto a 6% (wt/vol) polyacrylamide gel (acrylamide/*N*,*N*'-methylene bisacrylamide ratio of 37:1 [w/w]) in 1x TAE buffer (pH 8.3). The gel contained a linear gradient of denaturant, 30-60% (100% denaturant = 7 M urea plus 40% [v/v] formamide). Electrophoresis was performed at 200V and 60ºC for 5 h. The gel was stained with ethidium bromide and documented with a Fluor-S Multi Imager (BioRad). The DGGE fragments were excised and each fragment was eluted in sterile 10 mM Tris-HCl (pH 8.0) at 2ºC for 24 h. For the sequencing reaction, 1 µl of each eluted archaeal 16S rRNA gene was re-amplified using the PCR conditions as described above but with only 25 cycles and using archaeal primes without a GC clamp.

4.2.3.4 Sequencing of the DGGE fragments. The re-amplified DGGE fragments were purified using the QIAquick PCR Purification Spin Kit (Qiagen, Hilden, Germany) and the amount of DNA was quantified by PicoGreen. The cycle sequencing reaction contained 3 µl Big Dye terminator (BDT), 5x buffer (400mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$), 2 µl ABI Prism Big Dye Terminator V3.0 (Applied Biosystems, CA, USA), forward or reverse primer [final concentration 0.2 µM] (without GC clamp) and 10 ng of DNA. For the sequence reaction, the volume was adjusted to 20 µl with ultrapure water (Sigma) and the following reaction conditions were applied: 1 min initial denaturing at 96ºC, followed by 25 cycles of 10 s at 96ºC, 5 s at 50ºC and 4 min at 60ºC.

4.2.3.5 Phylogenetic analyses. For comparative analysis of the sequences, the ARB software was used [*Ludwig et al.,* 2004]. The sequences were aligned with complete length sequences of closest relatives obtained from the ribosomal database project II [*Maidak et al.,* 2001] and GenBank [*Benson et al.,* 2000] using the ARB FastAlinger utility. Matrices of similarity, distance and phylogenetically corrected distance values were generated with the maximum parsimony option in ARB. Sequences obtained in this study were submitted to the GenBank sequence database.

4.2.3.6 Real time quantitative PCR (Q-PCR). Real time PCR was performed in an iCycler system (BioRad) using a method modified from *Coolen et al.,* [2002]. To quantify the 16S rDNA copy numbers, the PCR conditions and primers (without GC-clamp) were used as described above. Accumulation of newly amplified rDNA was followed online as the increase in fluorescence due to the binding of the fluorescent dye SYBRGreen (Molecular Probes). Reaction mixtures (20 µl) contained 1 U of Picomaxx^{TM} High Fidelity DNA polymerase, 2 µl of 10x Picomaxx PCR buffer (both Stratagene), 200 µM of each dNTP, 20 µg of BSA, 0.2 µM of primers, 50,000 times diluted SYBRGreen, 3 mM of $MgCl_2$ and ultra-pure sterile water (Sigma). A melting curve analysis, performed between 60ºC and 96ºC at the end of the PCR reactions revealed that the melting temperature of the amplification products ranged between 82ºC and 91ºC. Dimer formation of the primer was very low and eventually occurred at 78ºC. In order to prevent biases in quantification of amplification products due to dimer formation, the fluorescent signal was read in each cycle during an additional step holding the temperature at 80ºC for 25 s. Hence, all PCR products were double stranded during detection, whereas

primer dimers were melted. Known amounts of template DNA from each sample were added to each Q- PCR reaction.

Calibration of the samples was done using *Sulfolobus* sp. DNA of *Sulfolobus* sp. served as template to generate 396-bp PCR fragments using the primers Parch519f and Arch915r. Primers and salts were removed from the PCR product using QIAquick Spin Columns (Qiagen) and the exact DNA concentrations were determined fluorometrically (PicoGreen, Molecular Probes) in order to calculate the number of 16S rDNA copies per µl PCR-purified PCR product. For the calibration of the samples, known 16S rDNA copy numbers of the latter PCR products (ranging between $5.3x10^0$ and $5.3x10^8$ copies) were subjected to Q- PCR to quantify Archaea. Control reactions were performed and included a reaction without DNA as a control for potential contamination during sample processing. A second reaction contained 4,000 16S rDNA copies of *E.coli* as a control for the specificity of the reactions.

4.2.4 Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) Heterotrophic picoplankton were stained with DAPI [*Porter and Feig,* 1980] and marine Archaea using the CARD-FISH approach [*Pernthaler et al.,* 2002]. According to the improved CARD-FISH protocol for marine Archaea [*Teira et al.,* 2004], the cell wall permeabilization was done with proteinase-K at 37ºC for 1 h. Hybridization was done at 35ºC for 12-15 h. Specific probes for marine Crenarchaeota Group I, Cren537 (5'-TGACCACTTGAGGTGCTG-3') and marine Euryarchaeota Group II, Eury806 (5'-CACAGCGTTTACACCTAG-3') were applied [*Teira et al.,* 2004]. Enumeration of the Bacteria and Archaea was performed with an epifluorescence microscope (Zeiss Axioplan 2) at 1,250x magnification. Twenty fields (50x50 µm) per filter were counted to determine picoplankton abundance. For archaeal abundance, 20 fields of a 100x100 µm square were counted. At least 200 cren- and euryarchaeotal cells were counted per filter.

4.2.5 Lipid extraction and analysis. The GF and C/A filters were freeze-dried and the filters were ultrasonically extracted. For GF filters, methanol, dichloromethane (DCM)/methanol (1:1, v/v) were used followed by three extractions with DCM. The C/A filters were extracted three times with methanol and hexane. The total lipid extracts were combined and an aliquot was cleaned over an activated Al_2O_3 column by eluting with methanol/DCM (1:1, vol/vol).

For analysis of intact glycerol dibiphytanyl glycerol tetraethers (GDGTs), the solvent was removed from the eluent under a stream of nitrogen and the residue was dissolved by sonication (5 min) in hexane/propanol (99:1). The resulting suspension was filtered through a 0.45 µm pore-size, 4 mm diameter Teflon filter prior to injection. The intact GDGTs were analyzed by high performance liquid chromatography – atmospheric pressure chemical ionization – mass spectrometry (HPLC – APCI/MS) using conditions modified from *Hopmans et al.* [2000]. Analyses were performed using an HP (Palo-Alto, CA, USA) 1100 series LC-MS equipped with an auto-injector and Chemstation chromatography manager software. Separation was achieved on a Prevail Cyano column (2.1 x 150 mm, 3 µm; Alltech, Deerfield, IL, USA), maintained at 30°C. Injection volumes varied from 1 µl to 5 µl. GDGTs were eluted isocratically with 99% A and 1% B for 5 min, followed by a linear gradient to 1.8% B in 45 min, where A = hexane and B = propanol. Flow rate was 0.2 ml/min. After each analysis, the column was cleaned by back-flushing hexane/propanol (90:10, v/v) at 0.2 ml/min for 10 min. Detection was achieved using APCI/MS of the eluent. Conditions for APCI/MS were as follows: nebulizer pressure 60 psi, vaporizer temperature 400°C, drying gas (N_2) flow 6 l/min and 200°C, capillary voltage -3 kV, corona 5 µA (~ 3.2 kV). GDGTs were detected by single ion monitoring of their $[M+H]^+$ ions and quantified by integration of the peak areas and comparison with a standard curve of a GDGT-0 standard.

4.2.6 Statistical analyses. The fragment patterns obtained by DGGE were converted into an absence/presence matrix compiling all the sampling dates. From this binary matrix a similarity matrix was calculated using the Jaccard's dichotomy coefficient. A hierarchical clustering procedure was performed on this similarity matrix.

Correlation analysis using physical and chemical parameters such as temperature and inorganic nutrient concentrations, Chl-*a*, picoplankton abundance, cren-and euryarchaeotal abundance was performed by converting the data to rank numbers. From these ranked numbers, a correlation matrix was made using Pearson's algorithm. To determine the significance of the obtained correlations, the Bonferroni probability test was used. All statistical analyses were done with SYSTAT 10.

4.3 Results

4.3.1 Seasonal dynamics in environmental parameters.

Inorganic nutrient concentrations exhibited the typical seasonal dynamics with high concentrations during the fall and winter and decreasing concentrations towards the summer. Nitrate concentrations were highest in February with about 80 µmol l^{-1} and decreased thereafter until June and ranging between 2-5 µmol l^{-1} until October (Fig. 2a). Highest ammonium concentrations were measured in late spring and fall (Fig. 2a). Nitrite concentrations were generally low over the seasonal cycle. Only in late November, a minor peak in nitrite was detectable coinciding with the decline in ammonium and the increase in nitrate, thus indicating a period of elevated nitrification (Fig. 2a). As indicated by the high Chl-*a* concentrations, a smaller spring phytoplankton bloom (March 02) was followed by a massive bloom in June reaching 35 mg Chl-*a* m^{-3} (Fig. 2b). During the summer and fall, Chl-*a* concentrations were about 5 mg m^{-3} declining to about 2 mg m^{-3} in the winter (Fig. 2b). In 2003, the main phytoplankton bloom occurred from March to May and reached slightly lower peak Chl-*a* concentrations but lasted somewhat longer than in the previous year.

The water temperature varied from 0-22ºC. Highest temperatures were measured during August 2002 and by mid December 2002, the temperature dropped to 0ºC and remained below 6ºC until mid March 2003 and then increased again (Fig. 2b).

4.3.2 Seasonal dynamics in cren- and euryarchaeotal richness as revealed by DGGE.

The DGGE fingerprint pattern indicated a seasonal succession in archaeal phylotypes (Fig. 3). All of the recovered and sequenced DGGE fragments from February 2002 belonged to the marine Crenarchaeota Group I except one closely related to a soil Euryarchaeote (Fragment 8) (Figs. 3a and 4). The archaeal 16S rDNA concentration was below the detection limit from April until June 2002. From July until the end of October 2002, most of the recovered DGGE fragments belonged to the marine Group II Euryarchaeota (Figs. 3a and 4).
In October, the archaeal community structure changed within two weeks from a dominance of Eury- to Crenarchaeota. In November 2002, nearly all of the recovered and sequenced DGGE fragments belonged to the Group I marine Crenarchaeota (Figs. 3 and 4). The marine Crenarchaeota were present until mid March 2003. Similar to the spring of 2002,

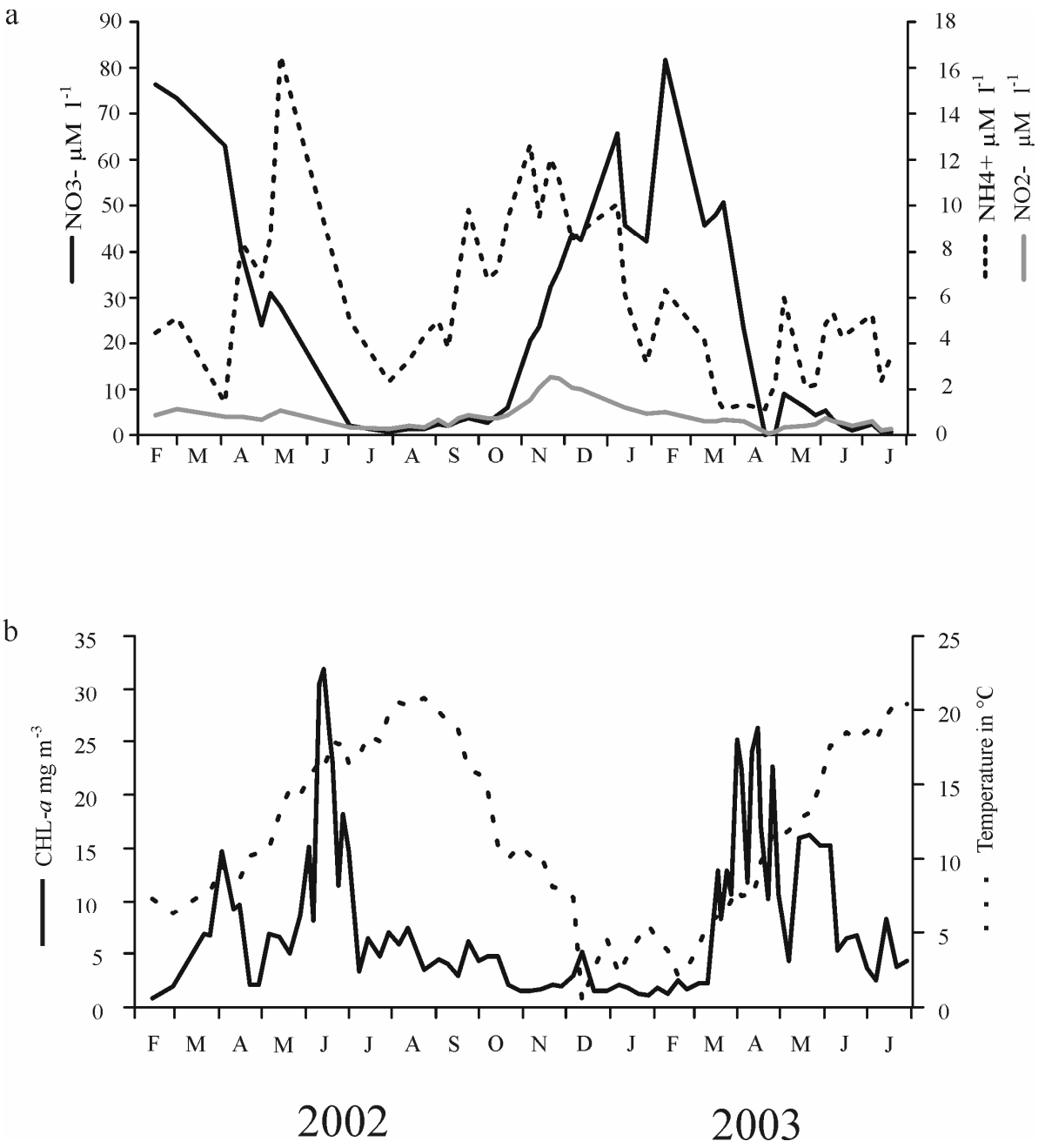

Figure 2. Nutrient concentrations (a) and, Chlorophyll-*a* and temperature development (b) at the investigated sampling site during February 2002 till July 2003.

also in spring of 2003 the archaeal 16S rDNA concentration was below the detection limit and from mid June 2003 onwards, marine Euryarchaeota became again the most dominant Archaea in coastal North Sea waters (Figs. 3 and 4).

The similarity tree revealed a clear pattern with two major branches (Fig. 3b). The archaeal community from winter and early spring 2002 and 2003 formed one cluster (A)

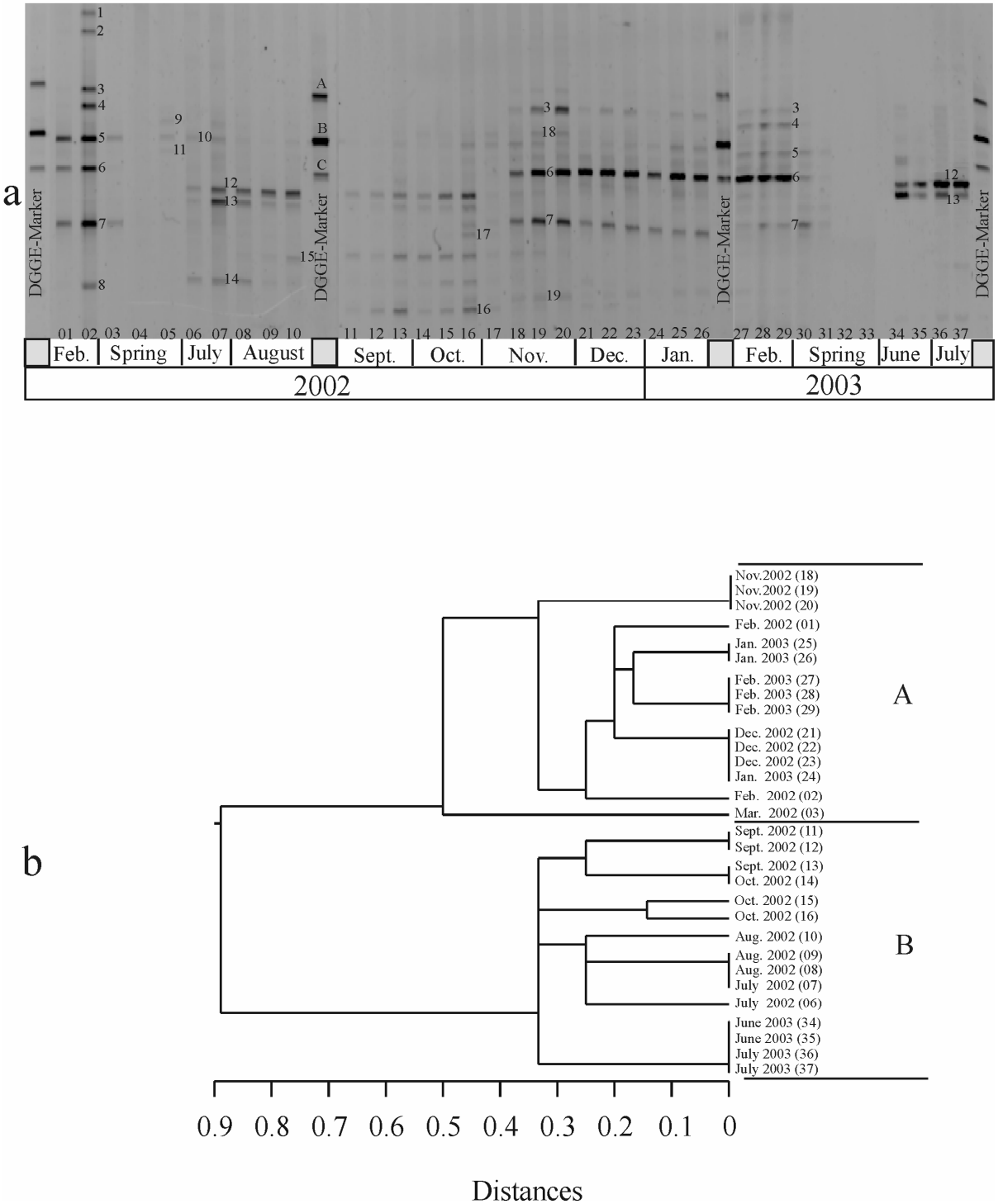

Figure 3. (a) DGGE analyses of 16S rRNA gene fragments of Archaea obtained from shallow North Sea surface waters over different seasons. Nineteen dominant partial 16S rDNA gene fragments of marine Archaea were detected. The DGGE marker is a PCR product from previous mesocosm experiments and used as a ruler to monitor the melting position of the archaeal amplicons within the gel.(b) Similarity tree of the phylogenetic composition of the archaeal community collected over the annual cycle. Custer A corresponds to the predominance of marine Crenarchaeota and cluster B corresponds to the predominance of marine Euryarchaeota

corresponding to the dominance of marine Crenarchaeota (Fig. 3b). The other cluster (B) consisted of samples collected in early summer 2002/03 and fall 2002 corresponding to the dominance of marine Euryarchaeota (Fig. 3b).

The recovered crenarchaeotal sequences exhibited between 96 and 100% sequence similarity to other marine Crenarchaeota (Fig. 4) and, according to the classification of *Massana et al*. [2000], they all cluster within the group 1α of the marine Crenarchaeota Group I. The recovered sequences obtained during summer were closest affiliated to environmental clones of the Euryarchaeota marine Group II (IIα and IIβ) [*Massana et al.,* 2000]. The sequence similarity among the recovered marine Euryarchaeota was lower than for Cenarchaeota (between 85 and 97%) (Fig. 4). In addition, three sequences related to soil Euryarchaeota were detected during winter (Fig. 4).

4.3.3 Seasonal dynamics in cren- and euryarchaeotal abundance as revealed by CARD-FISH. The seasonal shift from Crenarchaeota dominating in winter to Euryarchaeota during summer as revealed by DGGE was also observed with CARD-FISH. From late summer to fall 2002, Euryarchaeota reached an abundance of up to 6 x 10^4 cells ml^{-1} comprising about 3% of DAPI-stained cells (Fig. 5a). Crenarchaeota were detectable during the first week in November 2002 (Fig. 5a) and by the end of November about 8 x 10^4 crenarchaeotal cells ml^{-1} were detected (6 % of DAPI-stained cells). Most of the Crenarchaeota were attached to non-living suspended particles. During late November, euryarchaeotal abundance decreased to about 2 x 10^4 cell ml^{-1} and remained between 2-3 x 10^4 cell ml^{-1} until mid March 2003. During the spring phytoplankton bloom (March-May) archaeal abundance remained below the detection limit. In early June 2003, Euryarchaeota increased sharply again reaching an abundance of 10 x 10^4 euryarchaeotal cells ml^{-1} (3 % of DAPI-stained cells) (Fig. 5a).

4.3.4 Seasonal dynamics in relative abundance of marine Archaea as revealed by Q-PCR. In February 2002, the copy numbers of archaeal 16S rDNA were 5 x 10^4 copies ml^{-1} (Fig. 5b). By the end March to early April 2002, archaeal copy numbers decreased and remained below about 5 x 10^3 copies ml^{-1} from April to mid May. The archaeal copy numbers ml^{-1} remained low between June and August with 1-2 x 10^4 copies ml^{-1} and increasing to 3 x 10^4 copy numbers ml^{-1} in September and October 2002. In early November, a substantial

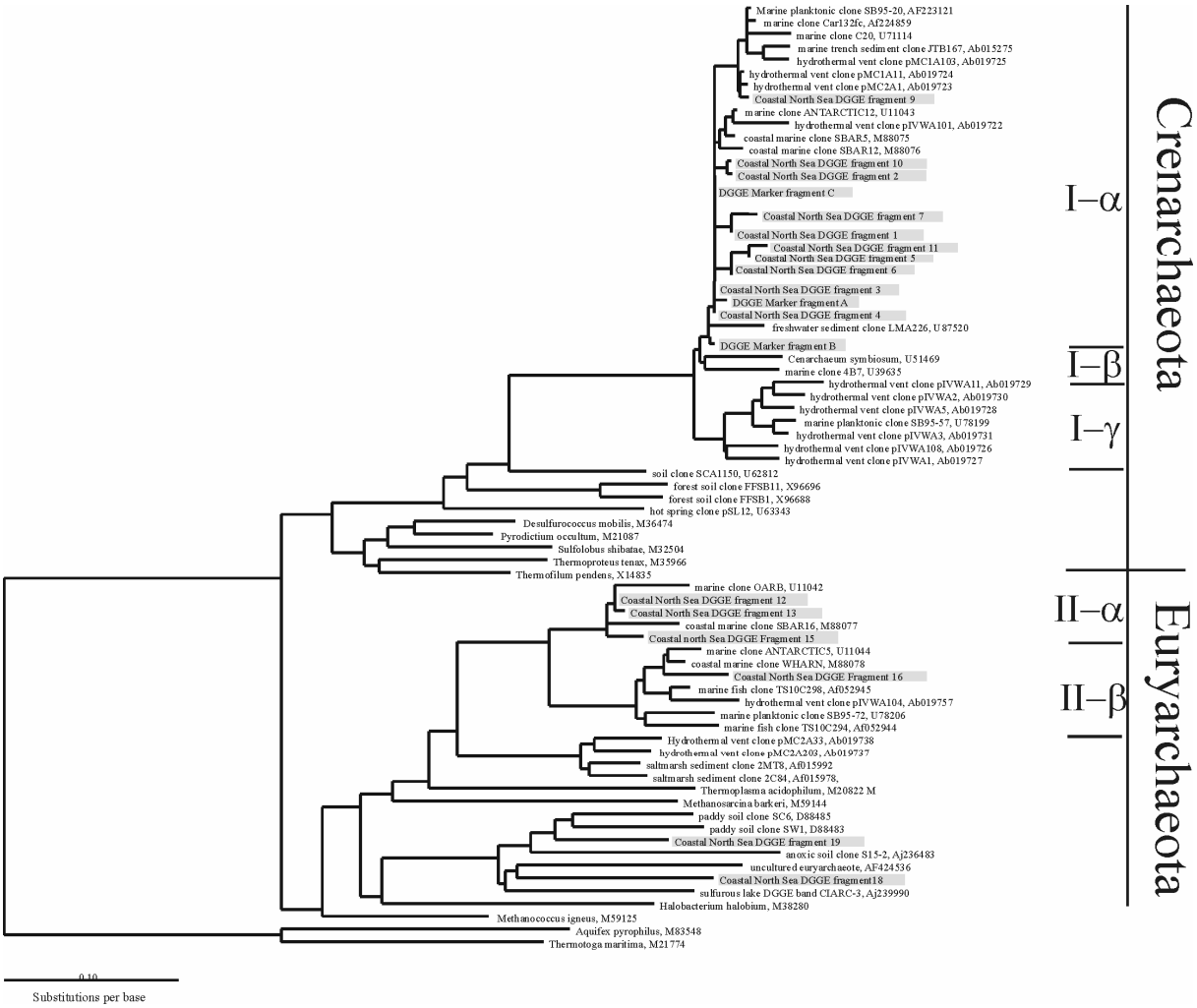

Figure 4. Phylogenetic tree showing the affiliation of the 19 partial archaeal 16S rDNA gene sequences recovered from the shallow coastal North Sea surface waters during 2002/2003 to reference sequences obtained from the GenBank database. All sequences are affiliated to the marine Crenarchaeota and Euryarchaeota.

increase in the archaeal 16S rDNA was detected reaching 9 x 10^4 copy numbers ml^{-1} at the end of November 2002 (Fig. 5b). The archaeal copy numbers ml^{-1} remained high throughout the winter season and decreased in mid March 2003 as in the previous year (Fig. 5b). In late March 2003, the numbers of archaeal 16S rDNA copies were below the detection limit and remained very low with 2-4 x 10^3 copies ml^{-1} until the end May 2003. During mid June 2003, the number of archaeal 16S rDNA copies increased again and reached up to 7 x 10^4 copies ml^{-1} (Fig. 5b).

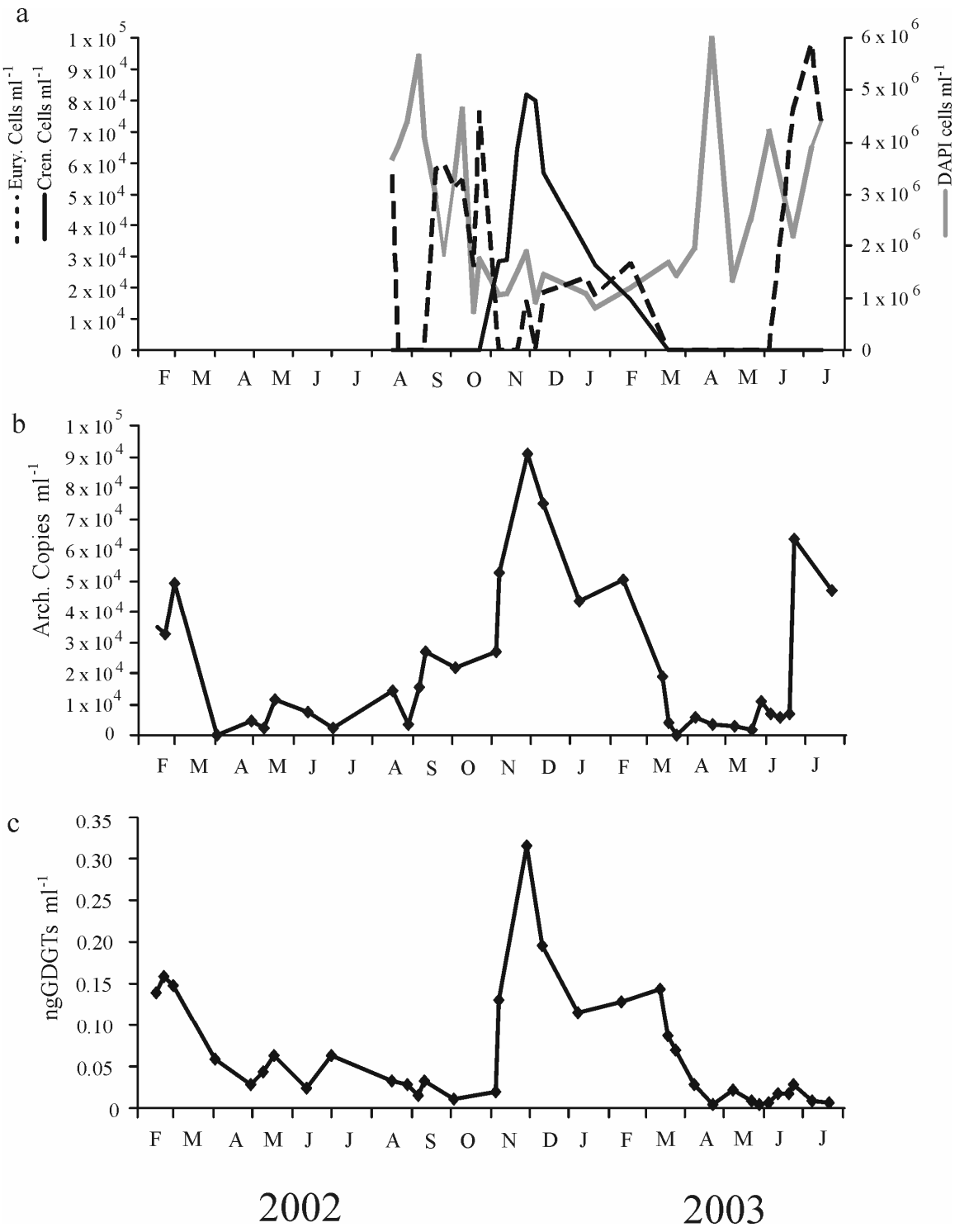

Figure 5. Seasonal dynamics in archaeal abundance (a), archaeal copy numbers (b), and archaeal tetraether lipid concentration (c) at the sampling site from August 2002 to July 2003 (a) and from February 2002 to July 2003 (b, c).

4.3.5 Seasonal dynamics in abundances of marine archaeal membrane lipids.

In February 2002, the concentration of GDGTs was 0.15 ng ml^{-1} (Fig. 5c). During March and April 2002, a substantial decrease in the GDGT concentration was apparent and the GDGT concentration remained low during the summer and early fall 2002. A sharp increase of the GDGT concentration was observed at the beginning of November 2002 reaching its maximum of 0.31 ng ml^{-1} at the end of November 2002 (Fig. 5c). The GDGT concentration remained high during the winter season 2002/03 and decreased during early spring 2003 and remained at relatively low levels throughout the summer 2003.

4.4 Discussion

Our combined 16S rDNA and lipid approaches enabled us to monitor the seasonal dynamics in abundance and the succession of the two major marine archaeal groups. This allows us to compare the potential of the individual approaches to detect marine Archaea. Furthermore, the distinct patterns in occurrence and abundance of Eury- vs. Crenarchaeota allow insights into the ecological role and physiological capabilities of these two archaeal groups in temperate coastal marine environments.

4.4.1 Comparison of methods for the identification and quantification of marine Archaea.

4.4.1.1 DGGE and CARD-FISH. The DGGE fingerprint technique and the CARD-FISH approach resulted in a similar pattern of archaeal dynamics in the North Sea waters. During the winter season, the marine Crenarchaeota Group I were the dominant Archaea with euryarchaeotal sequences affiliated to clones recovered previously from soil being present in presumably low numbers. During summer and fall, marine Euryarchaeota Group II dominated (Figs. 3, 4, 5a). These results demonstrate the specificity of the primer combination used for the archaeal PCR/DGGE and of the probes used for CARD-FISH.

***4.4.1.2 Q-PCR versus CARD-FISH*.** The total archaeal cell counts obtained by CARD-FISH and the copy numbers of archaeal 16S rDNA by the Q-PCR approach showed similar seasonal dynamics in archaeal abundance (Fig. 5a, b). During fall and winter 2002, archaeal cells and

copy numbers were readily detectable and decreased during early spring 2003. Both, the archaeal cells and archaeal 16S rDNA copy numbers increased again in early summer 2003 (Fig. 5a, b). The similarity in the seasonal archaeal dynamics revealed by the CARD-FISH and Q-PCR approach indicates that the archaeal 16S rDNA was selectively amplified during Q-PCR.

Based on the number of detected total archaeal cells and the number of quantified archaeal 16S rDNA copies, we calculated that marine Archaea in the North Sea waters have, on average, 0.9 ± 0.6 copies of the 16S rRNA gene per genome. This is in good agreement with earlier studies showing that the number of ribosomal genes in hyperthermophilic Crenarchaeota as well as in methanogens is fairly stable and varies between one and two copies of the rRNA genes per genome and thus per cell [*Fogel et al.,*1999] . Nevertheless, the total archaeal cell counts as determined by CARD-FISH were generally slightly higher than the archaeal 16S rDNA copy numbers as determined with Q-PCR. The determination of the copy numbers of genes by Q-PCR depends on the efficiency of the DNA extraction and the efficiency of the PCR reaction. The DNA extraction efficiency can be tested by counting the remaining intact cells after DAPI staining in the supernatant following the lysis step for DNA extraction. No DAPI-stained cells were detected indicating that the DNA extraction efficiency was high. However, specific substances in the DNA extracts can inhibit the PCR reaction and lower the PCR efficiency. Based on our standard curve, the Q-PCR efficiency was 89 % which might explain the lower copy number than cell counts. Thus, we conclude that the offset between the archaeal cell counts and the archaeal 16S rDNA copy numbers is mainly caused by the less than 100% PCR efficiency.

4.4.1.3 GDGTs versus CARD-FISH. The total GDGT concentration correlated well with the number of Crenarchaeota in the surface waters during winter months determined by CARD-FISH (Fig. 6a), while the abundance of Euryarchaeota was not correlated with the amount of GDGTs (Fig. 6b). Thus, marine pelagic Euryarchaeota most likely do not biosynthesize GDGTs. Based on the relation between GDGT concentration and the number of Crenarchaeota, the average content of GDGTs per crenarchaeotal cell is about 3 ±0.5 fg GDGTs. *Sinninghe Damsté et al.* [2002a] estimated that one crenarchaeotal cell contains on average 1 fg GDGTs assuming that the cell dimensions of marine Crenarchaeota are identical

to those of *Crenarcheum symbiosum.* Our GDGT concentrations per crenarchaeotal cell are slightly higher but well within the range of the previous estimates. At the sampling site, the crenarchaeotal cells were of similar size as reported for *C. symbiosum*, therefore this offset is probably caused by a mixed GDGT signal derived from living and non-living cells. While the archaeal copy numbers and the archaeal cell counts declined substantially in March 2003, the GDGT concentration was still high and decreased to background values only by beginning of April 2003 (Fig. 5c). Since nucleic acids are likely more labile than GDGTs, it is likely that the GDGTs detected in March 2003 were predominantly derived from dead and decaying archaeal cells with their nucleic acids already degraded.

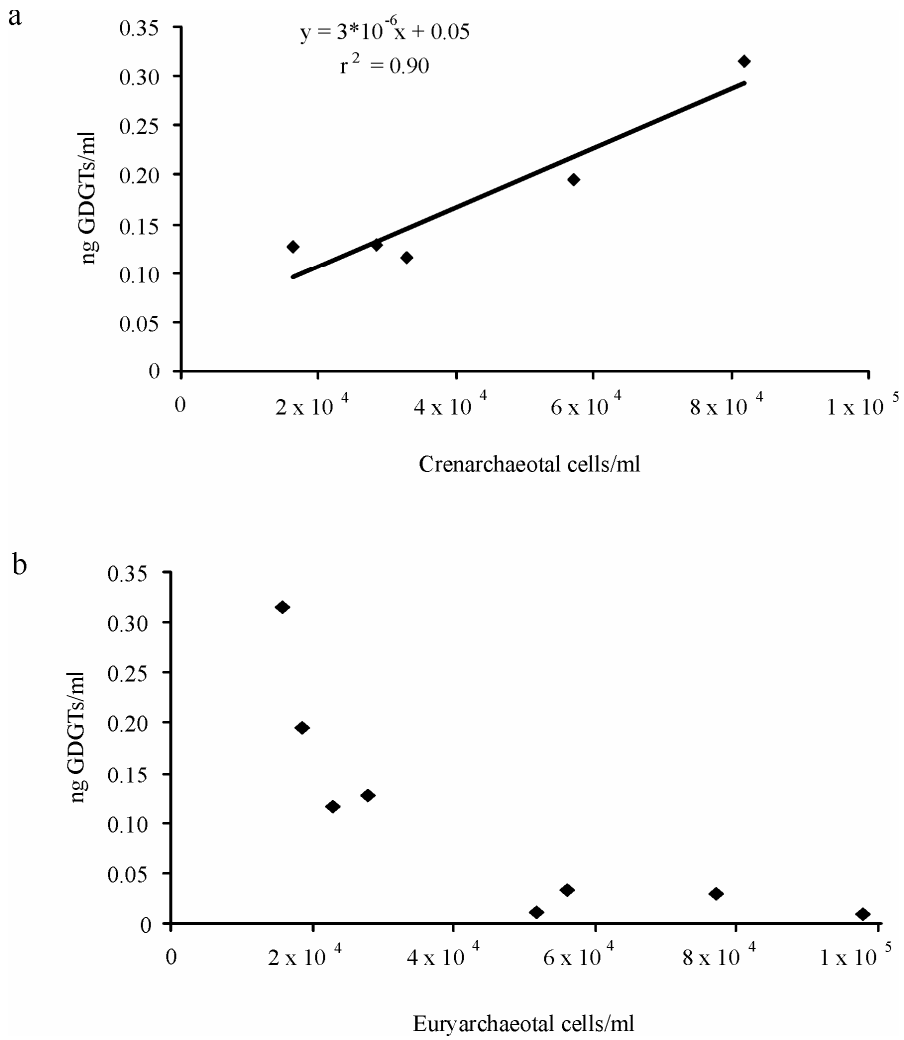

Figure 6. Relation between the (a) amount of GDGTs and marine crenarchaeotal cell numbers between Nov. 2002 to Feb. 2003, and (b) amount of GDGTs versus marine euryarchaeotal abundance between Aug. and Oct. 2002 and Jun. to- Jul. 2003.

4.4.2 Seasonality of marine Archaea in coastal North Sea surface waters.

In the coastal North Sea waters, marine Crenarchaeota dominate the archaeal population during winter and early spring while marine Euryarchaeota are the most dominant archaeal group in summer and early fall (Figs. 3, 4, 5a). All recovered crenarchaeotal sequences were closely related to each other with sequence similarities > 96% and all affiliated to the I-α group of the marine Crenarchaeota. The recovered euryarchaeotal sequences were more diverse with a sequence homology varying between 85-97% and grouped in the II-α and II-β marine Euryarchaeota. This is consistent with the previously described sequence homologies for the I-α marine Crenarchaeota and II-α and II-β marine Euryarchaeota cluster which are mainly found in the mixed layer of surface waters [*Massana et al.,* 2000]. The distinct successional occurrence of the two pelagic marine archaeal groups suggests different metabolic requirements and distinct ecological niches of those two groups.

Marine Crenarchaeota dominated in the winter to early spring when inorganic nutrient concentrations were high and temperature, Chl-*a* and overall prokaryotic abundance low (Fig. 2a, 5a). During the spring phytoplankton bloom, both archaeal groups decreased in abundance below the detection limit of Q-PCR and CARD-FISH (Figs. 3, 4, 5a). The marine Euryarchaeota increased again when Chl-*a* concentrations and total prokaryotic abundance decreased again after the phytoplankton bloom towards the summer (Figs. 3, 4, 5a). The abundance of marine Crenarchaeota correlates significant with inorganic nutrient concentrations especially with nitrite (r=0.82), and correlates inversely with Chl-*a* and temperature (Table 1). Marine Euryarchaeota, however, do not exhibit these significant correlations as the marine Crenarchaeota (Table 1).

	EURY.	CREN.
DAPI	0.13	-0.60*
NH_4^+	0.17	0.58*
NO_2^-	-0.07	0.82***
NO_3^-	-0.32	0.62*
CHL-*a*	-0.08	-0.64**
TEMP.	0.21	-0.63**

Table 1. Correlation of nutrients, chlorophyll-*a*, temperature and DAPI-stained cells with the abundance of marine Crenarchaeota and Euryarchaeota. Numbers in table represent r values. $p > 0.1$ not significant, $^{*}p = 0.1\text{-}0.01$, $^{**}p = 0.01\text{-}0.001$, $^{***}p < 0.001$. Number of cases n=28.

Pronounced seasonal dynamics of marine pelagic Archaea in surface waters were also reported previously. A negative correlation between Chl-*a* concentrations and archaeal rRNA concentration was reported by *Murray et al.,* [1998,1999] and with archaeal cell counts [*Wells and Deming* 2003]. Also, an increase in archaeal abundance, especially of marine Crenarchaeota Group I, was found coinciding with a decrease in picoplankton abundance [*Murray et al.,* 1998;1999; *Massana et al.,* 2000]. A positive correlation between crenarchaeotal abundance and suspended particle concentration was reported previously for the nepheloid layers of Artic waters [*Wells and Deming* 2003], confirming our notion that marine Crenarchaeota are frequently associated to particles in North Sea coastal waters. Similar to our study, a positive correlation between the abundance of Crenarchaeota and NO_2^- concentrations was found in the Santa Barbara Channel [*Murray et al.,* 1999], the Arabian Sea [*Sinninghe Damsté et al.,* 2002a] and with particulate organic nitrogen in Arctic waters [*Wells and Deming* 2003]. These authors suggest that marine Crenarchaeota may be involved in the marine nitrogen cycle, possibly as denitrifiers [*Sinninghe Damsté et al.,* 2002a] or as nitrifiers [*Wells and Deming* 2003].

Mesocosm experiments using stable isotopes [*Wuchter et al.,* 2003] and microautoradiography on North Atlantic deep waters [*Herndl et al.,* 2005] showed that, at least some marine Crenarchaeota and Euryarchaeota are using bicarbonate or CO_2 as their carbon source and are thus chemoautotroph. However, other microautoradiography experiments showed that some marine Archaea can take up amino acids [*Overney and Fuhrman,* 2000; *Teira et al.,* 2004]. Recent studies on mesocosm experiments [*Wuchter et al.,* 2004] and crenarchaeotal enrichment cultures (enrichment > 70%) [*Wuchter et al.,* submitted] show a strong negative correlation between the occurrence of marine Crenarchaeota and NH_4^+ concentrations and a positive correlation with NO_2^-, hence suggesting that some marine Crenarchaeota oxidize ammonium. This would agree with previous field observations [*Murray et al.,*1999] and the identification of an ammonium monooxygenase encoding gene, presumably of archaeal origin via genome shotgun cloning [*Venter et al.,* 2004]. In the present study, NH_4^+ concentrations started to decrease and NO_2^- concentrations increased when crenarchaeotal abundance increased (Fig. 2a, 5a). Recently, *Könneke et al.,* [2005] presented also evidence from an archaeon, isolated from a tropical aquarium, that marine Crenarchaeota are capable to oxidize ammonium.

Thus, we conclude that at least some marine Crenarchaeota appear to be chemolithoautotrophic and become abundant in winter when inorganic nutrient concentrations are high and competition for nutrients, especially with phytoplankton is low.

Marine pelagic Euryarchaeota are more abundant during the summer and early fall when inorganic nutrient concentrations are relatively low (Fig. 2a) suggesting that they might use other electron donors to meet their energy requirements, or alternatively, have other substrate affinity than Crenarchaeota.

4.5 Summary and Conclusions

The 16S rDNA approach to detect Archaea in coastal marine waters showed that the combined qualitative and quantitative DGGE, CARD-FISH, Q-PCR and archaeal lipid methods used in this study are highly specific for the identification and/or the quantification of marine Crenarchaeota and Euryarchaeota. In this study, we demonstrated that one archaeal copy number equals on average one archaeal cell and that the Q-PCR approach is a rapid and efficient tool to monitor relative, but not absolute archaeal cell abundance in environmental samples. The archaeal lipid approach indicated that GDGT lipids in oxygenated waters are exclusively derived from marine Crenarchaeota and thus, serve as qualitative biomarkers for these organisms. However, the more refractory nature of the GDGT lipids makes it difficult to use them directly to estimate absolute crenarchaeotal abundances.

The strong seasonality in the abundance of both Crenarchaeota and Euryarchaeota detected in the coastal North Sea confirms previous findings from other marine surface waters. The seasonal alteration in the occurrence of these two marine archaeal groups suggests different metabolic requirements of Cren- and Euryarchaeota. Marine Crenarchaeota are probably essentially chemolithoautotrophic organisms while marine Euryarchaeota may be mostly heterotrophic, however, further experimental work is required to allow firm conclusions on the metabolic requirements of these two marine archaeal groups.

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4.7 Literature list

Amann R.I., L. Krumholz, and D.A. Stahl (1990), Fluorescent-oligonicleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology, *J. Bacteriol. 172*, 762-770.

Benson D.A., I. Karsch-Mizrachi, D.J. Lipman, J. Ostell, B.A.Rapp and D.L.Wheeler (2000), GenBank, *Nucleic Acid Res. 28,*15-18.

Buckley, D.H., J.R. Graber, and T.M. Schmidt (1998), Phylogenetic analysis of nonthermophilic members of the kingdom Crenarchaeota and their diversity and abundance in soils, *Appl.Environ.Microbiol. 64*, 4333-4339.

Church, M.J., E.F. DeLong, H.W. Ducklow, M.B. Karner, C.M. Preston, and D.M. Karl (2003), Abundance and distribution of planktonic Archaea and Bacteria in the waters west of the Antarctic Peninsula, *Limnol.Oceanogr. 48*, 1893-1902.

Coolen, M.J.L., H. Cypionka, A.M. Sass, H. Sass, and J. Overmann (2002), Ongoing modification of Mediterranean Pleistocene sapropels mediated by prokaryotes, *Science 296*, 2407-2410.

Coolen, M.J.L., E.C. Hopmans, W.I.C. Rijpstra, G. Muyzer, S. Schouten, J.K. Volkman, and J.S. Sinninghe Damsté (2004), Evolution of the methane cycle in Ace Lake (Antarctica) during the Holocene: Response of methanogens and methanotrophs to environmental change, *Org.Geochem. 35,* 1151-1167.

DeLong, E.F., G.S. Wickham, and N.R.Pace (1989), Phylogenetic strains: ribosomal RNA-based probes for the identification of single cells, *Science 243*, 1360-1363.

DeLong, E.F. (1992), Archaea in coastal marine environments, *P.Natl.Acad.Sci.USA 89*, 5685-5689.

DeLong, E.F., K.Y. Wu, B.B. Prézelin, and R.V.M. Jovine (1994), High abundance of Archaea in Antarctic marine picoplankton, *Nature 371*, 695-697.

DeLong, E.F., L.L. King, R. Massana, H. Cittone, A. Murray, C. Schleper, and S.G. Wakeham (1998), Dibiphytanyl ether lipids in nonthermophilic crenarchaeotes, *Appl.Environ.Microbiol. 64*, 1133-1138.

DeLong E.F., L.T. Taylor, T.L. Marsh, and C.M. Preston (1999), Visualization and enumeration of marine planktonic archaea and bacteria by using polynucleotide probes and fluorescence in situ hybridization, *Appl. Environ. Microbiol. 65,* 5554-5563

Fogel G.B., C.R. Collins, J. Li, and C.F. Brunk (1999), Prokaryotic Genom Size and SSU rDNA Copy Number: Estimation of Microbial Relative Abundances from a Mixed Population, *Microb.Ecol. 38*, 93-113.

Fuhrman, J.A., K. McCallum, and A.A. Davis (1992), Novel major archaebacterial group from marine plankton. *Nature 356*, 148-149.

Herndl, G.J., T. Reinthaler, E. Teira, H. van Aken, C. Veth, A. Pernthaler and J. Pernthaler (2005), Contribution of Archaea to total prokaryotic production in the deep Atlantic Ocean, *Appl. and Environ. Microbiol. 71*, No.5.

Hoefs, M.J.L., S. Schouten, J.W. deLeeuw, L.L. King, S.G. Wakeham, and J.S. Sinninghe Damsté (1997), Ether lipids of planktonic archaea in the marine water column, *Appl.Environ.Microbiol.63*, 3090-3095.

Hopmans, E.C., S. Schouten, R.D. Pancost, M.T.J. Van der Meer, and J. S. Sinninghe Damsté (2000), Analysis of intact tetraether lipids in archaeal cell material and sediments by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, *Rapid.Commun.Mass Spectrom. 14*, 585-589.

Inagaki, F., U. Tsunogai, M. Suzuki, A. Kosaka, H. Machiyama, K. Takai, T. Nunoura, K. H. Nealson, and K. Horikoshi (2004), Characterization of C-1-metabolizing prokaryotic communities in methane seep habitats at the Kuroshima Knoll, southern Ryukyu Arc, by analyzing *pmo*A, *mmo*X, *mxa*F, *mcr*A, and 16S rRNA genes, *Appl.Environ.Microbiol. 70,* 7445-7455.

Karner, M.B., E.F. DeLong, and D.M. Karl (2001), Archaeal dominance in the mesopelagic zone of the Pacific Ocean, *Nature 409*, 507-510.

Keough, B.P., T.M. Schmidt, and R.E. Hicks (2003), Archaeal nucleic acids in picoplankton from great lakes on three continents, *Microb.Ecol. 46*, 238-248.

Könneke, M., A.E. Bernhard, J.R. de la Torre, C. B. Walker, J.B. Waterbury, and D.A. Stahl (2005), Isolation of an ammonia-oxidizing archaeon implies deep ocean autotrophy and nitrification, *Nature*, In press.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A.W.Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig , T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, K.H. Schleifer (2004), ARB: a software environment for sequence data, *Nucleic Acids Res.32*, 1363-1371.

MacGregor, B.J., D.P. Moser, E. Wheeler-Alm, K. H. Nealson, and D.A. Stahl (1997), Crenarchaeota in Lake Michigan sediment, *Appl.Environ.Microbiol. 63*, 1178-1181.

Maidak, B.L., J.R. Cole, T.G. Lilburn, C.T. Parker, P.R. Saxman, R.J. Farris, G.M. Garrity, G.J. Olsen, T.M. Schmidt, J.M. Tiedje (2001), The RDP-II (Ribosomal Database Project), *Nucleic Acids Res. 29*, 173-174.

Massana, R., L.T. Taylor, A.E. Murray, K.Y. Wu, W.H. Jeffrey, and E.F. DeLong (1998), Vertical distribution and temporal variation of marine planktonic archaea in the Gerlache Strait, Antarctica, during early spring, *Limnol.Oceanogr. 43,* 607-617.

Massana, R., E.F. DeLong, and C. Pedrós-Alió (2000), A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces, *Appl.Environ.Microbiol. 66,* 1777-1787.

Murray, A.E., C.M. Preston, R. Massana, L.T. Taylor, A. Blakis, K. Wu, and E. F. DeLong (1998), Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica, *Appl.Environ.Microbiol. 64*, 2585-2595.

Murray, A.E., A. Blakis, R. Massana, S. Strawzewski, U. Passow, A. Alldredge, and E.F. DeLong (1999), A time series assessment of planktonic archaeal variability in the Santa Barbara Channel, *Aquat.Microb.Ecol. 20*, 129-145.

Murray, A.E., K.Y. Wu, C.L. Moyer, D.M. Karl, and E.F. DeLong (1999), Evidence for circumpolar distribution of planktonic Archaea in the Southern Ocean, *Aquat.Microb.Ecol. 18*,263-273.

Muyzer, G., E.C. De Waal, and A.G. Uitterlinden (1993), Profiling of complex microbialpopulations by denaturing gradient gelelectrophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rRNA, *Appl.Environ.Microbiol. 59*, 695-700.

Ochsenreiter, T., D. Selezi, A. Quaiser, L. Bonch-Osmolovskaya, and C. Schleper (2003), Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR, *Environ.Microbiol. 5*, 787-797.

Ouverney, C.C., and Fuhrman, J.A. (2000), Marine planktonic archaea take up amino acids, *Appl. Env. Microbiol. 66,* 4829-4833.

Øvreas, L., Forney, L., Daae, F.L., Torsvik, V. (1997), Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol. 63*, 3367-3373.

Parsons, T., Y. Maita, and C. Lalli (1984), A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press.

Pernthaler A., J. Pernthaler and R. Amann (2002), Fluorescence in situ hybridization and Catalyzed Reporter Deposition for the Identification of Marine Bacteria. *Appl. Environ. Microbiol. 68*, 3094-3101.

Pesaro, M and F. Widmer (2002), Identification of novel Crenarchaeota and Euryarchaeota clusters associated with different depth layers of forest soil. *FEMS Micobiol. Ecol. 42*, 89-98.

Postma, H. (1954), Hydrography of the Dutch Wadden Sea. *Arch. Néerl. Zool. 10*, 405-511.

Porter K.G., and Y.S. Feig (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr. 25*, 943-948.

Powers, L.A., J.P. Werne, T.C. Johnson, E.C. Hopmans, J.S. Sinninghe Damsté, and S. Schouten (2004) Crenarchaeotal membrane lipids in lake sediments: A new paleotemperature proxy for continental paleoclimate reconstruction?, *Geology. 32*, 613-616.

Stahl, D.A., and R.I. Amann (1991), Nucleic acid techniques in bacterial systematics. Wiley, New York, N.Y. ed. By E. Stackebrandt, M. Goodfellow. 205

Sambrook, J., E.F. Fritsch and T. Maniatis (1989), Molecular Cloning: A Laboratory Manual. 2d ed. 3 vol., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schouten, S., E.C. Hopmans, R.D. Pancost, and J.S. Sinninghe Damsté (2000), Widespread occurrence of structurally diverse tetraether membrane lipids: Evidence for the ubiquitous presence of low-temperature relatives of hyperthermophiles, *P.Natl.Acad.Sci.USA. 97*, 14421-14426.

Sinninghe Damsté, J.S., W.I.C. Rijpstra, E.C. Hopmans, F.G. Prahl, S.G. Wakeham, and S. Schouten (2002a), Distribution of membrane lipids of planktonic Crenarchaeota in the Arabian Sea. *Appl.Environ.Microbiol. 68,* 2997-3002.

Sinninghe Damsté, J.S., S. Schouten, E.C. Hopmans, A.C.T. Van Duin, and J.A.J. Geenevasen (2002b), Crenarchaeol: the characteristic core glycerol dibiphytanyl glycerol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota, *J.Lipid Res. 43*, 1641-1651.

Teira, E., T. Reinthaler, A. Pernthaler, J. Pernthaler, and G.J. Herndl (2004), Combining catalyzed reporter deposition-fluorescence in situ hybridization and microautoradiography to detect substrate utilization by bacteria and archaea in the deep ocean. *Appl.Environ.Microbiol. 70*, 4411-4414.

Venter J.C, K. Remington, J.F. Heidelberg, A.L.Halpern, D.Rusch, J.A.Eisen,D.Y. Wu, I. Paulsen, K.E. Nelson, W. Nelson, D.E. Fouts, S. Levy, A.H.Knap, M. W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y.H. Rogers, and H.O.Smith (2004), Environmental genom shotgun sequencing of the Sargasso Sea. *Science. 304,* 66-77.

Vetriani, C., H.W. Jannasch, B.J. MacGregor, D.A. Stahl, and A.L. Reysenbach (1999), Population structure and phylogenetic characterization of marine benthic Archaea in deep-sea sediments, *Appl.Environ.Microbiol. 65*, 4375-4384.

Weijers, J.W.H., S. Schouten, M. Van den Linden, B. Van Geel, and J.S. Sinninghe Damsté (2004), Water table related variations in the abundance of intact archaeal membrane lipids in a Swedish peat bog, *FEMS Microbiol.Lett. 239*, 51-56.

Wells L.E., and J.W. Deming (2003), Abundance of Bacteria, the Cytophaga – Flavobacterium cluster and archaea in cold oligotrophic waters and nepheloid layers of the Northwest Passage, Canadian Archipelago, *Aquat. Microb. Ecol. 31*, 19-31.

Wuchter, C., S. Schouten, H.T.S. Boschker, and J.S. Sinninghe Damsté (2003), Bicarbonate uptake by marine Crenarchaeota, *FEMS Microbiol.Lett. 219*, 203-207.

Wuchter, C., S. Schouten, M.J.L. Coolen, and J.S. Sinninghe Damsté (2004), Temperature-dependent variation in the distribution of tetraether membrane lipids of marine Crenarchaeota: Implications for TEX_{86} paleothermometry, *Paleoceanography.19*, PA4028, doi:10.1029/2004PA001041

Wuchter, C., L. Herfort, M.J.L. Coolen, P. Timmers, M. Strous, G.J. Herndl, J.J. Middelburg, S. Schouten, and J. S. Sinninghe Damsté (2005), Archaeal nitrification in the ocean. *Submitted.*