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Ecology and membrane lipid distribution of marine Crenarchaeota: Implications for TEX_{86} paleothermometry

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Ecology and membrane lipid distribution of marine Crenarchaeota: Implications for TEX_{86} paleothermometry

Ecologie en membraanlipiden samenstelling van mariene Crenarchaeota: Implicaties voor TEX_{86} paleothermometrie

(met een samenvatting in het Nederlands)

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To my parents

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Chapter 1

Introduction

1.1 Archaea: the third domain of life

Besides the Bacteria and the Eukarya, a third domain of life on Earth is formed by the Archaea [*Woese et al*., 1990]. The Archaea are prokaryotes like Bacteria and also have some genes and metabolic sequences in common. However, Archaea also share some similarities in the genome replication and expression systems with the Eukarya and, like the Eukarya, Archaea lack muramic acid in their cell wall [see *Woese et al*., 1990 and references therein]. The cell membrane of the Archaea is unique and differs from the cytoplasm membrane of both Bacteria and Eukarya (see 1.3).

Based on the composition of the 16S ribosomal (rRNA) gene the domain of the Archaea is subdivided in two major phyla, the Crenarchaeota and Euryarchaeota [*Woese et al*., 1990] and one smaller phylum the Korarchaeota [*Barns et al*., 1996] (Fig. 1). Traditionally Archaea were viewed as organisms that thrive under extreme conditions, such as anoxic, hypersaline, extremely warm (> 60 ºC) and acidic (< pH 2) environments [*Tindal et al*., 1992]. About a decade ago, however, phylogenetic analyses of environmental 16S rRNA genes showed that Archaea are far more widespread than previously thought and that they occur in more temperate environments [*Fuhrman et al*., 1992; *DeLong*, 1992]. This discovery changed the traditional view on the Archaea dramatically and over the following years, small-subunit archaeal rRNA genes and unique archaeal membrane lipids were retrieved from the pelagic realm of tropical, temperate and polar seas [*DeLong*, 1994; 1998; *Hoefs et al*., 1997; *Sinninghe Damsté et al*., 2002], marine sediments [*Vetriani et al*., 1999; *Schouten et al*., 2000], lake waters and sediments [*Keough et al*., 2003; *McGregor et al*., 1997; *Powers et al*., 2004], soils [*Buckley et al*., 1998; *Ochsenreiter et al.*, 2003; *Pesaro et al*., 2002] and peat bogs [*Weijers et al*., 2004]. Thus, Archaea are organisms occurring ubiquitously on this planet in a wide variety of settings ranging from ice seas to hydrothermal vents.

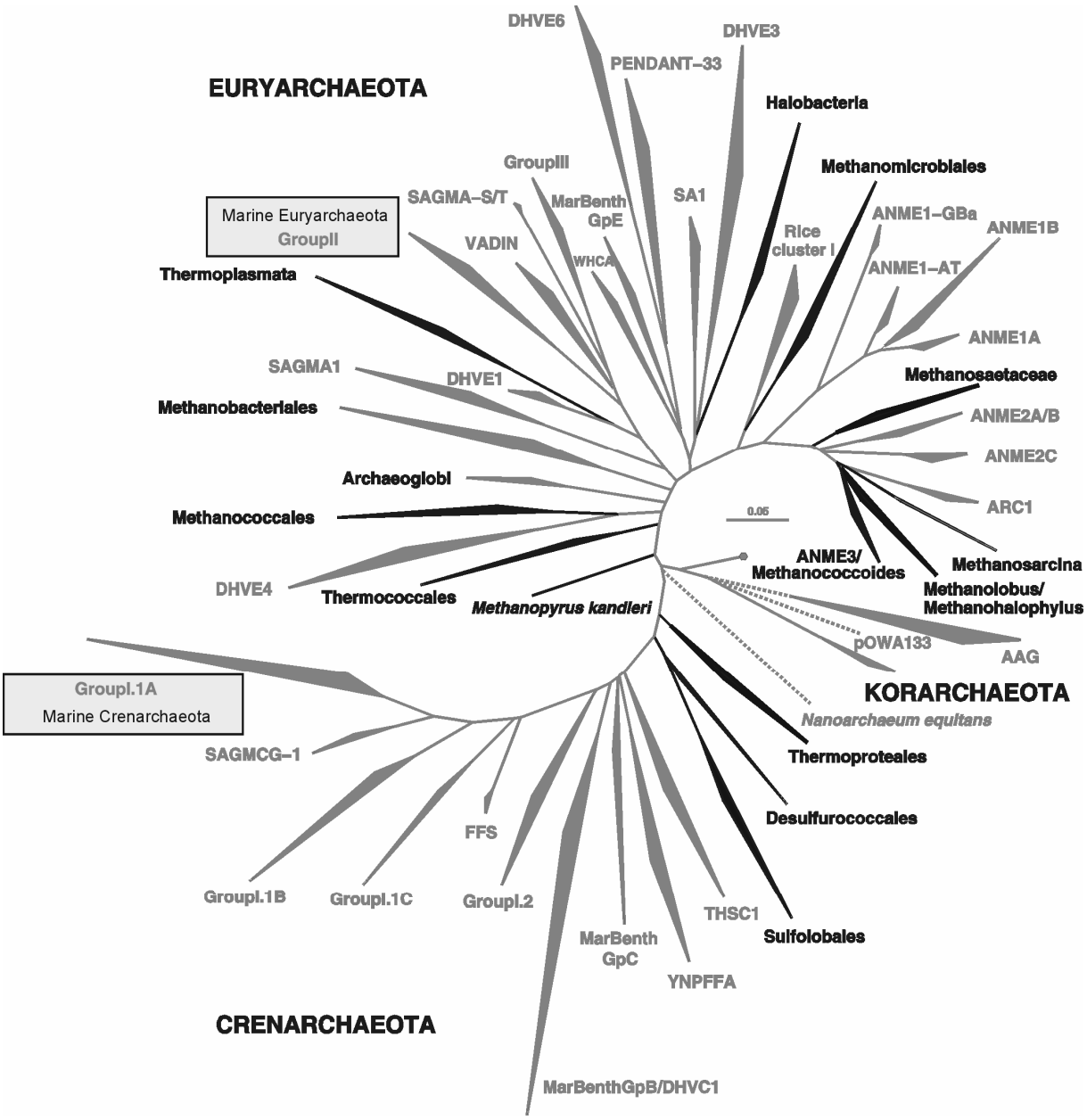

Figure 1. Phylogenetic tree based on the 16S rRNA gene of Achaea showing the three phyla Crenarchaeota, Euryarchaeota and Korarchaeota [after *Schleper et al.*, 2005]. Triangles in grey represent branches containing exclusively uncultivated species. The black triangles correspond to branches containing at least one cultivated species. Overall, the tree reveals the predominance of environmental clones representing groups for which no cultured close relatives are available. The marine Crenarchaeota group and the marine Euryarchaeota group are highlighted.

1.2 Ecology and physiology of marine mesophilic Archaea

Archaea in temperate aquatic environments appear to be widespread and a very abundant group. In the marine environment they form two major planktonic archaeal groups in the crenarchaeotal and euryarchaeotal phyla (Fig. 1). Recent studies revealed that marine Crenarchaeota comprise almost 20% of the picoplankton in the world oceans [*Karner et al*., 2001]. Generally, marine Crenarchaeota are relative more abundant than marine Euryarchaeota in deeper layers of the neritic waters and in the meso- and bathypelagic zone of the ocean [*DeLong et al*., 1999; *Karner et al*., 2001; *Church et al*., 2003; *Herndl et al*., 2005] (Fig. 2). In contrast, marine Euryarchaeota are relatively more abundant than the marine Crenarchaeota in surface waters of open oceans and coastal systems [*Murray et al*., 1999; *Massana et al*., 2000; *Pernthaler et al*., 2002].

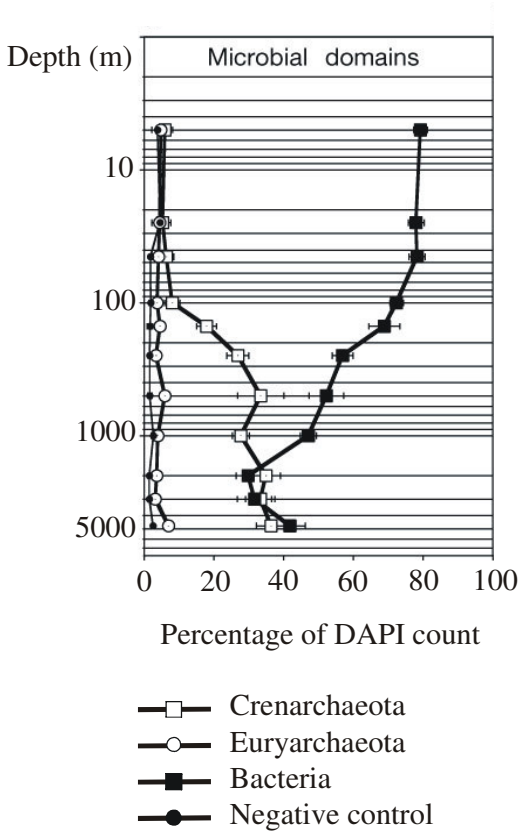

Figure 2. The mean annual depth profile of microbial domains from the North Pacific subtropical gyre obtained by fluorescence *in situ* hybridization [after *Karner et al*., 2001]. Depth profiles for bacteria (solid squares), pelagic Crenarchaeota (open squares), pelagic Euryarchaeota (open circles) and non-specific control probe ('negative', solid circles) are shown.

Despite the fact that marine Archaea constitute a substantial fraction of the world ocean picoplankton, little is known about the basic physiology of these organisms. A positive correlation between the abundance of Crenarchaeota and nitrite concentrations was reported from field studies in the Santa Barbara Channel [*Murray et al*., 1999], the Arabian Sea [*Sinninghe Damsté et al*., 2002] and with particulate organic nitrogen in Arctic waters [*Wells and Deming*, 2003]. This suggested that marine Crenarchaeota may in some way be involved in the marine nitrogen cycle.

Stable isotope analyses of the archaeal membrane lipids (see 1.3) showed that the $\delta^{13}C$ values of these lipids are relatively invariant in comparison to phytoplankton lipids [*Hoefs et al*., 1997; *Schouten et al*., 1998]. These findings suggested that marine Crenarchaeota may utilize bicarbonate as their carbon source [*Hoef et al*., 1997; *Kuypers et al*., 2001]. Radiocarbon analyses, done on biphytanes derived from sedimentary archaeal lipids, suggested that Archaea utilize "old" ^{14}C depleted dissolved inorganic carbon from below the photic zone in the water column [*Pearson et al*., 2001]. Direct experimental evidence for the metabolic requirements of pelagic Archaea is, however, limited. Microautoradiography showed that some marine Crenarchaeota and Euryarchaeota are capable to utilize bicarbonate or CO_2 as their carbon source [*Herndl et al*., 2005] and that some marine Archaea are capable to take up amino acids [*Overney and Fuhrman*, 2000, *Teira et al*., 2004]. Thus, the metabolic requirement of pelagic Archaea remains still enigmatic.

1.3 Archaeal membrane lipids and their application as paleothermometer

The Archaea biosynthesize unique membrane lipids compared to Bacteria and Eukarya. This has been, in addition to their unique gene composition, a major argument to propose the Archaea as the third domain of life [*Woese et al*., 1990]. While the core membrane lipids of Bacteria and Eukarya consist predominantly of fatty acids esterified to glycerol, the Archaea synthesize lipids with ether-linkages, which consist of long-chain isoprenoid diphytanyl glycerol diethers (DGDs) and glycerol dibiphytanyl glycerol tetraethers (GDGTs) (Fig. 3).

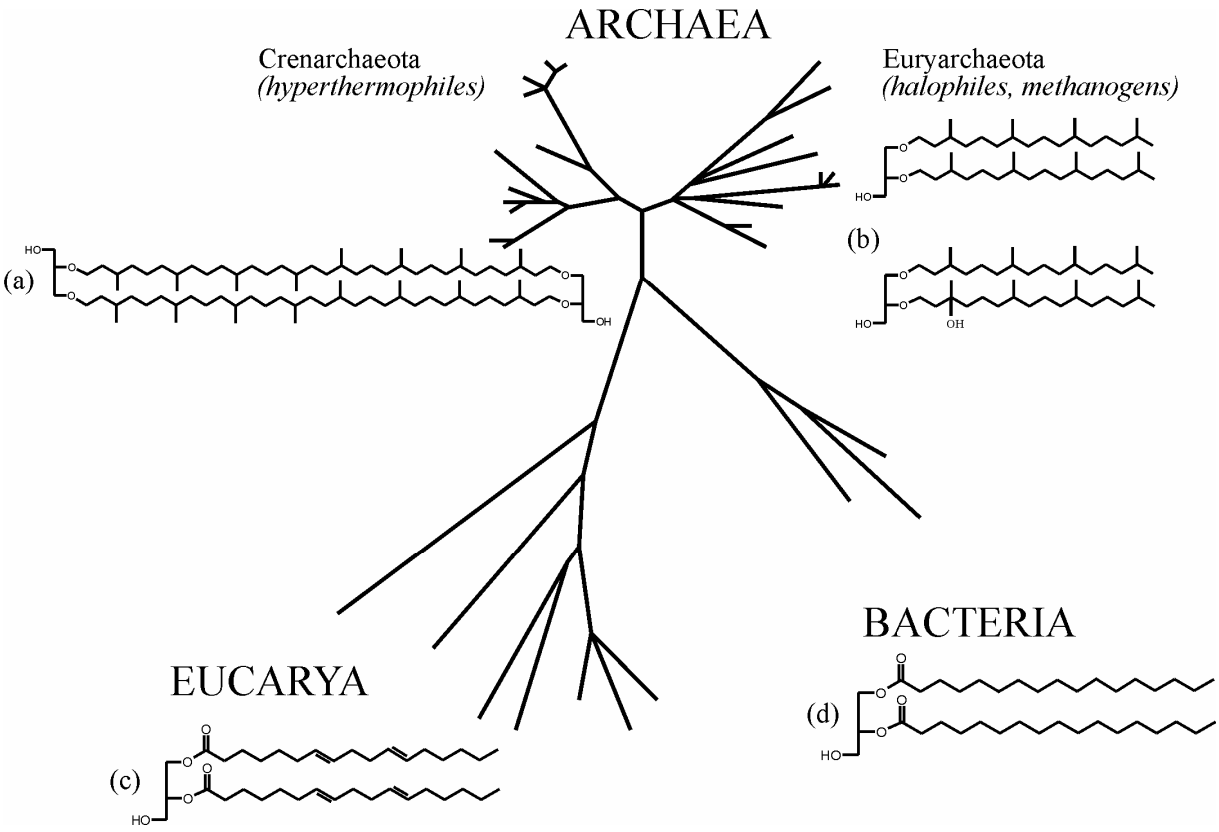

Figure 3. The different membrane lipid composition in the three domains of life (a) glycerol dibiphytanyl glycerol tetraethers, (b) diphytanyl glycerol diethers, (c) and (d) fatty acids glycerol diesters.

Some Archaea, especially the hyperthermophilic Crenarchaeota, biosynthesize only GDGTs, which form a lipid monolayer in the membrane. In the membrane this lipid monolayer is less sensitive to denaturation at high temperatures than a lipid bilayer composed from for example DGDs [*van de Vossenberg*, 1999 and references therein]. The GDGTs of Archaea can contain cyclopentane moieties (Fig. 4). Hyperthermophilic Crenarchaeota can increase the amount of cyclopentane ring-containing GDGTs as a response to changes in temperature [*Gliozzi et al*., 1983; *DeRosa and Gambacorta* 1988; *Uda et al*., 2001] to keep their cytoplasmic membrane at a liquid crystalline state and reduce their proton permeabilisation rate [*Albers et al*., 2000]. The biosynthesis of cyclopentane moieties in the membrane lipids of hyperthermophilic Archaea is, therefore, generally considered to be a temperature adaptation mechanism of the membrane.

Marine Crenarchaeota are close relatives to the hyperthermophilic Crenarchaeota and chemical degradation studies suggested that marine Crenarchaeota also biosynthesize GDGTs

[*Hoefs et al*., 1997; *DeLong et al*., 1998]. A new high performance liquid chromatography mass spectrometry (HPLC/MS) method was developed to analyze intact core GDGT membrane lipids of Archaea [*Hopmans et al*., 2000] and this method allowed a rapid detection of intact GDGTs in all kinds of environmental samples. With this HPLC/MS method intact GDGTs were found in *Cenarchaeum symbiosum* [*Sinninghe Damsté et al*., 2002], in marine water samples [*Sinninghe Damsté et al*., 2002] and sediments [*Schouten et al*., 2002]. A specific GDGT which contains four cyclopentane rings and one cyclohexane ring was detected with this new method (Fig. 4). This GDGT is exclusively attributed to the mesophilic Crenarchaeota and was, therefore, named crenarchaeol [*Sinninghe Damsté et al*., 2002]. It has been suggested that marine Crenarcheota have evolved from hyperthermophilic Crenarchaeota by building an additional ”kink” in their membrane lipid, i.e. the cyclohexane ring [*Sinninghe Damsté et al*., 2002]. Crenarchaeol is thought to have lowered the transition point of the membrane lipids and allowed the Crenarcheota to live in temperate environments such as the marine water column.

Analyses of core top sediments from different geographic settings showed that the distribution of marine crenarchaeotal GDGTs varies with temperature [*Schouten et al*., 2002]. In surface sediments of warmer parts of the ocean the GDGT distribution is dominated by crenarchaeol and relatively higher amounts of the 1-3 cyclopentane-containing GDGTs [*Schouten et al*., 2002] (Fig. 4). The GDGT distribution in surface sediments from cold areas consisted almost completely of GDGT I and crenarchaeol. Thus, it seems that marine Crenarchaeota inherited a temperature adaptation mechanism for their cytoplasm membrane similar to that of their hyperthermophilic relatives.

The change in the GDGT distribution in core top sediments was expressed in an index of GDGT isomers [*Schouten et al*., 2002]. This index was named Tetraether Index of lipids with 86 carbon atoms (TEX_{86}) and is defined as followed:

$$TEX_{86} = (III+IV+VI)/ (II+III+IV+VI) \quad (1)$$

The correlation of this index to the annual mean sea surface temperature (SST) at the position of the surface sediments gave the following linear correlation:

$$TEX_{86} = 0.015 * T + 0.28, (r^2 = 0.92) \quad (2)$$

With T = annual mean SST (in °C) (Fig. 5). The membrane lipids of marine Crenarchaeota are relatively stable components, and are found in sediments up to 140 million years old [*Kuypers*

et al., 2001; *Carrillo- Hernandez et al*., 2003]. Thus, by analysis of ancient GDGT distribution the TEX_{86} may have the potential to calculate ancient SST and may allow reconstruction of climatic changes in the past. SST is one of the most important parameter needed to reconstruct ancient environments [*Fischer and Wefer*, 1999].

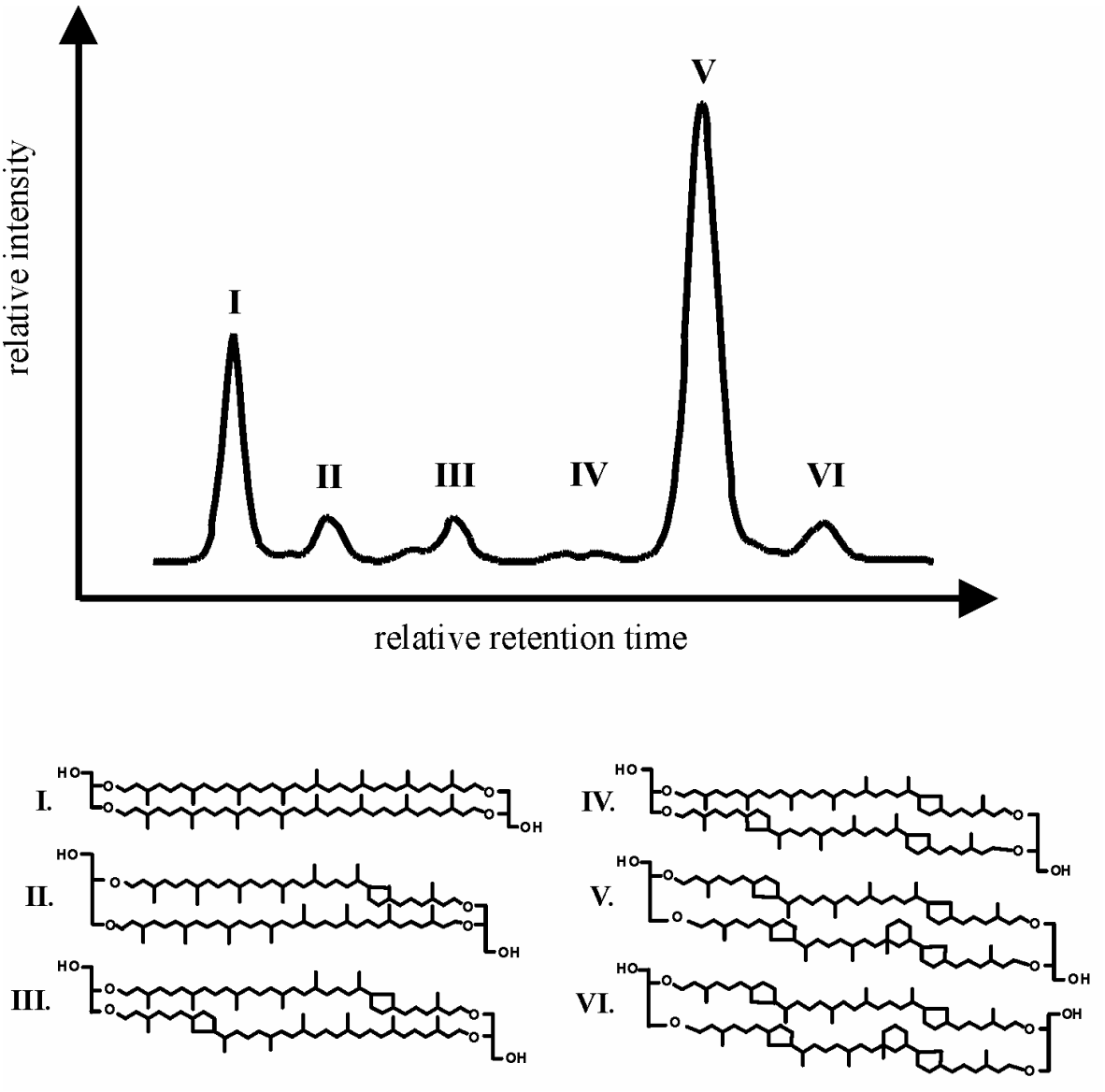

Figure 4. Partial HPLC/MS base peak chromatogram of an Arabian Sea surface sediment extract and structures of intact core tetraether membrane lipids of marine Crenarchaeota. Structure of the stereoisomer (VI) of crenarchaeol is likely a regio-isomer [*Sinninghe Damsté et al*, unpublished data 2004].

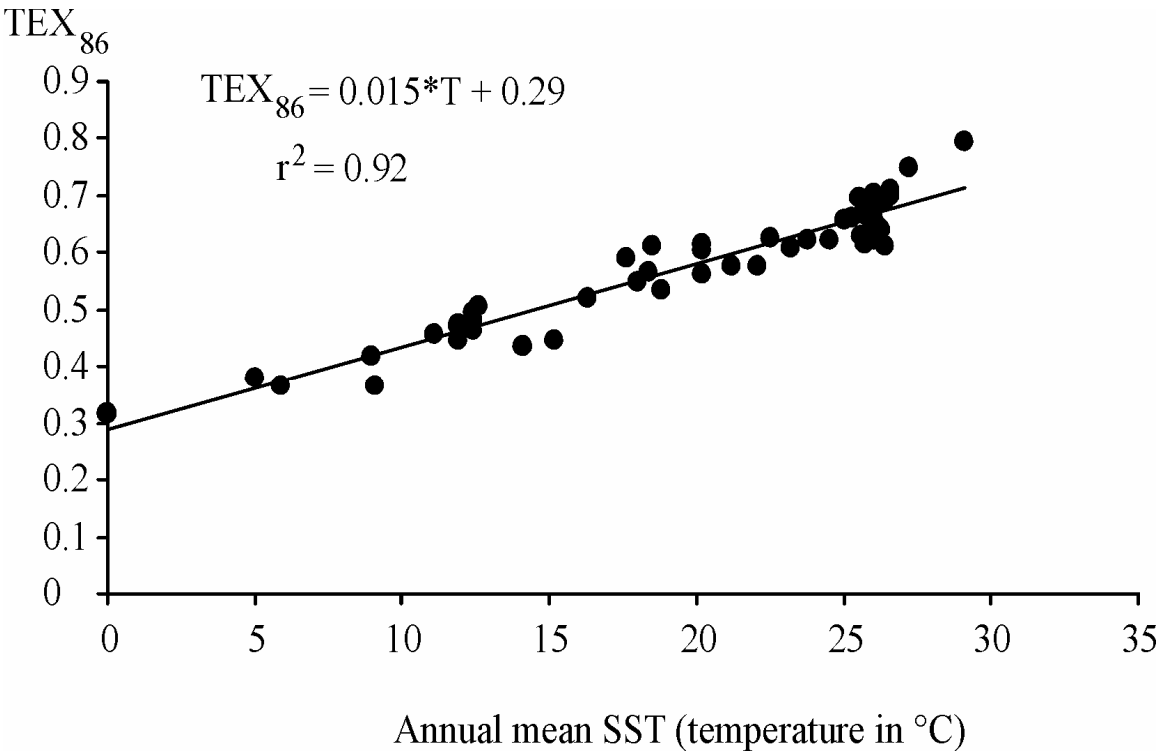

Figure 5. Updated correlation line of TEX_{86} in core top samples with annual mean SST [after *Schouten et al.*, 2002].

Marine Crenarchaeota may adjust their membrane lipids to temperature similar to their hyperthermophilic relatives but direct evidence that temperature is the only controlling factor is lacking. It is difficult to link the GDGT distribution only to temperature since little is known about the metabolic requirements of marine Crenarchaeota. For instance, salinity and nutrient levels can vary to a substantial degree in marine systems and may also influence the GDGT distribution. Furthermore, the new temperature proxy TEX_{86} was established on core top sediments and correlated best with SST. However, marine Crenarchaeota occur highly seasonal in surface waters and are generally relatively more abundant in the deeper layers of the neritic waters and in the meso-and bathypelagic zone of the ocean (see Fig. 2). It is as yet not clear during which season and at which depth in the water column the lipid signal used for TEX_{86} paleothermometry is biosynthesized. Thus, further research is required to validate the TEX_{86} as a new SST proxy.

1.4 Scope and Framework of the thesis

This thesis has two major objectives. The first objective was to shed more light on the basic physiology of marine Crenarchaeota, and the second objective was to validate the newly introduced temperature proxy $TEX_{86.}$ Hence, answers were sought for the following key questions:

(1) What is the basic physiology of marine Crenarchaeota and how does this determine their ecology?

(2) Is the GDGT distribution in the membrane lipids of marine Crenarchaeota mainly determined by temperature or are other factors such as nutrient levels or salinity also important?

(3) Does the GDGT signal which is found in the sediments reflect a physiological response to temperature of single species or is it an integrated signal of complex crenarchaeotal communities?

(4) Is the GDGT signal produced in the surface water over the whole annual cycle or is it a seasonal signal?

(5) Which GDGT signal is effectively transported in deeper waters and sediments and how does this influence the temperature calibration?

These questions address ecological and physiological aspects of the marine Crenarcheota and the mechanism and temperature calibration of the TEX_{86} proxy. Therefore, the thesis is divided into two parts. The first part of these thesis (**Chapter 2**, **3** and **4**) deals with studies concerning the physiology and ecology of marine Crenarchaeota. **Chapter 2** describes an *in situ* ^{13}C bicarbonate labeling experiment to study the carbon acquisition mechanism of marine Crenarchaeota and demonstrates that at least some pelagic marine Crenarchaeota are capable of light independent bicarbonate uptake and are thus autotrophic organisms. In **Chapter 3** an enrichment culture of marine Crenarchaeota obtained from mesocosm experiments showed that a crenarchaeotal species is involved in ammonium oxidation and is a chemolithoautotrophic organism. **Chapter 4** describes the seasonal dynamics of marine Archaea in costal North Sea surface waters during a 1.5 year long time series, using different molecular approaches, and demonstrates that cell densities of marine Crenarcheota strongly vary over the annual cycle and are highest during winter. This is in

agreement with the idea that marine Crenarchaeota are autotrophic ammonia oxidizing organismns.

The second part of this thesis (**Chapters 5**, **6** and **7**) deals with factors determining the GDGT distribution in marine Crenarchaeota and thus of the TEX_{86} proxy. **Chapter 5** describes the temperature dependent adaptation of marine crenarchaeotal GDGTs using mesocosm experiments, and shows that indeed temperature is the major controlling factor on the distribution of crenarcheotal tetraether membrane lipids. Nutrient levels and salinity do not play an important role in the GDGT distribution. The mesocosm temperature calibration shows a similar slope to the core top correlation but differs in the intersection probably caused by differences between the crenarchaeotal species enriched under laboratory conditions and the more complex crenarchaeotal communities in the field. In **Chapter 6,** lipid analysis of suspended particulate organic matter (POM) from surface and deep waters in different oceanic settings are presented. These data showed that the GDGT signal mainly reflects column temperatures of the upper 100 m. GDGTs from the upper parts of the ocean are probably more effectively transported to the sediment by grazing and repackaging in the upper water column since an active food-web exists there. The temperature calibration of the POM was similar to the core top correlation but with a lower correlation coefficient due to scatter of a mixed signal from living suspended Crenarchaeota and fossil sedimenting GDGTs. In **Chapter 7** sediment trap samples from the northeast Pacific and the Arabian Sea revealed that the TEX_{86} at all trap deployment depth reflects SST. At the shallow trap in the Arabian Sea the TEX_{86} followed the seasonal cycle, whereas the signal which ends up in the deeper traps reflects an integrated SST signal probably due lateral transport and mixing processes.

In summary, the experimental work and the field studies presented in the first part of the thesis revealed that, at least some, marine Crenarchaeota appear to be chemolithoautothrophic organisms and that they have a highly seasonal occurrence in surface waters. The experimental work and the field studies described in the second part of this thesis validates the newly introduced temperature proxy TEX_{86}. Thus, with this new proxy it is possible to reconstruct upper water column temperatures in ancient environments of up to 140 million years old.

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Chapter 2

Bicarbonate uptake by marine Crenarchaeota

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Abstract.

Biphytanyl membrane lipids and 16S rRNA sequences derived from marine Crenarchaeota were detected in shallow North Sea surface water in February 2002. To investigate the carbon fixation mechanism of these uncultivated archaea *in situ* ^{13}C bicarbonate tracer experiments were performed with this water in the absence of light. About 70% of the detected ^{13}C incorporation into lipids (including fatty acids and sterols) is accounted for by the crenarchaeotal biphytanyl membrane lipids. This finding indicates that marine Crenarchaeota can utilize bicarbonate or CO_2 derived thereof in the absence of light and are chemoautotrophic organisms.

2.1 Introduction

Archaea are one of the three domains of life. Until recently these were thought to be restricted to extreme environments such as high salinity or high temperature. However, by applying molecular biological techniques and lipid analyses it could be shown that archaea are far more widespread then previously thought and that they also occur in temperate environments such as the open ocean [*Fuhrman et al.*,1992; *De Long*, 1992; *Preston et al.*, 1996; *Massana et al.*, 1997; *Murray et al.*, 1998]. Recent molecular biological work showed that marine Crenarchaeota are distributed over a large depth range in both the photic and aphotic zones of the water column and make up about 20% of the picoplankton in the world's oceans [*Karner et al.*, 2001].

Membrane lipids of archaea are unique and consist of diphytanyl glycerol diethers and glycerol dibiphytanyl glycerol tetraethers (GDGTs) [*DeRosa and Gambacorta*, 1988]. Marine Crenarchaeota have a specific GDGT, containing four cyclopentane rings and one cyclohexane ring which occurs exclusively in non-thermophilic Crenarchaeota [*Schouten et al.*, 2000; *Sinninghe Damsté et al.*, 2002] and was called crenarchaeol. This GDGT was detected in ocean water over a large depth range [*De Long et al.*, 1998; *Schouten et al.*, 2000; *Wakeham et al.*, 2003] consistent with the 16S rRNA analyses [*Karner et al.*, 2001].

Despite the fact that marine Crenarchaeota make up an important part of the world's ocean picoplankton almost nothing is known about their basic physiology. *Ouverney and Fuhrmann* [2000] showed that marine archaea can utilize dissolved amino acids and appear to be heterotrophic organisms. However, $\delta^{13}C$ values of GDGTs are relatively invariant, in contrast to phytoplankton lipids [*Hoefs et al.*, 1997; *Bidigare et al.*, 1997; *Schouten et al.*, 1998]. This has led several authors to suggest that marine Crenarchaeota utilize bicarbonate [*Hoefs et al.*, 1997; *Kuypers et al.*, 2001]. Further evidence for autotrophy in marine Crenarchaeota was provided by radiocarbon analysis of biphytanes derived from sedimentary GDGTs, which suggested that archaea are not feeding on phytoplankton biomass, but rather use "old" ^{14}C-depleted dissolved inorganic carbon from below the photic zone in the water column [*Pearson et al.*, 2001].

Most studies, thus, suggest that marine Crenarchaeota use bicarbonate as a carbon source but solid experimental evidence is presently lacking. Here we show by a ^{13}C label experiment that pelagic marine Crenarchaeota are capable of light independent bicarbonate

uptake and are autotrophic organisms. This may have significant implications for our understanding of the marine carbon cycle.

2.2 Materials and methods

2.2.1 ^{13}C label experiment

An *in situ* ^{13}C bicarbonate label experiment was performed to study the carbon acquisition mechanism of the marine Crenarchaeota by measuring ^{13}C incorporation into their specific lipids. Since marine Crenarchaeota are not in culture yet, this approach [*Boschker et al*., 1998; *Middelburg et al*., 2000; *Boschker and Middelburg*, 2002] is a powerful tool to study *in situ* substrate utilization of these microorganisms in their natural environment. The experiment was performed in the dark to eliminate photosynthetic activity and thereby limiting the competition for marine Crenarchaeota. Fully labeled $H_2{}^{13}CO_3$ (99% ^{13}C) was continuously added over a 7 day time period to 20 l shallow North-Sea surface water, sampled in February 2002 at the NIOZ harbor. Measured average nutrient concentrations of the water in February were for nitrate 75 µmol l^{-1}, nitrite 1 µmol l^{-1}, ammonium 4,8 µmol l^{-1}, phosphate 1,4 µmol l^{-1} and silicate 39 µmol l^{-1} while salinity was at 26,5 ‰. The water was incubated in a sealed container at 15°C in the dark, and label was added to a concentration of 100 µM resulting in a final labeling of the natural dissolved inorganic carbon pool of about 5%. This small increase of *in situ* bicarbonate concentration had no significant influence on the pH or nutrient condition of the water used. A control tank was incubated under the same conditions without addition of labeled bicarbonate.

2.2.2 Lipid analyses

For lipid analyses the water was filtered through a 0.7 µm ashed glass fiber filter (GF) and subsequently through a 0.2 µm cellulose/acetate filter (C/A). The filters were freeze-dried and for GF filters ultrasonically extracted with methanol, methanol/dichloromethane (1:1, vol/vol) and three times with dichloromethane. C/A filters were extracted three times with methanol and three times with hexane. The extracts were combined and the water was removed with a small pipette Na_2SO_4 column and analyzed for GDGTs by high-performance liquid chromatography/mass spectrometry (HPLC/MS) [*Hopmans et al*., 2000]. Aliquots of the extracts were derivatized with boron trifluoride methanol complex and bis-

trimethylsilyltrifluoroacetamide (BSTFA)/pyridine to derivatize fatty acids and alcohols, respectively. The lipids were analyzed by gas chromatography (GC), GC/MS. The total lipid fraction was analyzed for label incorporation by isotopic-ratio-monitoring (IRM) GC/MS. To measure the ^{13}C incorporation in archaeal membrane lipids the GDGTs were treated with $HI/LiAlH_4$ [see *Schouten et al*., 1998] to release the biphytanes and measured by IRM-GC/MS. Label incorporation into lipids is expressed in $\Delta\delta^{13}C$ values (the difference between $\delta^{13}C$-label experiment and $\delta^{13}C$-control experiment).

2.2.3 DNA analyses

For molecular biological analysis the water was filtered through a 0.2 µm polycarbonate filter and total DNA was extracted with an ultra clean soil DNA kit (Mobio, Carlsbad, CA, USA) using the conditions as described by the manufacturer. Partial archaeal 16S rRNA genes were amplified by polymerase chain reaction (PCR) using primers Parch519f (complementary reverse sequence of primer PARCH519r [*Øvreas et al*., 1997] and GCArch915r [*Stahl and Amann*, 1991] including a 40-bplong GC clamp [*Muyzer et al*., 1993]. The amplification protocol after *Coolen et al*., [2002] includes initial denaturation at 96°C (4 min) followed by 35 cycles including a denaturation step at 94°C (30 s), primer annealing at 57°C (40 s) and primer extension at 72°C (40 s). A final extention was performed at 72°C for 10 min. A final concentration of 0.5 µM of each primer was used. The 436bp-long PCR products were separated by denaturing gradient gel electrophoresis (DGGE) [*Schäfer and Muyzer*, 2001]. DGGE conditions were applied as described by *Coolen et al*., [2002]. DGGE was carried out in a Bio-Rad D Gene system. PCR samples were applied directly onto 6% (wt/vol) polyamide gel (acrylamide/*N,N'*-methylene bisacrylamid ratio, 37:1 [wt/wt]) in 1x TAE buffer (pH 7.4). The gel contained a linear gradient of 30% to 60% denaturant (100% denaturant 7M urea plus 40% [vol/vol] formamide). Electrophoresis proceeded for 5 h at 200 V and 60°C. DGGE fragments were excised from the gel and the individual 16S rRNA genes were subsequently sequenced. Comparative analysis of the various sequences was done by using the ARB program package (developed by O. Strunk and W. Ludwig; available online at http:/www.biol.chemie.tu-muenchen.de/pub/ARB/). Evolutionary distances were calculated by using the Jukes-Cantor equation [*Jukes and Cantor*, 1969]. The phylogenetic tree was constructed by using the neighbor-joining algorithm [*Saitou and Nei*, 1987].

2.3. Results and Discussion

Several archaeal 16S rRNA gene sequences could be detected in shallow North Sea surface water sampled during the winter. The comparative analyses of the sequences showed that they all belong to the group of marine Crenarchaeota (Fig. 1). This is in good agreement with previous seasonal studies, which reported abundant marine Crenarchaeota in North Sea waters during the winter season [*Pernthaler et al*., 2002]. Analysis by HPLC/MS of the archaeal lipids from shallow North Sea surface water showed abundant GDGT-0 (I) and crenarchaeol (II) (Fig.2), the characteristic GDGT membrane lipids of marine Crenarchaeota [*De Long et al*., 1998, *Sinninghe Damsté et al*., 2002].

The lipids analyses of the label incorporation study revealed that hardly any ^{13}C label incorporation could be detected in the C_{32} hopanoic acids, $C_{24\text{-}26}$ fatty acids and C_{26} alcohol (Fig.3). Since these compounds are likely derived from dead bacterial and higher plant material, respectively, this is not surprising. Very low ^{13}C incorporation with $\Delta\delta^{13}C$-values of 7‰ could be measured for eukaryotic C_{27}-C_{29} sterols (Fig.3). This is probably due the lack of light, which inhibits photosynthesis by eukaryotes in the North Sea water and thereby uptake of ^{13}C-labeled bicarbonate, or CO_2. Minor ^{13}C enrichment could be detected in the *anteiso*-C_{15} fatty acid, a compound which occurs exclusively in bacteria, with $\Delta\delta^{13}C$-values of 10‰ (Fig.3). Compounds derived from both bacteria and eukaryotes such as the C_{14}–C_{20} fatty acids and C_{18}-C_{20} alcohols showed higher label incorporation. The $\Delta\delta^{13}C$-values for fatty acids were up to 80‰ for the C_{14} fatty acid and for the alcohols up to 35‰ for the C_{18} alcohol (Fig.3). In contrast to the eukaryotic and bacterial lipids, the crenarchaeotal membrane lipids were heavily labeled in ^{13}C with $\Delta\delta^{13}C$-values between 400‰ and 440‰ (Fig.3) for the crenarchaeotal biphytanes derived from GDGT-0 (I) and crenarchaeol (II).

Quantification of the ^{13}C uptake in lipids revealed that about 70% of the detected ^{13}C incorporation was accounted for by the crenarchaeotal biphytanes. The ^{13}C enrichment in the crenarchaeotal membrane lipids clearly shows that Crenarchaeota actively incorporate the inorganic carbon label into their biomass. Transfer of algal fixed carbon to bacteria or Crenarchaeota through a DOC pool is not likely because of several reasons: Firstly, the algal lipids show hardly any ^{13}C incorporation suggesting no substantial uptake by algae. Second, lipids of heterotrophic bacteria, specifically the *anteiso* C_{15} fatty acids [*de Bie et al*., 2002],

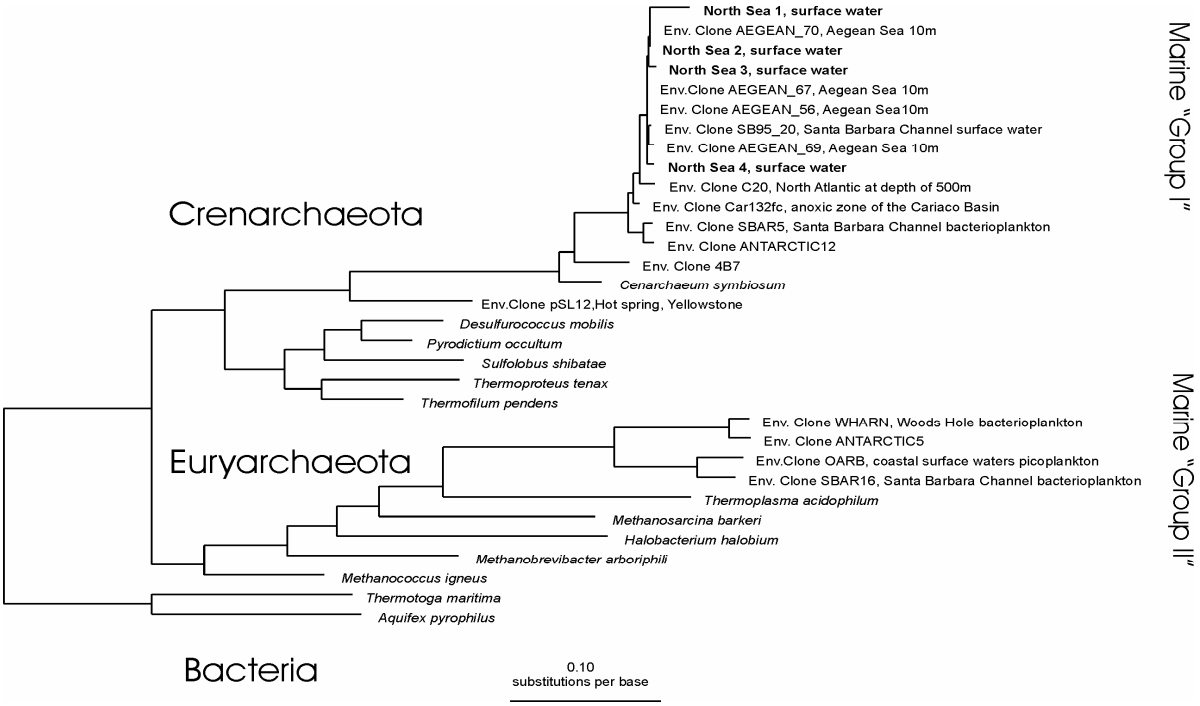

Figure 1. Phylogenetic positions of four partial 16S rRNA gene sequences of Crenarchaeota recovered from shallow surface North Sea water (North Sea 1-4) as well as their closest relatives. All sequences were affiliated to the marine Crenarchaeota Group I.

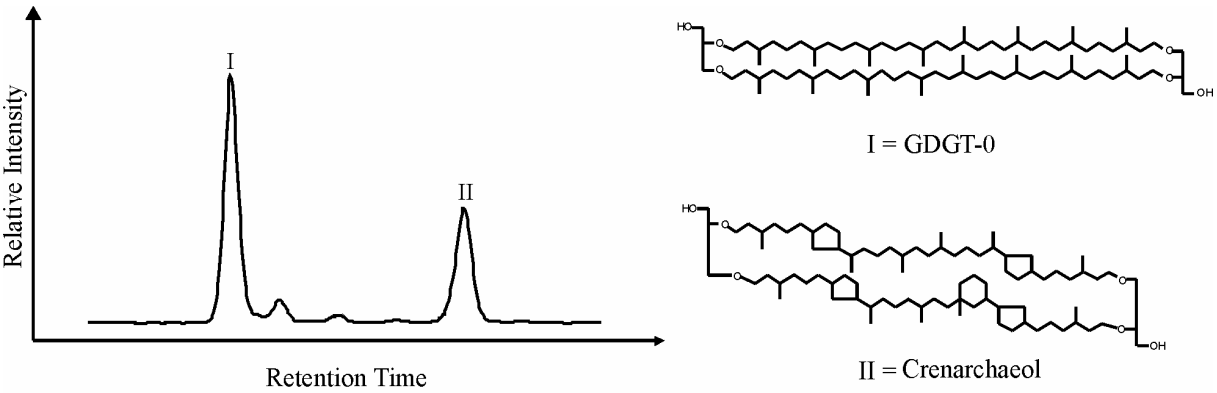

Figure 2. HPLC/MS base peak ion chromatogram of intact archaeal tetraether membrane lipids from the 20 l ^{13}C-label tank filled with North Sea water, showing a typical cold water lipid pattern with the main archaeal lipids GDGT-0 (I) and Crenarchaeol (II). These lipids are derived from marine pelagic Crenarchaeota [*Schouten et al.* 2000, *Sinninghe Damsté et al.,* 2002]

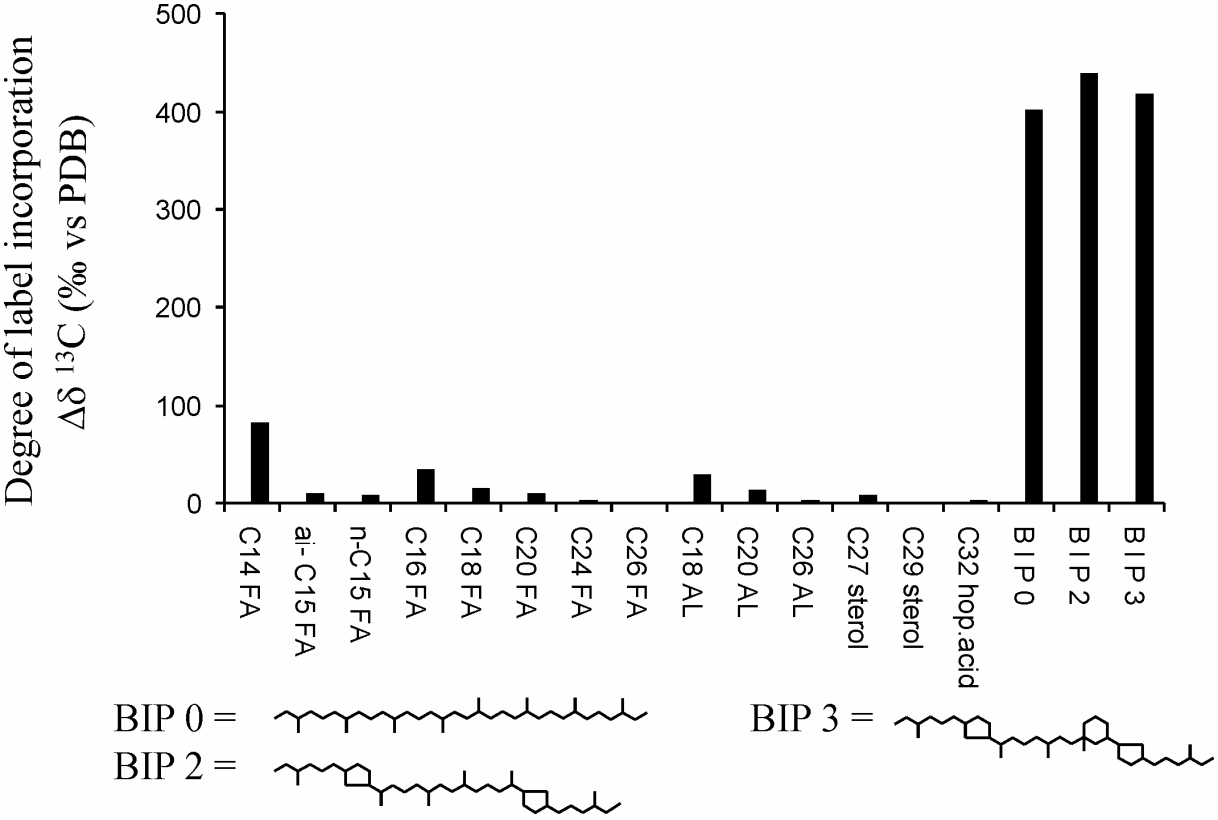

Figure 3. Results of ^{13}C label experiment with 20l shallow North Sea water incubated in the dark. The figure shows $\Delta\delta\ ^{13}C$ values from different lipids where $\Delta\delta\ ^{13}C$ is defined as $\delta\ ^{13}C$ of the label tank - $\delta\ ^{13}C$ of the control tank. There is minor ^{13}C incorporation into short chain bacterial and eukaryotic fatty acids (FA), C_{18}-C_{20} alkanols (AL) and eukaryotic $C_{27\text{-}C29}$ sterols. The main ^{13}C enrichment is found in the acyclic, bicyclic and tricyclic archaeal biphytanes (BIP) derived from GDGT – 0 (I) and Crenarchaeol (II).

also incorporated only minor amounts of label suggesting that no ^{13}C labeled organic matter was released. Our experiment thus indicates that an cosmopolitan group of marine picoplankton, the marine Crenarchaeota, are autotrophic organisms. Experiments are now underway to estimate the dark ^{13}C uptake rates of marine Crenarchaeota.

Our ^{13}C tracer experiment does not provide any information about the energy source of marine Crenarchaeota. Therefore, we have to rely on available ecological studies. Marine Crenarchaeota dwell in surface as well as in deep waters [e.g. *Massana et al.*, 1997, *Karner et al.*, 2001] and grow under dark conditions as our experiment shows and, thus, are not phototrophic organisms. Studies from the Arabian Sea [*Sinninghe Damsté et al.*, 2002] and Black Sea [*Wakeham et al.*, 2003] showed that marine Crenarchaeota do not seem to require oxygen for their metabolism and can thrive at very low levels of oxygen or even

anaerobically, suggesting they are aerotolerant anaerobes. A correlation of increasing abundance of marine Crenarchaeota and a distinct nitrate minimum and nitrite maximum in the oxygen minimum zone of the Arabian Sea led *Sinninghe Damsté et al*., [2002] to suggest that marine Crenarchaeota may reduce nitrate to nitrite. This metabolism is known in the kingdom Crenarchaeota from the hyperthermophilic autotrophic archaeon *Pyrobaculum aerophilum*, which reduces nitrate to nitrite using hydrogen. Nitrate is generally abundant in the water column and is only limited during the phytoplankton blooms, which may explain the reported negative correlation between chlorophyll *a* concentration and archaeal abundance [*Murray et al*., 1998] in Antarctic surface waters.

Further research will be needed to determine the source of energy and carbon fixation rates of marine Crenarchaeota. Since these organisms occur throughout the world's ocean over a large depth range and are one of the most abundant classes of picoplankton, it suggests that archaeal autotrophy may be a general and quantitatively important (deep) ocean process. Marine Crenarchaeota may represent a significant global sink for inorganic carbon and form an important but as yet unrecognized component of the global carbon cycle.

2.4 Acknowledgements.

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Chapter 3

Ammonia oxidation by a marine Crenarchaeote

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Abstract.

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

3.1 Introduction

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

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

3.2 Material and Methods

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

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

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

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

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

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

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

3.3 Results and Discussion

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

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

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

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

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

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

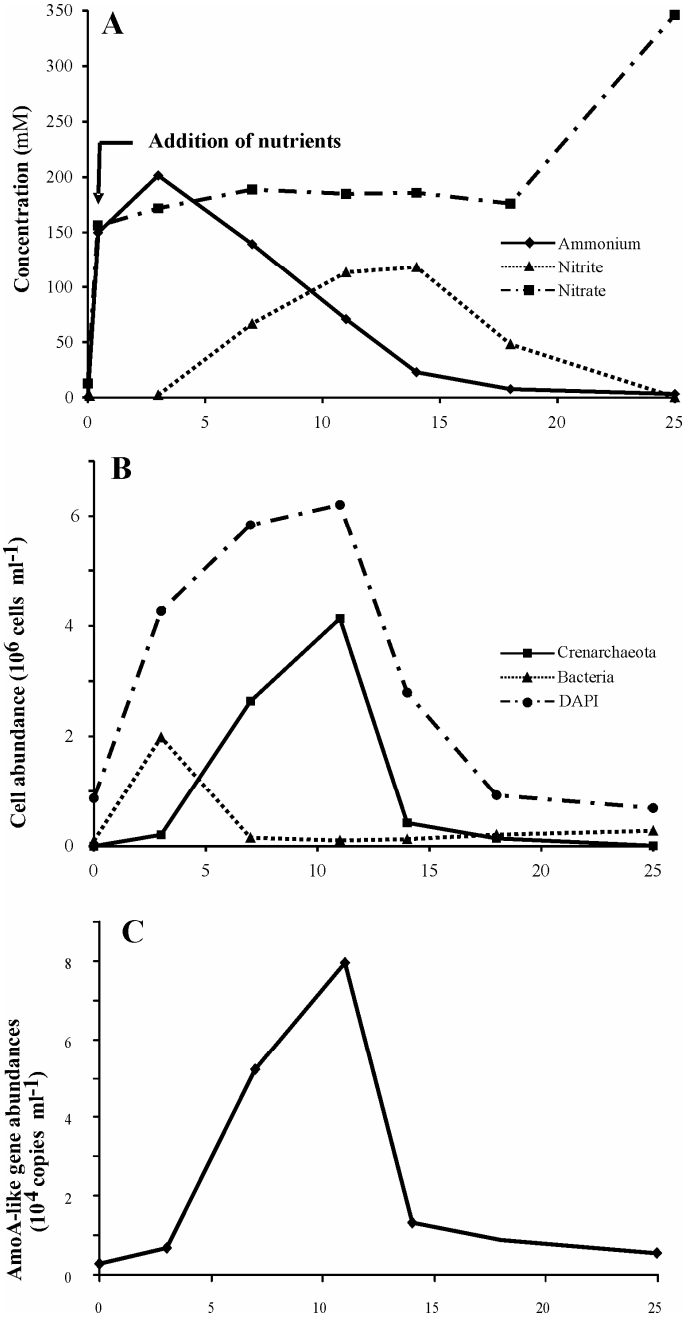

Figure 1. Archaeal nitrification in an enrichment culture. (**A**) Nutrient concentrations (μM) in the course of the experiment. (**B**) DAPI counts and archaeal and bacterial cell numbers (cells ml^{-1}) as determined by CARD-FISH (**C**) *amo*A-like gene copy numbers as determined by Q-PCR. Batches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

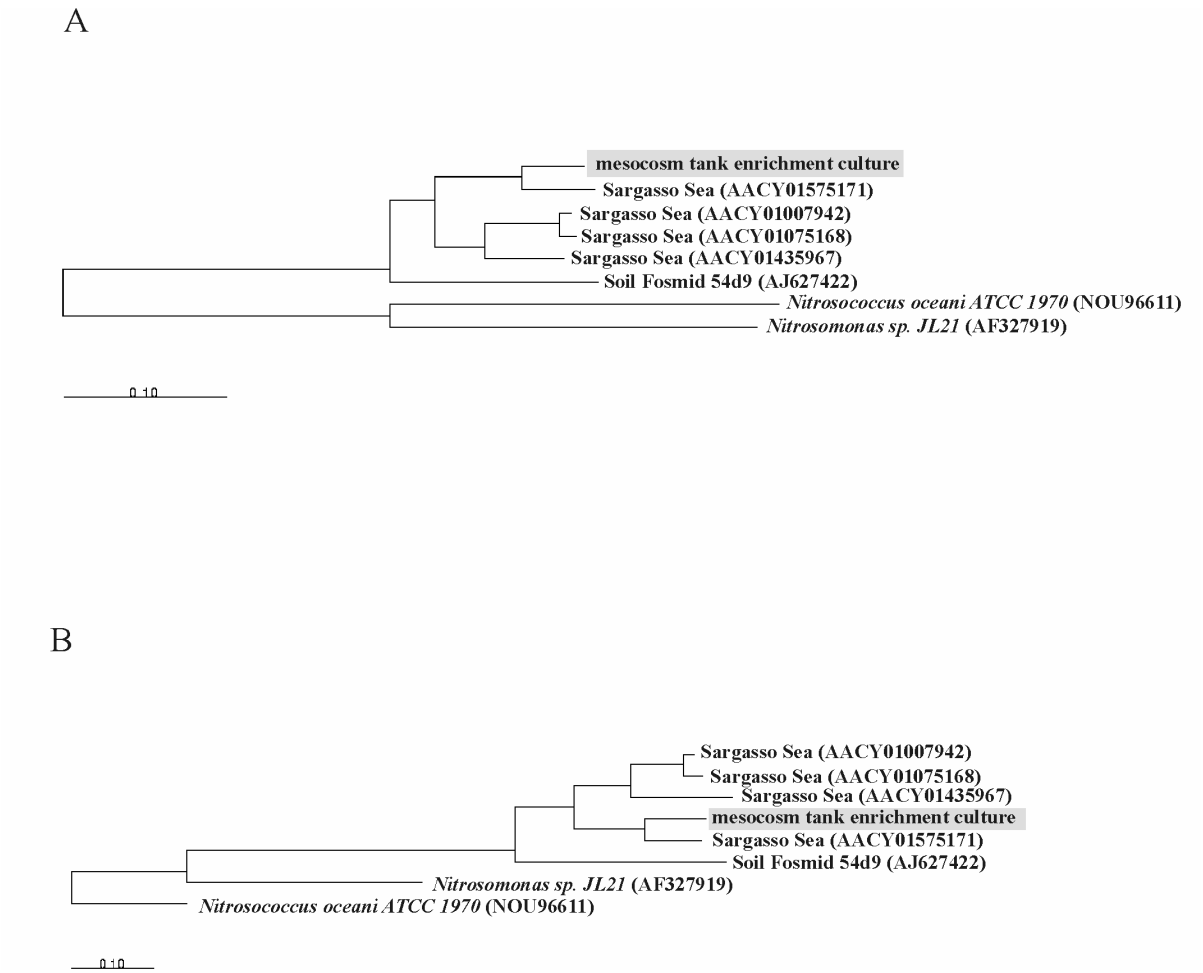

Figure 2. Phylogenetic analyses of *amo*A-like gene recovered from the enrichment culture. (**A**) Neighbour joining tree of 256 bp long *amo*A-like nucleotide sequence according to *Schleper at al.,* [2005]. The recovered *amo*A- like gene recovered from the incubation experiment is closely related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

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

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

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

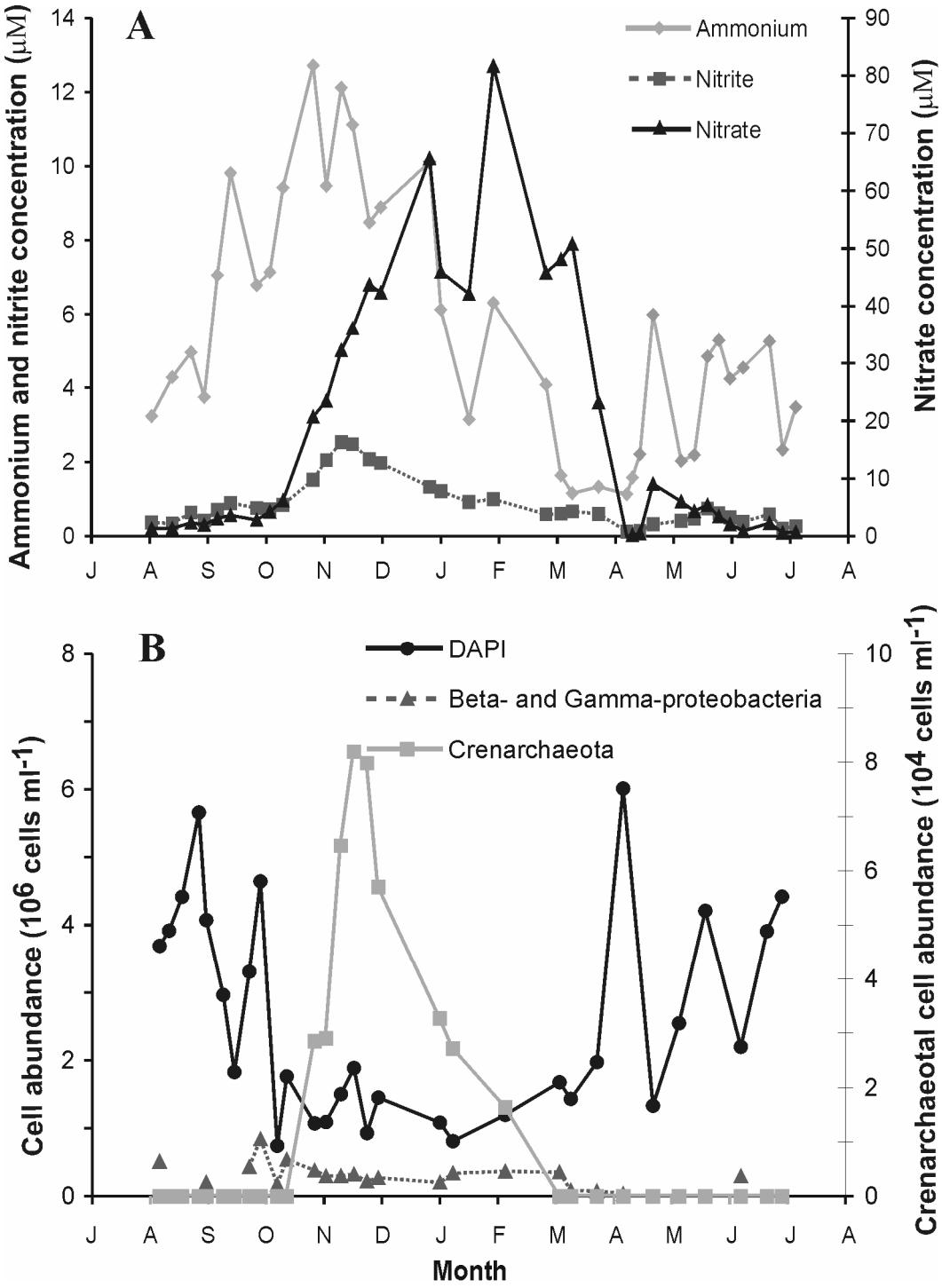

Figure 3. Crenarchaeotal abundance in the North Sea during 2002/2003 as a response to changing nutrient concentrations. (**A**) Nutrient concentrations (µM). (**B**) Cell abundances (cells ml^{-1}) of DAPI-stained cells and for Crenarchaeota and beta- and gamma-Proteobacteria as determined by CARD-FISH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

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

3.4 Acknowledgements. Analytical assistance was provided by Bouwe Kuipers, Govert van Noort, Karel Bakker and Judith van Bleijswijk (Royal NIOZ).

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3.5 Supplementary material

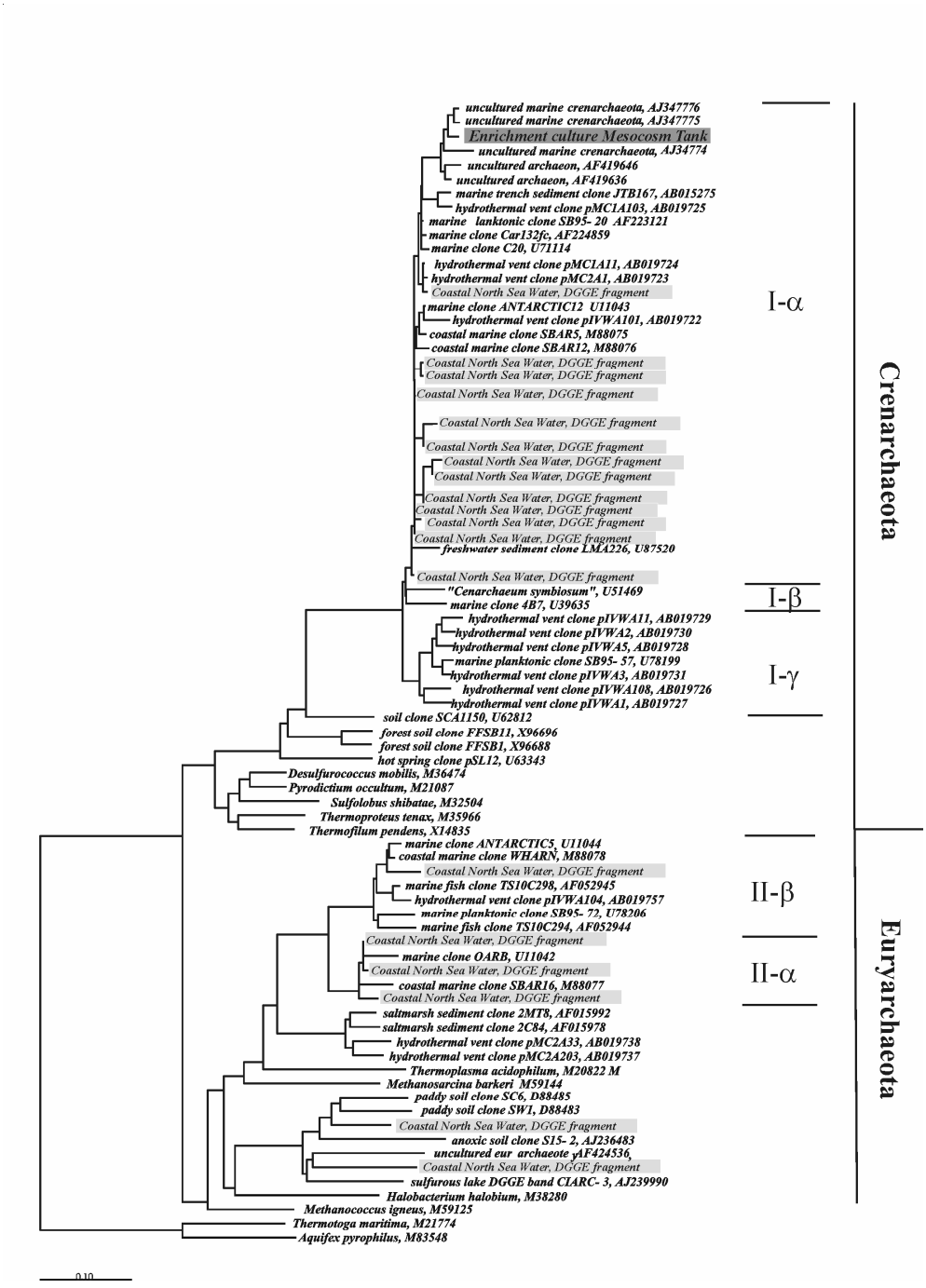

Figure S1. Partial phylogenetic tree of the Archaea showing the affiliation of the 15 partial crenarchaeal and 6 euryarchaeotal 16S rDNA gene sequences recovered from the North Sea waters time series 2002/2003 and the almost complete sequence of the Crenarchaeote in the enrichment culture (*E. coli*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

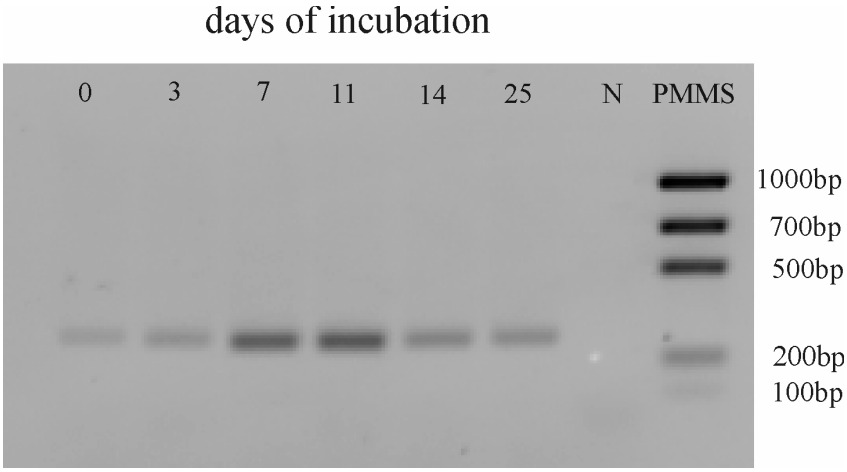

Figure S2. Agarose gel (2.5 wt%) with the Q-PCR product of the *amo*A like gene (256 bp) indicating the specificity of the Q-PCR reaction. Q-PCR was stopped after 39 cycles. N = negative control, PMMS = Precision Molecular Mass Ladder.

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

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

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

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

3.6 References

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Chapter 4

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

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Manuscript in preparation

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.

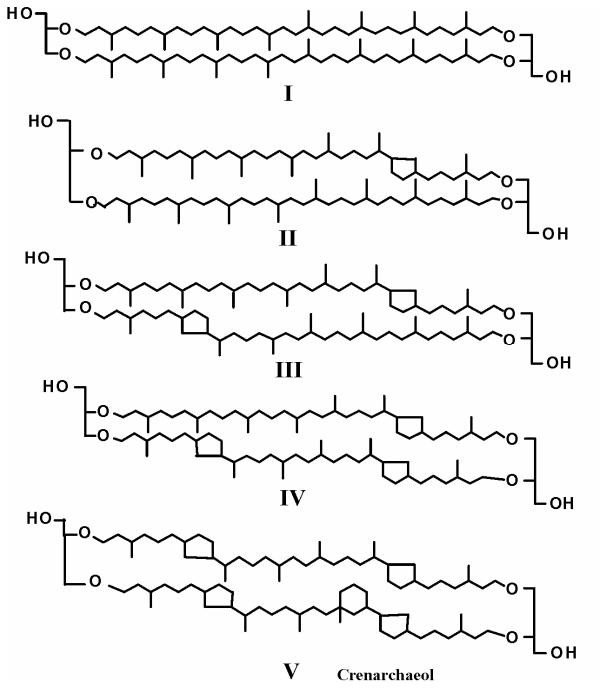

Figure 1. Structures of core tetraether membrane lipids of marine crenarchaeota.

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,

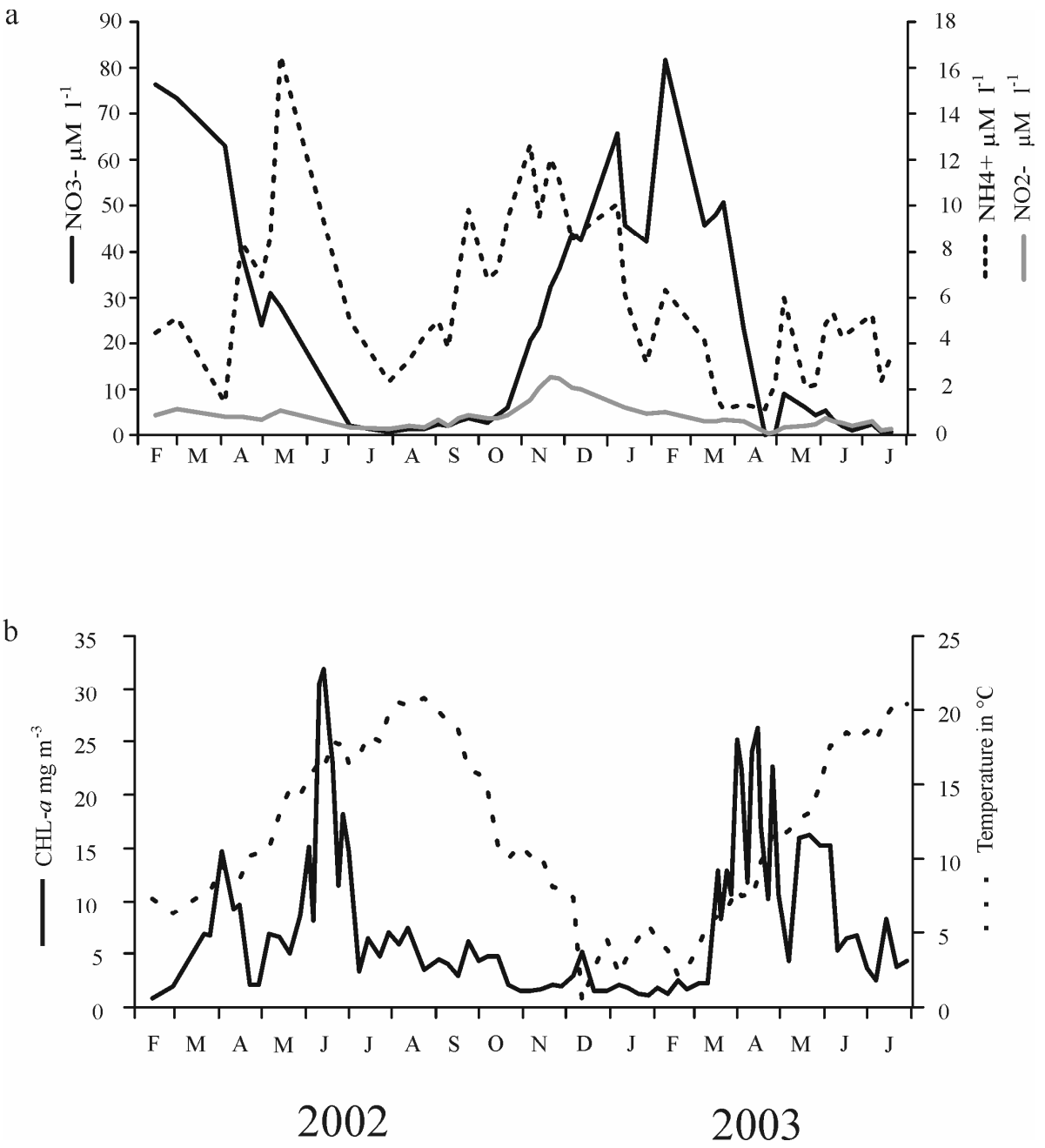

Figure 2. Nutrient concentrations (a) and, Chlorophyll-*a* and temperature development (b) at the investigated sampling site during February 2002 till July 2003.

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)

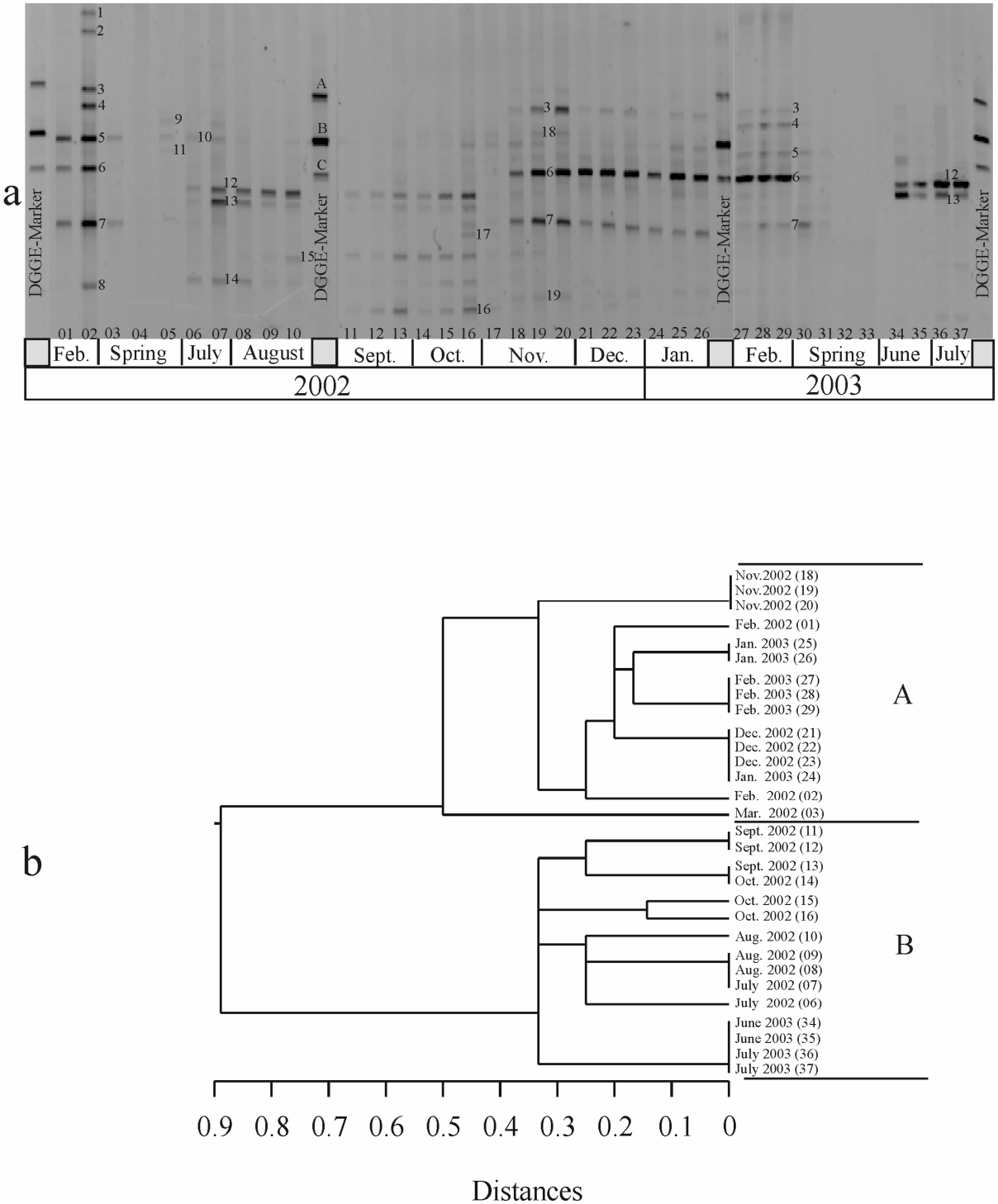

Figure 3. (a) DGGE analyses of 16S rRNA gene fragments of Archaea obtained from shallow North Sea surface waters over different seasons. Nineteen dominant partial 16S rDNA gene fragments of marine Archaea were detected. The DGGE marker is a PCR product from previous mesocosm experiments and used as a 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

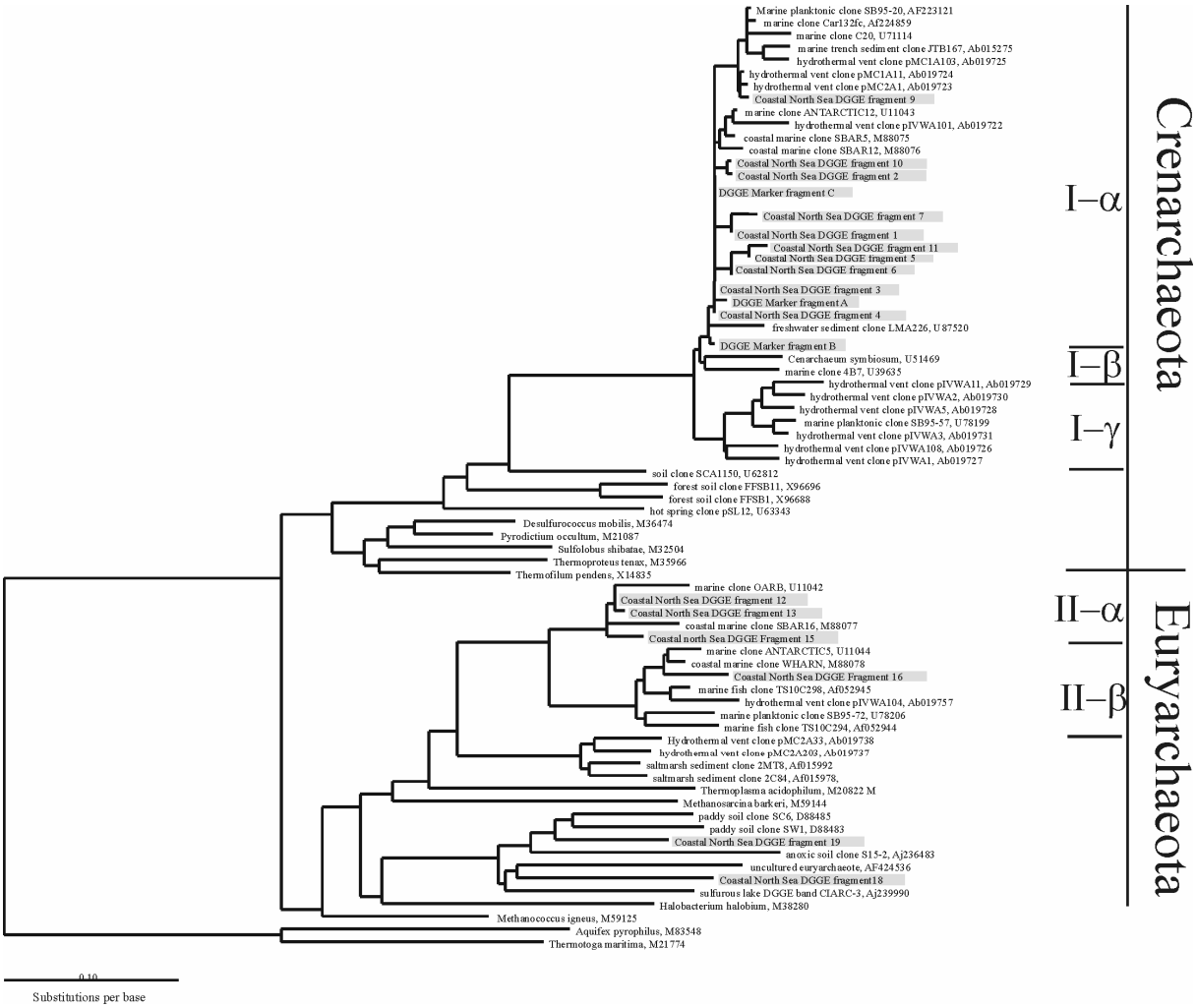

Figure 4. Phylogenetic tree showing the affiliation of the 19 partial archaeal 16S rDNA gene sequences recovered from the shallow coastal North Sea surface waters during 2002/2003 to reference sequences obtained from the GenBank database. All sequences are affiliated to the marine Crenarchaeota and Euryarchaeota.

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).

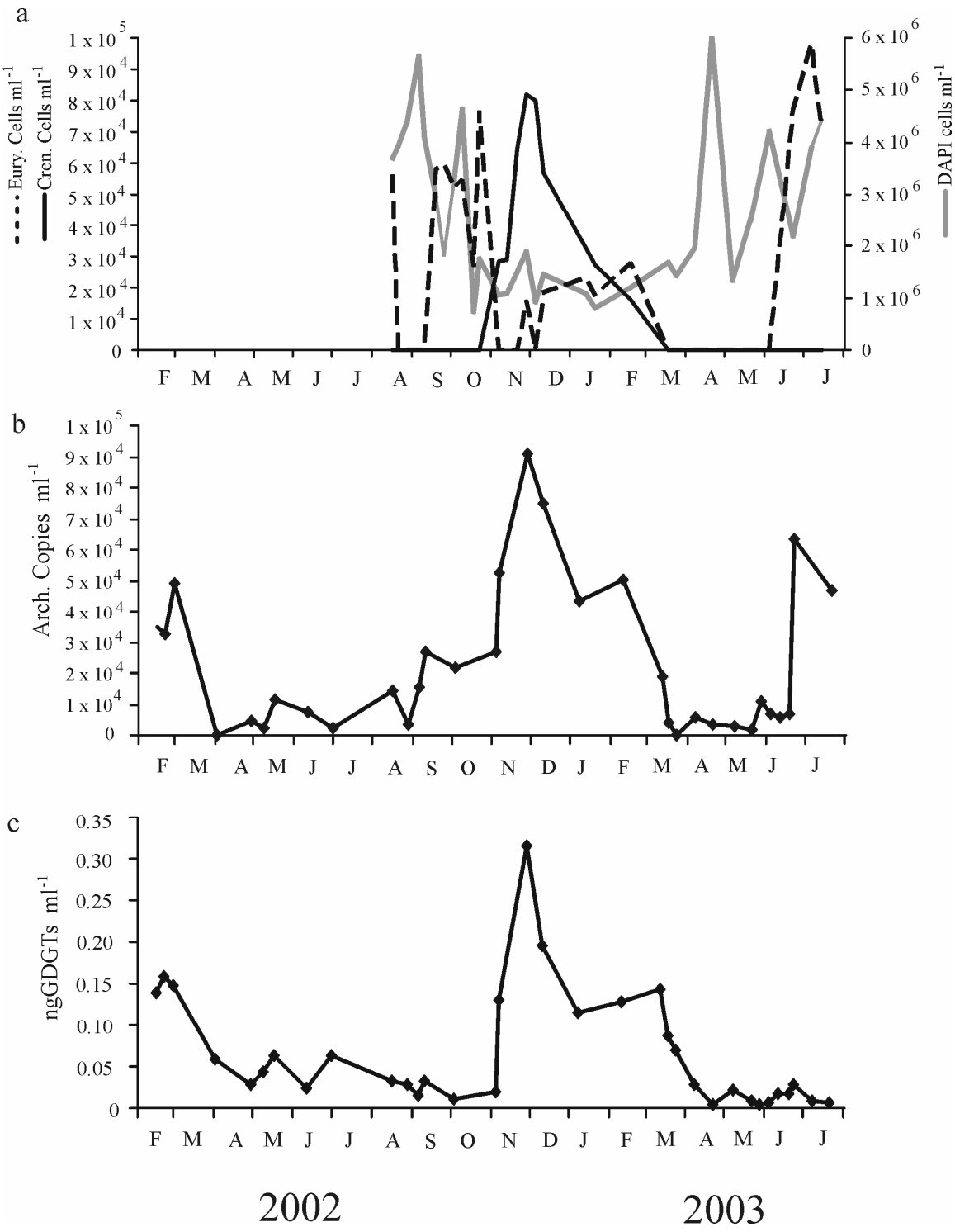

Figure 5. Seasonal dynamics in archaeal abundance (a), archaeal copy numbers (b), and archaeal tetraether lipid concentration (c) at the sampling site from August 2002 to July 2003 (a) and from February 2002 to July 2003 (b, c).

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.

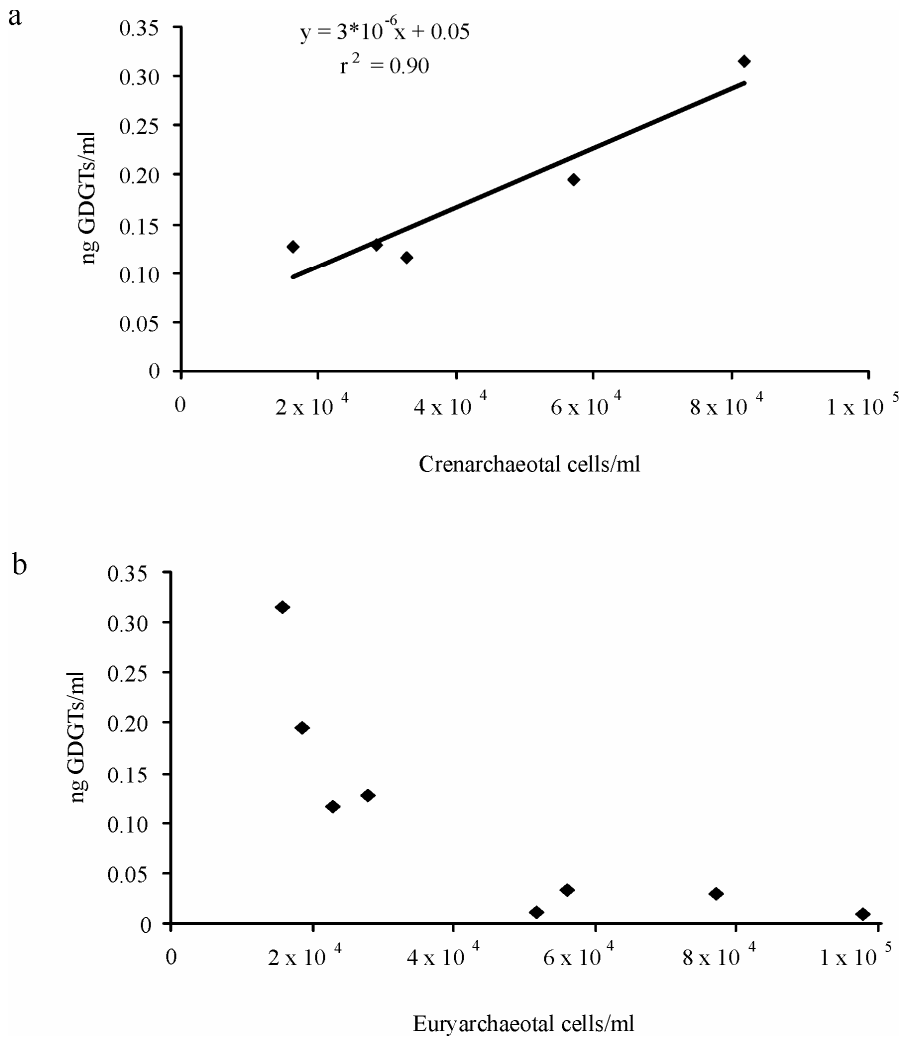

Figure 6. Relation between the (a) amount of GDGTs and marine crenarchaeotal cell numbers between Nov. 2002 to Feb. 2003, and (b) amount of GDGTs versus marine euryarchaeotal abundance between Aug. and Oct. 2002 and Jun. to- Jul. 2003.

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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Chapter 5

Temperature-dependent variation in the distribution of tetraether membrane lipids of marine Crenarchaeota: Implications for TEX_{86} paleothermometry

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Abstract.

Recently a new geochemical temperature proxy, the TEX_{86}, was introduced. This proxy is based on the number of cyclopentane moieties in the glycerol dialkyl glycerol tetraethers (GDGTs) of the membrane lipids of marine Crenarchaeota which changes as a response to temperature. However, until now only sediment data have been used to establish this proxy and experimental work is missing. We performed mesocosm studies with marine Crenarchaeota incubated at temperatures ranging from 5ºC to 35ºC and salinities of 27‰ and 35‰ to test the validity of the TEX_{86} proxy. Growth of marine Crenarchaeota in these mesocosms was evident from the substantial increase in the concentration of marine Crenarchaeotal membrane lipids with amounts up to 3400 ng/l. With increasing temperature an increase in the number of cyclopentane moieties in the crenarchaeotal membrane lipids was observed. Different salinities did not show any effect on the GDGT distribution. The TEX_{86} showed a significant linear correlation to incubation temperature: $TEX_{86} = 0.015*T + 0.10$ ($r^2 = 0.79$). This equation has a similar slope to the correlation obtained from core tops but differs in the intersection ($TEX_{86} = 0.015*T + 0.28$, $r^2 = 0.92$). This difference is mainly determined by the smaller amount of the regio-isomer of crenarchaeol in the incubation series compared to core top samples. These incubation experiments indicates that water temperature is indeed the major controlling factor for the membrane distribution of marine Crenarchaeota, and confirms that the TEX_{86} proxy depends on a physiological response to regulate membrane fluidity.

5.1 Introduction

One of the most important parameters to reconstruct climatic changes in ancient environments is the temperature distribution at the surface of the ocean. With the knowledge of past temperature conditions, it is possible to reconstruct climatic changes, ocean circulation and biogeography [*Fischer and Wefer,* 1999]. For the reconstruction of the sea surface temperature (SST) in ancient environments several geochemical proxies have been developed over the years. Commonly used proxies are the $\delta^{18}O$ and Mg/Ca ratio of planktonic foraminifera [*Erez and Luz,* 1983] and the $U^{K'}_{37}$ ratio of long-chain unsaturated ketones from haptophyte algae [*Brassell et al.,* 1986]. However, some problems exist using planktonic foraminifera or alkenones to reconstruct SST. For foraminifera, these uncertainties concerns the depth habitat of foraminifera species, the state of preservation of their shells, the carbonate concentration and the original oxygen isotope composition of the sea water [e.g *Spero et al.,* 1997]. Alkenones are more robust in this respect for SST reconstruction. However, until now it is not clear what the biochemical role of alkenones in haptophyte algae is and why the distribution of alkenones responds to temperature. An additional problem is that in some settings alkenones do not occur in sufficient amounts for paleothermometry. Furthermore, alkenones have generally not been used for SST reconstruction in sediments older than the Late Ouarternary [*Schneider* 2001].

Recently, a new organic geochemical SST proxy, the TetraEther Index of lipids with 86 carbon atoms, the TEX_{86}, was introduced [*Schouten et al.,* 2002]. This proxy is based on the number of cyclopentane rings in the membrane lipids of marine Crenarchaeota. Marine Crenarchaeota belong to the domain of Archaea. Until recently archaea were thought to dwell only in ecological niches characterized by extreme conditions such as high salinity, high temperature and anoxia. Ecological studies using 16S rDNA and lipid analyses showed, however, that Archaea are far more widespread and abundant than previously thought in temperate environments such as the open ocean [*Fuhrman et al.,* 1992; *DeLong,* 1992; *Hoefs et al.,* 1997; *Sinninghe Damsté et al.,* 2002a]. One of the three major groups of non-thermophilic archaea, which appear to be a widely distributed, abundant and ecologically diverse group, is found in the kingdom of Crenarchaeota [*DeLong et al.,* 1998]. Recent molecular biological work demonstrated that marine Crenarchaeota are distributed over a large depth range in the photic and aphotic zones of the water column and account for ca. 20%

of the picoplankton in the world's ocean [*Karner et al.,* 2001]. ^{13}C tracer experiments revealed that marine Crenarchaeota can utilize bicarbonate [*Wuchter et al.,* 2003] and may represent a significant sink for inorganic carbon in the global carbon cycle.

The membrane lipids of archaea are unique and consist of isoprenoid glycerol dialkyl glycerol diethers and glycerol dibiphytanyl glycerol tetraethers (GDGT's) (Fig. 1) [*DeRosa and Gambacorta,* 1988]. One specific GDGT, containing four cyclopentane rings and one cyclohexane ring, occurs exclusively in non-thermophilic Crenarchaeota and was named crenarchaeol [*Sinninghe Damsté et al.,* 2002b] (Fig. 1). The membrane lipids of Crenarchaeota have been detected in sediments of more than 112 million years old [*Kuypers et al.,* 2001]

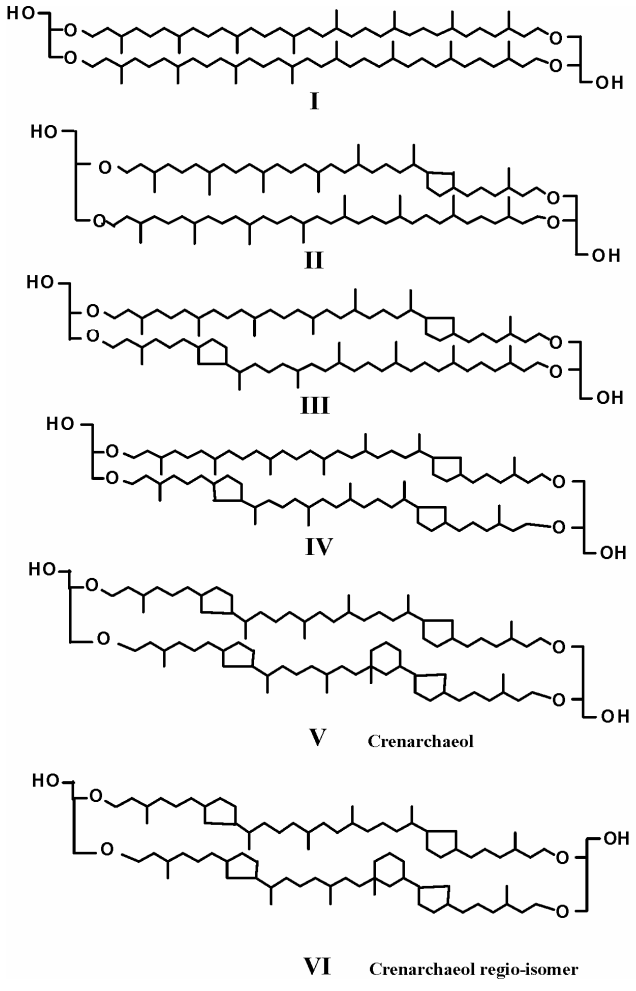

Figure 1. Structures of intact core tetraether membrane lipids of marine pelagic Crenarchaeota. The structure of the stereoisomer (VI) of crenarchaeol is likely a regio-isomer (Sinninghe Damsté et al., unpublished results).

The distribution of crenarchaeotal GDGT's in core top sediments from different geographical locations varies with SST [*Schouten et al.,* 2002]. The GDGT distribution in surface sediments from cold areas consist almost completely of GDGT I and crenarchaeol, whereas GDGT distribution in surface sediments from warmer areas are dominated by crenarchaeol and contain relatively high amounts of the 1-3 cyclopentane containing GDGT's (II-IV) and a regio-isomer of crenarchaeol (VI) [*Schouten et al.,* 2002] (Fig.1). The change in the GDGT distribution was expressed in an index of GDGT isomers, which was named the TEX_{86} and is defined as follows;

$$TEX_{86} = \frac{III+IV+VI}{II+III+IV+VI} \quad [1]$$

The correlation of this index to the annual mean SST gave the following linear equation;

$$TEX_{86} = 0.015*T+0.28, (r^2=0.92) \quad [2]$$

with T = annual mean SST (in ºC). The observed correlation of the GDGT distribution in core top sediments with SST does not provide direct evidence that marine Crenarchaeota adjust their membrane lipids to temperature or that temperature is the only controlling factor. Salinity and nutrient concentrations, for instance, can also vary to a substantial degree in marine systems. However, culture experiments with hyperthermophilic Crenarchaeota, close phylogenetic relatives of marine Crenarchaeota [*DeLong et al.,* 1998], have shown that the relative amount of cyclopentane moieties in GDGT's strongly depend on temperature [*Gliozzi et al.,* 1983; *DeRosa and Gambacorta,* 1988; *Uda et al.,* 2001]. With increasing temperature an increase in the relative amount of cyclopentane moieties in the membrane lipids was found. Temperature-dependent changes in the archaeal membrane lipid composition keeps their cytoplasmatic membrane at a liquid crystalline state and reduces their proton permeation rate [*Albers et al.,* 2000]. Therefore, the biosynthesis of cyclopentane rings in the membrane lipids of thermophilic archaea is considered to be a mechanism for temperature adaptation of the membrane. For marine Crenarchaeota, however, this experimental proof is still lacking. To the best of our knowledge, attempts to isolate and culture these microorganisms have not yet been successful, mainly because there is little known about their basic physiology. In this

study North Sea water was incubated at different temperature and salinities using mesocosm tanks to determine the response of the membrane lipid composition of marine Crenarchaeota to temperature and salinity. The TEX_{86} values were calculated from the GDGT distribution and correlated with the incubation temperatures in order to validate the new temperature proxy, TEX_{86}.

5.2 Material and Methods

5.2.1 Growth of marine Crenarchaeota in mesocosm tanks

Two mesocosm tanks with a volume of 850 l were filled with high-tide shallow North Sea water in July 2002. Initial nutrient concentrations were 0.5µM NO_3^-, 0.1µM NO_2^-, 1.2µM NH_4^+ and 0.1µM PO_4^{3-}. Initial temperature was 15ºC, salinity was 27‰ and pH was 8.2. The mesocosms were kept at a constant temperature by a water mantle filled with tap water which was continuously mixed by a pump system. A stirring wheel in each of the mesocosm tanks provided optimum mixing (0.5 rpm) of the North Sea water. The mesocosm systems were open so there was constant gas exchange with the open air. The North Sea water in the mesocosm tanks was incubated under different conditions. By using heating elements the water mantle in mesocosm tank I was slowly heated up to 27ºC within one week and kept constant for three months. In mesocosm tank II the water mantle was cooled down to 13ºC within one day with a cooling system and kept constant for three months. More detailed information of the mesocosm tank system is described in *Brussaard et al.,* [2004]. Both tanks were incubated in the dark. No additional nutrients were added to the North Sea water. Salinity and pH were regularly measured using a EUTECH CyberScan 510 pH meter and a salinity refractometer. Salinity and pH remained constant over the three month of incubation. Water samples were taken for nutrients, lipid and DNA analyses weekly for three months. Nutrients were measured spectro-photometrical using an auto-analyzer system Bran and Luebbe TRAACS 800+.

5.2.2 Incubation series

For temperature and salinity incubation experiments we used Nalgene Clearboy 20 l tanks. At the end of the 850 l mesocosm experiments two incubation series were started with water from mesocosm tank I or II, respectively. The tanks were incubated in the dark under different

temperature and salinity conditions i.e. 5, 10, 15, 22, 25, 30 and 35ºC at 27‰ and 15 and 25ºC at 35‰. The tanks at 5 to 22ºC were incubated in climate controlled rooms. For the higher incubation temperatures (25-35ºC), the tanks were heated with heating elements. To study the effect of salinity, the salinity of the water in the tanks was adjusted to 35‰ by adding sterile NaCl. Each tank was set on a stirring plate and was continuously stirred to provide good mixing of the water. The tanks were open during the experiment, allowing constant gas exchange with the air. Because of the oligotrophic water conditions at the end of the mesocosm experiments, additional nutrients were added before incubation in the 20l tanks. Initial added nutrient concentrations per liter were 150 µM $NaNO_3$, 150 µM NH_4Cl, 25 µM NaH_2PO_4 and 2666 µM $NaHCO_3$ according to *Stolte et al.,* [1994]. Also a sterile mix of 250 mg yeast and 100 mg pepton extract was added in each tank. Vitamins and trace elements were added as well according to *Veldhuis and Admiraal* [1987]. The temperature, pH and salinity were regularly measured for each tank and kept constant. The pH showed a variation between 7.9-8.5 and was regularly adjusted to 8.2, by addition of sterile 0.1M NaOH or HCl. Due to small evaporation effects in some tanks salinity was adjusted to 27‰ by adding de-mineralized H_2O. Lipid and DNA were sampled at the start and at the end of the experiments (i.e. three months).

5.2.3 DNA extraction and analysis

For DNA analysis 1 l of water was filtered through a 0.2 µm pore size polycarbonate filter in order to collect particulate organic matter for subsequent DNA extraction. DNA was extracted with phenol, phenol/chloroform/isoamyl-alcohol and chloroform and precipitated using ice cold ethanol according to *Sambrook* [1989]. Partial archaeal 16S rRNA genes were amplified by polymerase chain reaction (PCR) using primers Parch 519f (complementary reverse sequence of PARCH 519r [*Øvreas et al.,* 1997] and GCArch 915r [*Stahl and Amann* 1991], including a 40bp long GC clamp [*Muyzer et al.,* 1993] as described by *Coolen et al.,* [2002].The PCR protocol was applied as described previously [*Coolen et al.,* 2002; *Wuchter et al.,* 2003] and the PCR products were separated by denaturing gradient gel electrophoreses (DGGE) using conditions described previously [*Coolen et al.,* 2002; *Wuchter et al.,* 2003]. DGGE fragments were excised from the gel and the individual 16S rRNA genes were subsequently sequenced. The partial sequences were analyzed using BLAST at the NCIB

database (*http://ncbi.nlm.nih.gov/BLAST*) and added together with the most important BLAST hits, to alignment of all available archaeal 16S rRNA sequences by using the aligning tool of the ARB software package. Trees were generated by using maximum parsimony as implemented in ARB.

5.2.4 Lipid extraction and analysis

Water was filtered through a 0.7 µm ashed glass fiber filter. The filters were freeze dried and the filters were ultrasonically extracted with methanol, dichloromethane (DCM)/methanol (1:1, v/v) and three times with DCM. An aliquot of the total lipid extract was cleaned over an activated Al_2O_3 column by eluting with methanol/DCM (1:1, vol/vol). For analysis of intact GDGT's 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 trough a 0.45-µm-pore-size, 4 mm diameter Teflon filter prior to injection. The intact GDGT's were analyzed by high performance liquid chromatography (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 to 5 µl. GDGT's 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 atmospheric pressure positive ion chemical ionization mass spectrometry (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 temperature 200 °C, capillary voltage -3 kV, corona 5 µA (~ 3.2 kV). GDGTs were detected by Single Ion Monotoring of their $[M+H]^+$ ions and quantified by integration of the peak areas and comparison with a standard curve of a GDGT-0 standard.

5.3 Results

5.3.1 Growth of marine Crenarchaeota in mesocosm tanks

Two mesocosm tank experiments were set up to monitor the response of marine crenarchaeotal GDGT's to different temperature conditions, i.e. 13˚C and 27˚C. The tanks were incubated in the dark because marine Crenarchaeota are relatively more abundant in the aphotic zone of the water column [*Karner et al.,* 2001, *Sinninghe Damsté et al.,* 2002a] and recent incubation experiments revealed that marine Crenarchaeota can grow chemoautotrophically in the dark [*Wuchter et al.,* 2003]. In the initial shallow North Sea water (July 2002) the total GDGT concentration was low at 18 ng/l and the GDGT distribution was dominated by GDGT-0 (I) and crenarchaeol (V) (Fig. 2a). No archaeal 16S rDNA was detected in the initial water as the archaeal 16S rDNA concentration was under the detection limit.

During three months of incubation, the GDGT abundance and distribution and the 16S rDNA pattern changed substantially in the two different mesocosm tanks. After about 5 weeks an increase of GDGTs was measured and archaeal 16S rDNA was detected in both mesocosm tanks (e.g. Fig.3). In mesocosm tank I, incubated at 27˚C, a substantial increase in the total GDGT concentration was measured with values reaching 650 ng/l, i.e. a ca. 35 fold increase (Fig.3). The initial GDGT distribution changed into a distribution dominated by crenarchaeol and with relatively higher amounts of cyclopentane containing GDGT's (II-IV) and the regio-isomer of crenarchaeol (VI) (Fig. 2b). In both mesocosm tanks the nutrient concentrations changed in a similar way. Nitrate concentrations increased and ammonium concentrations decreased when GDGTs concentrations increased (e.g. Fig. 3 for 27˚C tank). A weaker, negative correlation with $[NO_2]^-$ and GDGT abundance was also observed in both tanks (Fig. 3). Phosphate concentrations did not change substantially during the incubation time in both tanks and ranged between 0.1 µmol/l and 1.0 µmol/l. During the three months of incubation the TEX_{86} values for mesocosm tank I increased and was at 0.56 at the end of the experiment. Once the exponential growth took place the GDGT distribution, and therefore the TEX_{86} values, did not change any more. DGGE analyses of archaeal 16S rDNA for mesocosm tank I revealed that there were two archaeal phylotypes. However, at the end of the experiment there was only one archaeal phylotype present in the mesocosm water (Fig. 3). Sequencing of these

16S rDNA fragments showed that both phylotypes belong to the marine Crenarchaeota (Fig. 4b).

In mesocosm tank II, incubated at 13˚C, a five-fold increase in the GDGT concentration was measured with concentrations reaching 90 ng/l. However, in contrast to mesocosm tank I the relative distribution of the GDGT's did not change as it was still dominated by GDGT-0 (I) and crenarchaeol (V) (Fig. 2c). The TEX_{86} value for Tank II after three months of incubation was 0.26. Two dominant archaeal rDNA fragments were detected by DGGE analyses, pointing towards two dominant archaeal phylotypes in this tank (Fig. 4a). Both phylotypes belong to the marine Crenarchaeota (Fig. 4b). Both Crenarchaeotal phylotypes in mesocosm tank II were identical to the Crenarchaeotal phylotypes of mesocosm tank I (Fig. 4b). These mesocosm waters were subsequently used for smaller scale incubation experiments.

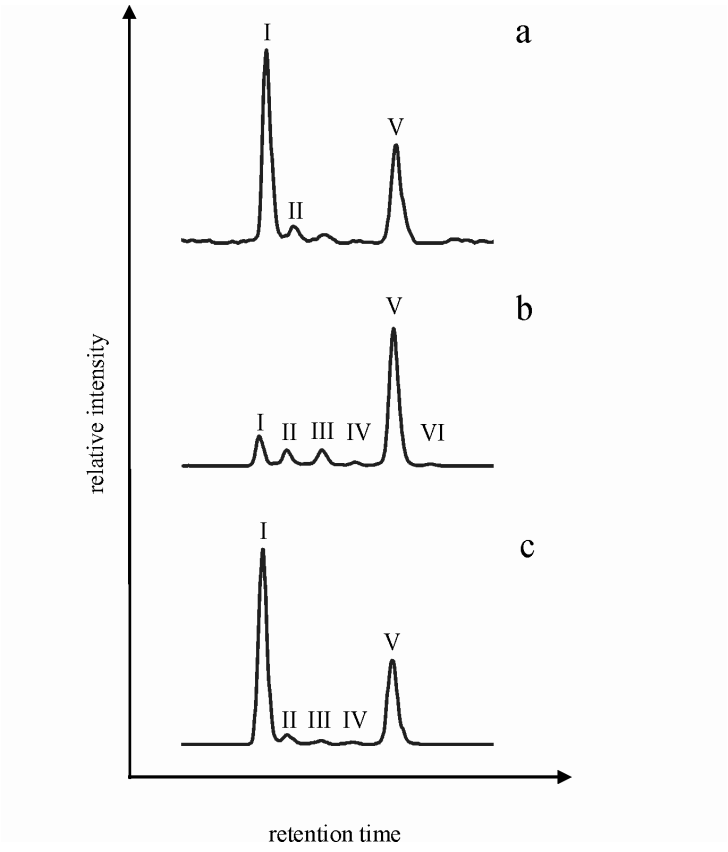

Figure 2. Partial HPLC-APCI/MS base peak chromatograms of particulate organic matter derived from the mesocosm tank experiments: (a) GDGT pattern of the initial water used for the mesocosm experiment, (b) final GDGT pattern of the water from mesocosm tank I incubated at 27ºC for three month and (c) final GDGT pattern of the water from mesocosm tank II incubated at 13ºC for three month. Numbers indicating structures drawn in Fig. 1.

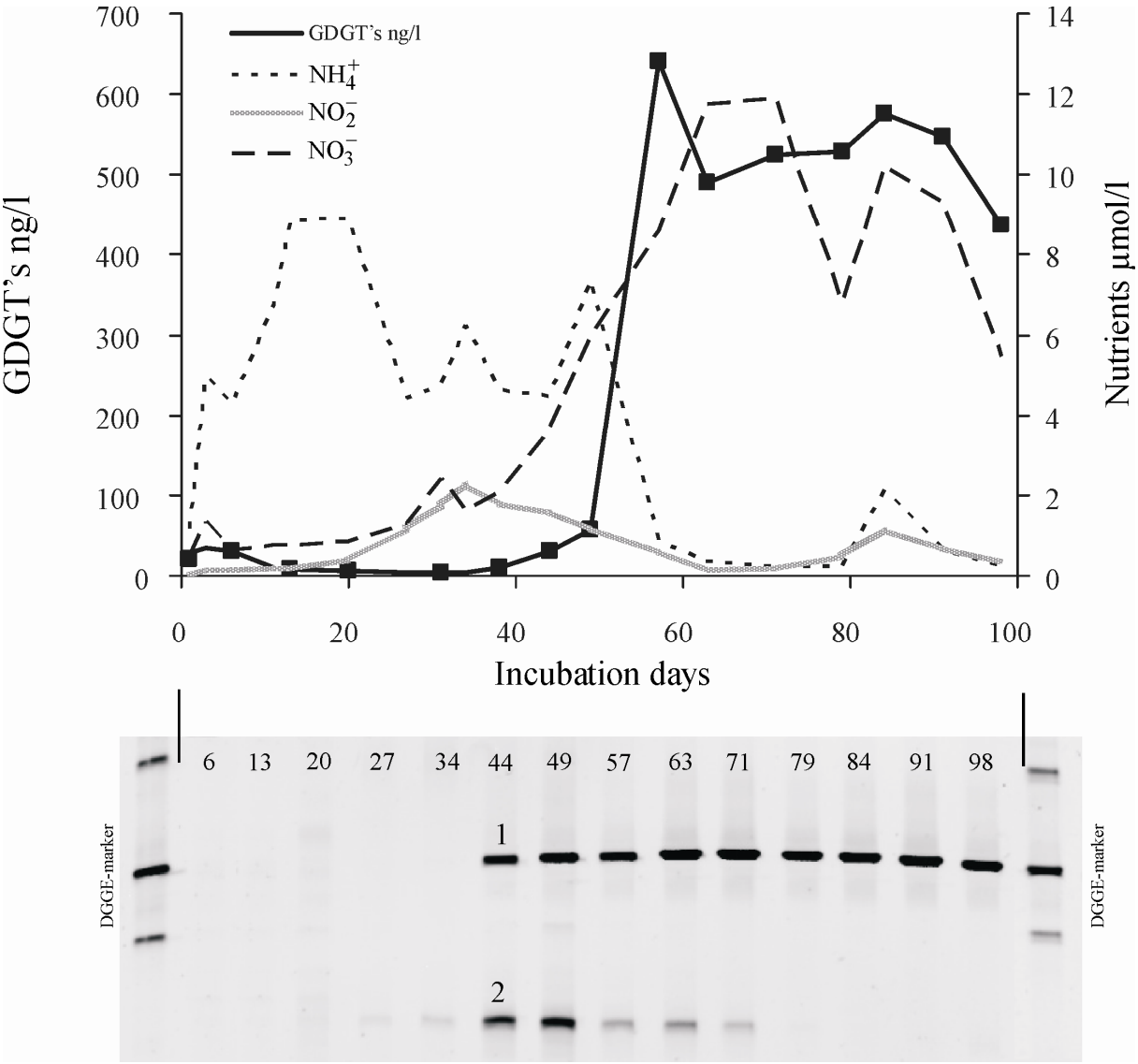

Figure 3. (top) Nutrient and GDGT concnetrations in mesocosm tank I incubated at 27°C with time. Results for mesocosm tank II incubated at 13ºC were similar (data not shown). Phosphate concentration in both mesocosm tanks remained constant and ranged between 0.1 µmol/l and 1.0 µmol/l during the incubation time.
(bottom) DGGE analysis of PCR amplified 16S rRNA genes fragments of Archaea obtained from mesocosm tank I incubated at 27°C. Both sequences obtained from both mesocosm tanks are affiliated to the marine Crenarchaeota (see Fig.4).

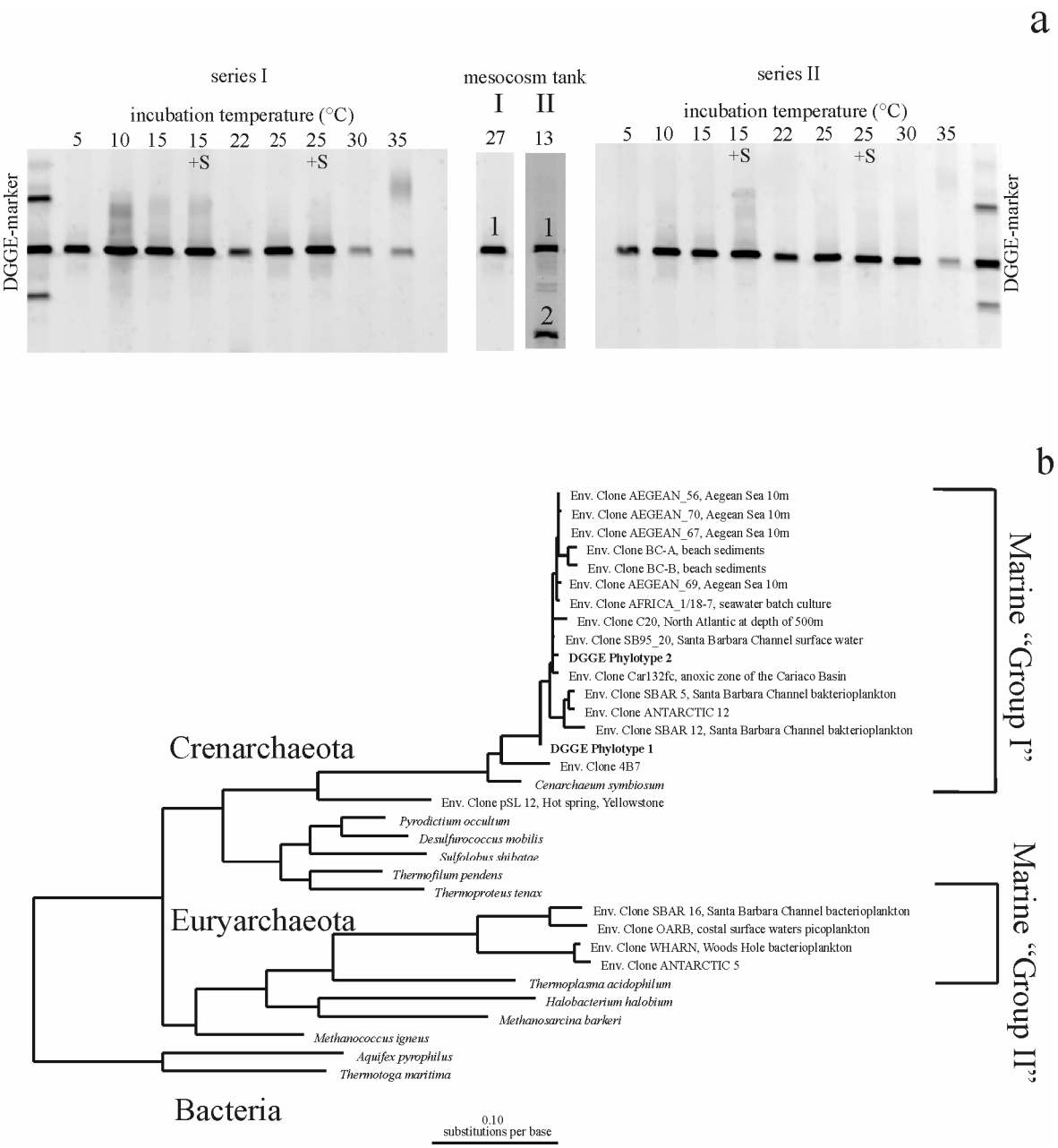

Figure 4. (a) DGGE analysis of PCR amplified 16S rRNA genes fragments of Archaea obtained from mesocosm tank I and II and incubation series I and II, all after three month incubation at the indicated temperature. Experiments performed at elevated salinity are denoted by +S. One dominant partial 16S rDNA fragment (phylotype 1) was detected in the mesocosm tank I and II and also for incubation series I and II at all incubation temperatures. In mesocosm tank I and II a second partial 16S rDNA fragment was detected and is called phylotype 2. The DGGE marker is an archaeal PCR product from previous mesocosm experiments and used as a ruler to monitor the melting position of the archaeal amplicons within the gel. (b) Phylogenetic tree showing the affiliation of two partial 16S rDNA gene sequences of Crenarchaeota recovered from the mesocosm tanks and incubation series (phylotype 1 and 2) to reference sequences obtained from the GenBank database. Both sequences are affiliated to the marine Crenarchaeota.

5.3.2 Incubation series

Smaller scale incubation experiments were set up to monitor the response of marine crenarchaeotal GDGT's to a wider range of temperature and salinity conditions. Water from both mesocosm tank I and tank II was incubated in the dark at temperatures ranging from 5-35˚C and salinities of 27 and 35‰. The initial GDGT concentration for each tank was ca. 30 ng l^{-1}. After three months in both incubation series a substantial increase in GDGT concentration was observed. In both series I and II the highest amount of GDGT's was detected in the 25ºC experiment, reaching almost 1800 and 3400 ng l^{-1}, respectively (Fig. 5). Generally, the highest GDGT concentrations were measured in the temperature range between 15 and 30ºC (Fig. 5) with sometimes a more than 100 fold increase in GDGT concentration relative to the initial water.

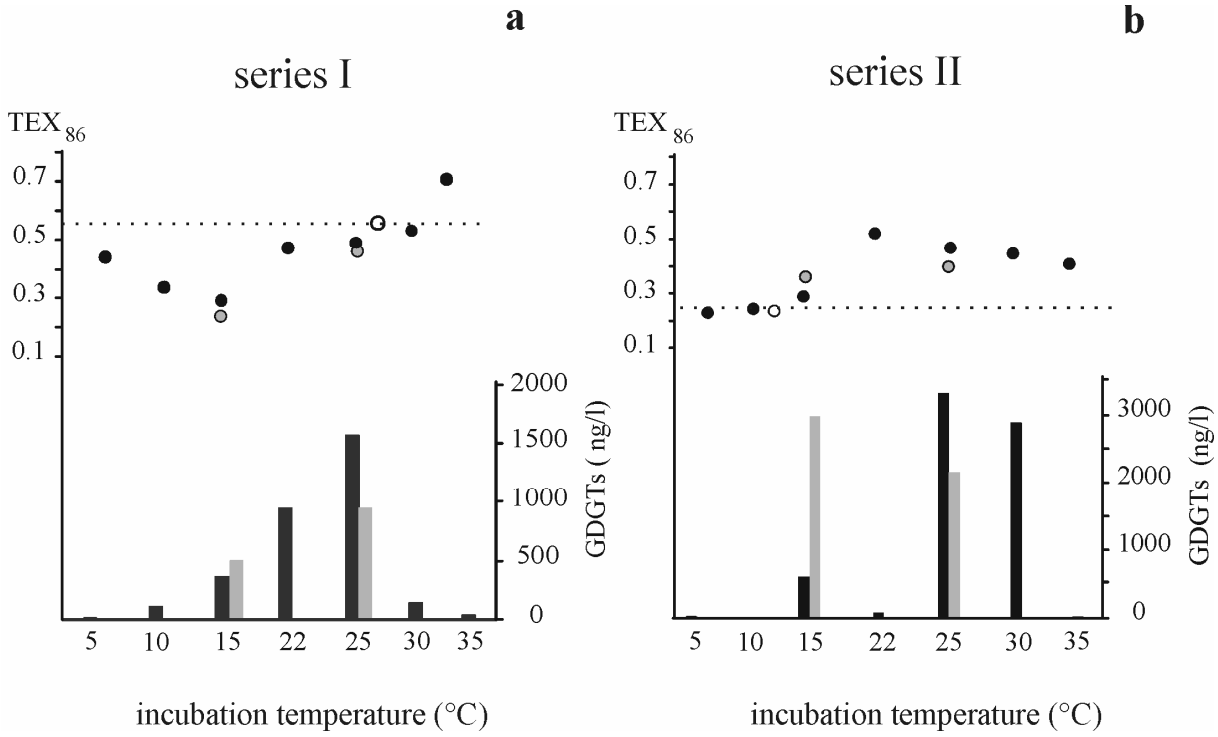

Figure 5. Concentration of newly produced GDGT's (i.e. corrected for the initial concentration) in tanks incubated at different temperatures and salinities for three months for (a) incubation series I and (b) incubation series II. In the upper parts of the graph TEX_{86} values calculated from the GDGT distribution are plotted. Black bars and dots represent incubation at 27‰ salinity and gray bars and dots incubation at 35‰ salinity. Open dots and stippled lines represent TEX_{86} values from the initial water used for incubation series I and II. For incubation series I, the initial water is derived from mesocosm tank I incubated at 27ºC. For incubation series II the initial water is derived from mesocosm tank II incubated at 13ºC.

The distributions of GDGT's in the different tanks changed from the GDGT pattern of the initial mesocosm tank water, especially in the cases where a substantial increase in GDGTs was observed. For both incubation series, GDGT distributions in tanks with temperatures <20ºC were dominated by GDGT-0 (I) and, to a lesser extent, crenarchaeol (Fig. 6a). In tanks with higher temperatures (>20ºC) crenarchaeol was the most prominent GDGT and relatively higher amounts of cyclopentane ring-containing GDGT's (II-IV) were observed (Fig. 6b-d). The tanks incubated at different salinities but the same temperature did not show any major difference in the distributions of GDGT's (Fig. 6 b,c).

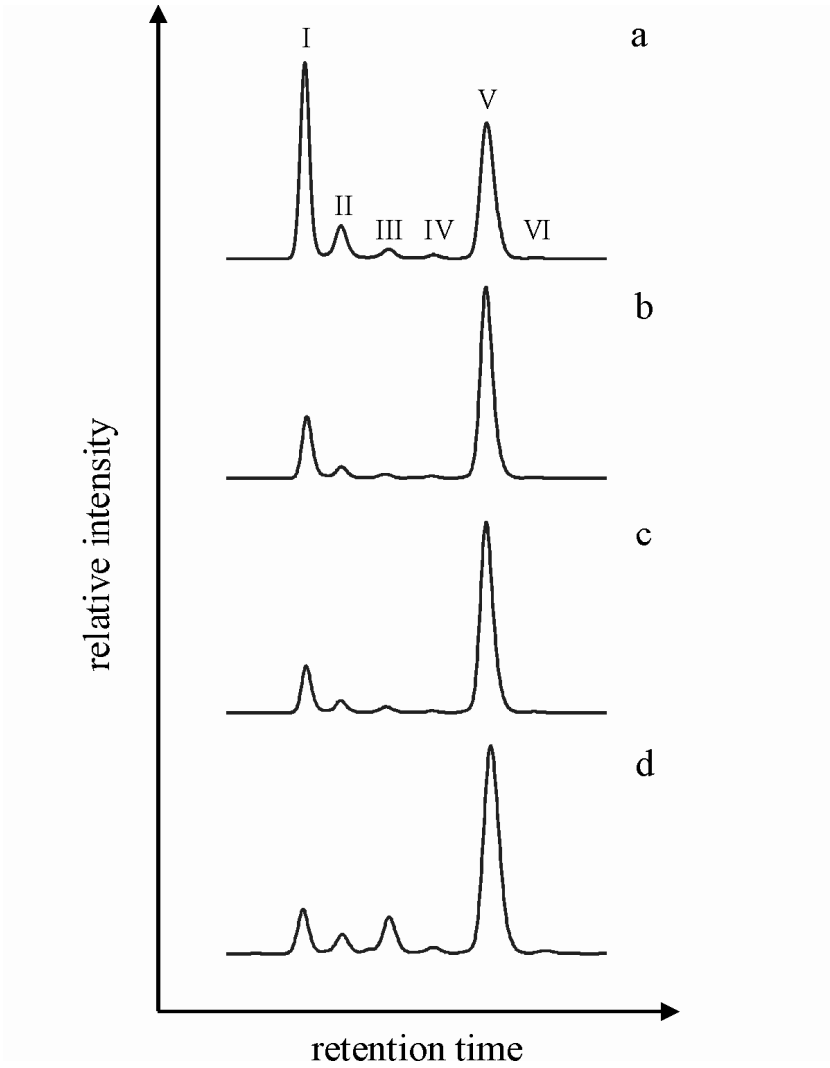

Figure 6. HPLC-APCI/MS base peak chromatograms of particulate organic matter from incubation series I at different temperatures and salinities, i.e. (a) 10ºC, 27‰; (b) 25ºC, 27‰; (c) 25ºC, 35‰; (d) 35ºC, 27‰. Numbers indicate structures drawn in Fig. 1.

For both mesocosm tank series the TEX_{86} values changed from the initial TEX_{86} (indicated by the dashed line in Fig. 5) and followed a similar pattern, i.e. TEX_{86} values were higher at higher temperatures (Fig. 5). In contrast, no substantial difference for the TEX_{86} values were observed in water incubated at different salinities but with the same temperatures, showing an average variation of 0.02 ±0.06 (Fig.5, grey open dots and grey bars).

In each tank of both incubation series one dominant 16S rDNA fragment was detected by DGGE analysis at the end of the experiments (Fig. 4a). This rDNA fragment belonged to the marine Crenarchaeota and was identical to phylotype-1 detected at the start of the incubation experiments. Phylotype-2 present in mesocosm tank II disappeared in the course of the incubation series (Fig. 4a). Different salinities and temperatures did not show any effect on the archaeal species distribution (Fig. 4a).

5.4 Discussion:

5.4.1 Origin of change in GDGT distribution

One of the major problems to validate the temperature proxy TEX_{86} is the fact that until now no pure culture is available of marine Crenarchaeota. However, our data show that GDGT concentrations strongly increase at the same time that 16S rDNA from marine Crenarchaeota could be amplified (Fig. 3). Furthermore, GDGTs are very unique membrane lipids and until now they only have been detected in the domain of the Archaea. This strongly suggests that the GDGT signal measured in the mesocosm tanks is derived from marine Crenarchaeota. This is supported by the fact that the two archaeal species found in these incubation experiments belonged to the marine Crenarchaeota (Fig. 4b). These two crenarchaeotal phylotypes were closely related and showed a sequence homology of 99%. The sequence homology between the two phylotypes and *Cenarchaeum symbiosum*, which is known to produce GDGTs I-VI [*DeLong et al.,* 1998.], was between 96.3-96.6%. The most prominent marine Crenarchaeota was phylotype-1 and was found in the incubation series at all temperatures, indicating that the GDGT's are probably mostly derived from this species. Thus, the observed changes in GDGT distribution and abundance are mostly due to the growth and physiological response of a single crenarchaeotal species.

In the mesocosm tank experiments, and in most of the small scale incubation series, GDGT concentrations increased substantially. Since GDGT are membrane lipids, this

indicates that Crenarchaeota were actually growing under the applied conditions. The largest increase in GDGT concentration occurred at temperatures between 15 and 30ºC (Fig. 5). This seems to be the most effective growth range for the species of marine Crenarchaeota which proliferated under our laboratory conditions.

The GDGT distribution changed substantially with temperatures. At higher temperatures a relatively higher amount of cyclopentane ring-containing GDGT's were detected. The relative amount of the isomers of the GDGT II-V increased also at higher temperatures. The relative distribution of GDGT's did not depend on the concentration of GDGT's. For example, in incubation series I and II at 25ºC no substantial difference in the relative GDGT distribution was apparent at concentrations at 1000 or 3400 ng l^{-1}. This suggests that, once substantial growth took place, the relative distribution is not determined by growth of the organism and, hence, nutrient conditions. This is supported by the nutrient and lipid data of the mesocosm tank experiments. As GDGT abundance increases the nutrients followed the same pattern in both mesocosm tanks with a decrease in $[NH_4]^+$ and $[NO_2]^-$ and an increase in $[NO_3]^-$ (Fig.3). Despite this identical change in nutrient conditions TEX_{86} values were different and stayed different during the stationary phase between the two tanks. This strongly suggests that growth rate and nutrients do not influence the TEX_{86} values. Different salinities did not substantially influence the GDGT distribution either as was show by the similar TEX_{86} values in tanks of different salinities (Fig. 5).

Therefore, our experiments suggest that temperature is the major controlling factor for the relative GDGT distribution in the membranes of marine Crenarchaeota. Since the incubation experiments are dominated by one crenarchaeotal phylotype the response of the membrane composition to temperature must be a physiological adaptation. This is in good agreement with culture experiments of hyperthermophilic Crenarchaeota, genetically close relatives of marine Crenarchaeota, which showed that the relative amount of cyclopentane moieties in GDGT's mainly depend on culture temperature [*Gliozzi et al.,* 1983; *DeRosa and Gambacorta,* 1988; *Uda et al.,* 2001].

5.4.2 TEX_{86} calibration

The calculated TEX_{86} values for the incubation experiments are clearly correlated with the incubation temperature. In calculating the relationship of TEX_{86} with temperature we only used TEX_{86} values from tanks in which at least a doubling of the initial amount of GDGT's took place within three months. The TEX_{86} values obtained at the end of the initial mesocosm experiments were also included in this correlation. The correlation line that fitted the data best is a polynome:

$$TEX_{86} = 0.0003*T^2 + 0.0037T + 0.21 \text{ with } r^2 = 0.80, (n=15) \quad [3]$$

However, the r^2 of a linear fit was similar (Fig.7a)

$$TEX_{86} = 0.015*T + 0.10 \text{ with } r^2 = 0.79, n=15 \quad [4]$$

This equation can now be compared to the core top calibration line (Fig.7b) as reported by *Schouten et al.,* [2002]. The data set of *Schouten et al*. [2002] has been extended by 18 additional core top sediments which did not substantially change the original linear correlation.

$$TEX_{86} = 0.015*T + 0.29 \text{ with } r^2 = 0.92 , n = 58 \quad [5]$$

Equations 4 and 5 have the same slope but differ in the intersection with the y–axes. The most obvious difference between the GDGT distribution of the incubation series and the core top samples is the relative amount of crenarchaeol isomer. In the incubation series the percentage of crenarchaeol isomer from the total GDGTs is substantially lower than in the core top sediments. For instance, at 27°C the crenarchaeol isomer comprises ca. 9% of the total amount of GDGTs in the core top samples, whereas in the incubation series at 25°C the crenarchaeol isomer reaches only 0.6% of the total amount of GDGTs.

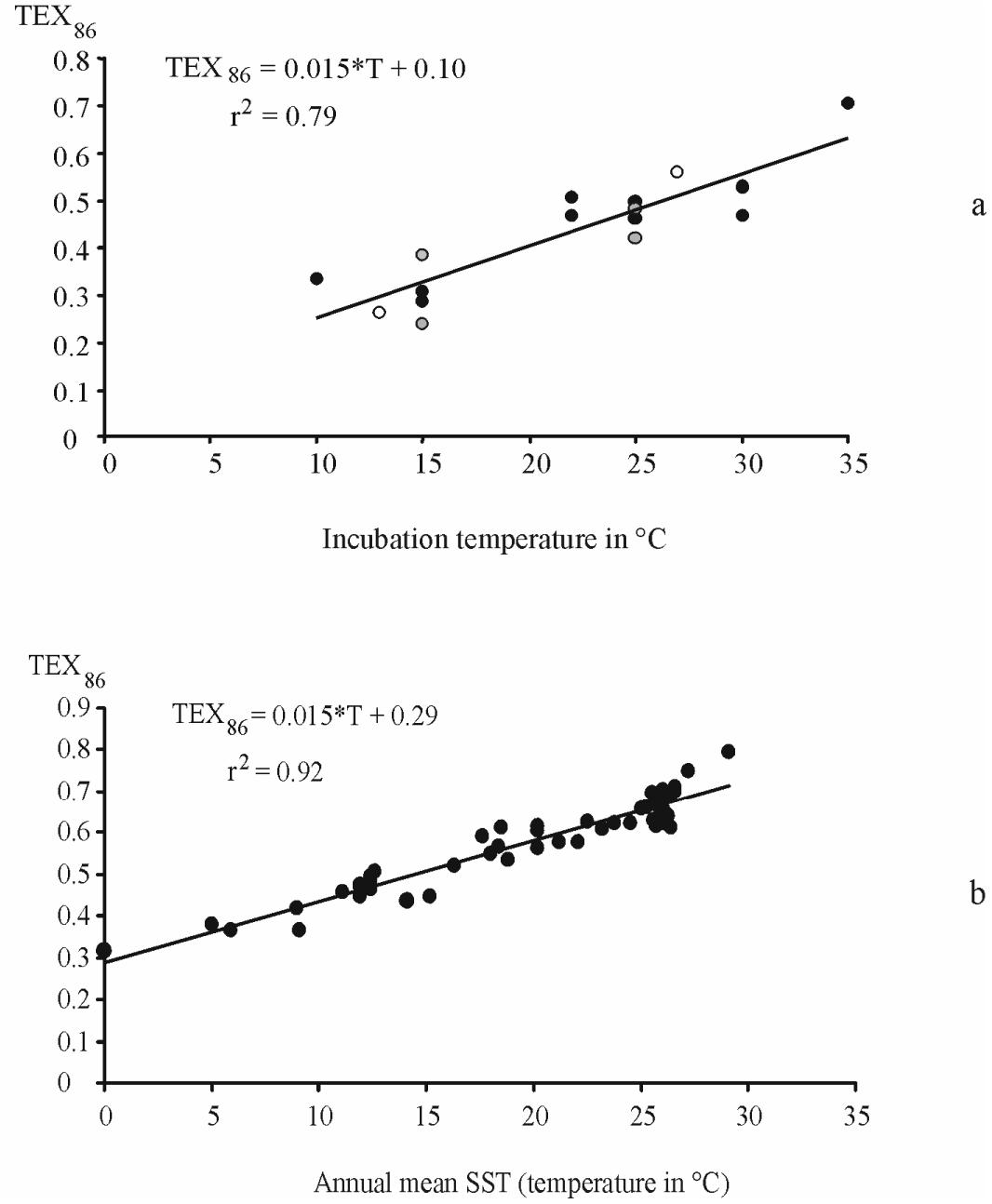

Figure 7. Correlation of the GDGT distribution with temperature. A: Correlation of the incubation series. Black dots represent TEX_{86} values from incubation at 27‰ salinity and grey dots represent TEX_{86} values from incubation at 35‰ salinity. Open dots represent TEX_{86} values from the initial mesocosm water. 5°C TEX_{86} value from incubation series I and 5°C, 10°C and 35°C TEX_{86} values from incubation series II are excluded in this correlation because no substantial GDGT increase could be measured.

B: Updated correlation line of TEX_{86} in core top samples with annual SST according to *Schouten et al.,* [2002]. The additional 18 data points are derived from core tops from Washington margin (7 samples), Arabian Sea (10 samples) and Santa Monica basin (1 sample).

To minimize this difference we omitted the crenarchaeol isomer from the TEX_{86} equation.

$$TEX'_{86} = \frac{III+IV}{II+III+IV} \quad [6]$$

Omission of the crenarchaeol isomer from the TEX_{86} equation result in the following relationship for core top samples and incubation series, respectively:

$$TEX'_{86} = 0.010*T+0.29 \ (r^2=0.68) \quad [7]$$

and

$$TEX'_{86} = 0.015*T+0.09 \ (r^2=0.72) \quad [8]$$

The exclusion of the crenarchaeol isomer changes the slope but not the intersection for the core top equation. For the incubation series no substantial difference with the initial equation is noted due to the low abundance of the crenarchaeol isomer. Hence, exclusion of the crenarchaeol isomer from the TEX_{86} definition does not reconcile the different equations.

The relative amount of crenarchaeol isomer is ca. 14 fold lower in the incubation series compared to the core tops. Interestingly, by adjusting the amount of crenarchaeol isomer in the incubation series to levels found in surface sediments, i.e. multiplication with a factor 14, a new linear correlation was obtained:

$$TEX''_{86} = \frac{III+IV+(14*VI)}{II+III+IV+(14*VI)} \quad [9]$$

$$TEX''_{86} = 0.016*T+0.29 \text{ with } r^2=0.85. \quad [10]$$

The slope stays nearly the same but now the intersection changes and is similar to the core top equation. The difference in the core top equation and the incubation series equation seems mainly determined by the low relative amount of crenarchaeol isomer in the incubation series.

Recent ^{13}C NMR analysis of this compound indicates that it is a regio-isomer of crenarchaeol (Fig. 1) (Sinninghe Damsté, unpublished data). Interestingly, regio-isomers of GDGT II-V are also observed in increasing amounts with temperature in the core top samples. This suggests that, besides an increase in the relative amount of cyclopentane rings, also an increase in the relative amount of regio-isomers takes place in the archaeal membrane with increasing growth temperature. This may be an additional temperature adaptation of the membrane of marine Crenarchaeota. It seems that this additional mechanism of temperature adaptation for membrane fluidity is less pronounced in the incubated marine crenarchaeotal species obtained from cold coastal North Sea waters than in the crenarchaeotal species dominantly present in tropical oceans. This may explain the discrepancy between incubation series correlation and core tops correlation. Further research involving tropical crenarchaeota may shed light on this phenomenon.

5.5 Conclusions

Our laboratory experiments suggest that temperature is a major controlling factor for the GDGT distribution of marine Crenarchaeota and that, therefore, TEX_{86} values are mainly determined by temperature. With these findings we support the newly introduced geochemical temperature proxy TEX_{86} because this strongly suggests that the TEX_{86} values indeed mainly reflect growth temperature of marine Crenarchaeota in the field. Comparison of the correlation line from the incubation series with the core top correlation shows that there is a difference in the intersection to the y- axes, which is mainly determined by the relative amount of the crenarchaeol regio-isomer. The TEX_{86} correlation for core top samples, however, may also be influenced by more complex factors such as population dynamics, seasonal occurrence and depth distribution of marine Crenarchaeota.

5.6 Acknowledgements

We thank Bouwe Kuipers (NIOZ) for his logistic support with the mesocosm tank experiments, Ellen Hopmans (NIOZ) for assistance and advice for the HPLC/MS analyses and Martijn Woltering (NIOZ) for assistance in sample processing. We thank Dr. Sloan and two anonymous reviewers for critical comments on an initial draft of this paper.

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Chapter 6

Temporal and spatial variation in tetraether membrane lipids of marine Crenarchaeota in particulate organic matter: Implications for TEX_{86} paleothermometry

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Abstract.

The TEX_{86} is a new temperature proxy which is based on the number of cyclopentane moieties in the glycerol dialkyl glycerol tetraether (GDGT) lipids of the membranes of Crenarchaeota which occur ubiquitously in oceans and shelf seas. This proxy was calibrated by core top sediments but it is as yet not clear during which season and at which depth in the water column the GDGT signal used for TEX_{86} paleothermometry is biosynthesized. Here, we analyzed >200 particulate organic matter (POM) samples from 11 different marine settings for TEX_{86}. This revealed that the GDGTs occur seasonally in surface waters and occur in higher abundances during the winter and spring months. The depth distribution showed that GDGTs generally appeared in higher amounts below 100 m depth in the water column. However, the TEX_{86} values for waters below the photic zone (150-1500 m) did not correlate with the in situ temperature but rather correlated linearly with surface temperature. The TEX_{86} for POM from the upper 100 m showed a linear correlation with in situ temperature, which was nearly identical to the previously reported core top equation. The correlation of all POM samples with surface temperature was also strikingly similar to the core top correlation. These findings demonstrate that the GDGT signal which reaches the sediment is mainly derived from the upper 100 m of the water column. This may be caused by the fact that GDGTs from the photic zone are much more effectively transported to the sediment by grazing and repackaging in large particles than GDGTs from deeper waters.

6.1 Introduction

Organic components in sediments are useful tools to reconstruct paleo-environments and climates. The most prominent example is sea surface temperature (SST) reconstruction using C_{37} alkenones. Alkenones are produced by marine haptophytes including the cosmopolitan coccolithophorids such as *Emiliania huxleyi* [*Volkman et al.,* 1980] and *Geophyrocapsa oceanica* [*Volkman et al.,* 1994]. In these algae the ratio of di- and tri-unsaturated C_{37} alkenones (expressed in the U^{K}_{37} ratio) changes as a function of temperature [*Brassell et al.,* 1986].

A new organic geochemical SST proxy, the TetraEther Index of lipids with 86 carbon atoms (TEX_{86}) was recently introduced [*Schouten et al.,* 2002]. This proxy is based on the number of cyclopentane moieties in the tetraether membrane lipids of marine Crenarchaeota. Marine Crenarchaeota are Prokaryotes that belong to the domain of Archaea. Molecular biological work using ribosomal DNA and the analysis of membrane lipids showed that marine Archaea are ubiquitously distributed in the world's ocean and occur in polar as well as in tropical regions [*Fuhrman et al.,* 1992; *DeLong,* 1992; *Hoefs et al.,* 1997; *Sinninghe Damsté et al.,* 2002a]. Recent molecular biological work revealed that the group of the marine Crenarchaeota comprises ca. 20% of the picoplankton in the world's ocean [*Karner et al.,* 2001]. The membrane lipids of Archaea consist of isoprenoid glycerol dialkyl glycerol diethers and glycerol dibipytanyl glycerol tetraethers (GDGTs) [*DeRosa and Gambacorta,* 1988]. Marine Crenarchaeota biosynthesize a specific GDGT, named crenarchaeol, which contains four cyclopentane ring and one cyclohexane ring, in addition to some more generally occurring GDGTs (Fig. 1) [*Sinninghe Damsté et al.,* 2002b]. It has been suggested that marine Crenarchaeota have evolved from hyperthermophilic Crenarchaeota by building an additional "kink" in their membrane lipid, i.e. the cyclohexane ring [*Sinninghe Damsté et al.,* 2002b]. This component is thought to have lowered the transition point of the membrane lipids and allowed the Crenarchaeota to live in temperate environments. Culture experiments and field studies have shown that membrane lipids of hyperthermophilic Archaea change their relative distribution of cyclopentane moieties as a function to temperature [*Gliozzi et al.,* 1983; *Ward et al.,* 1985*, DeRosa and Gambacorta,* 1988; *Uda et al.,* 2001]. With this mechanism Archaea keep their cytoplasm membrane at a liquid crystalline state and reduce their proton permeation

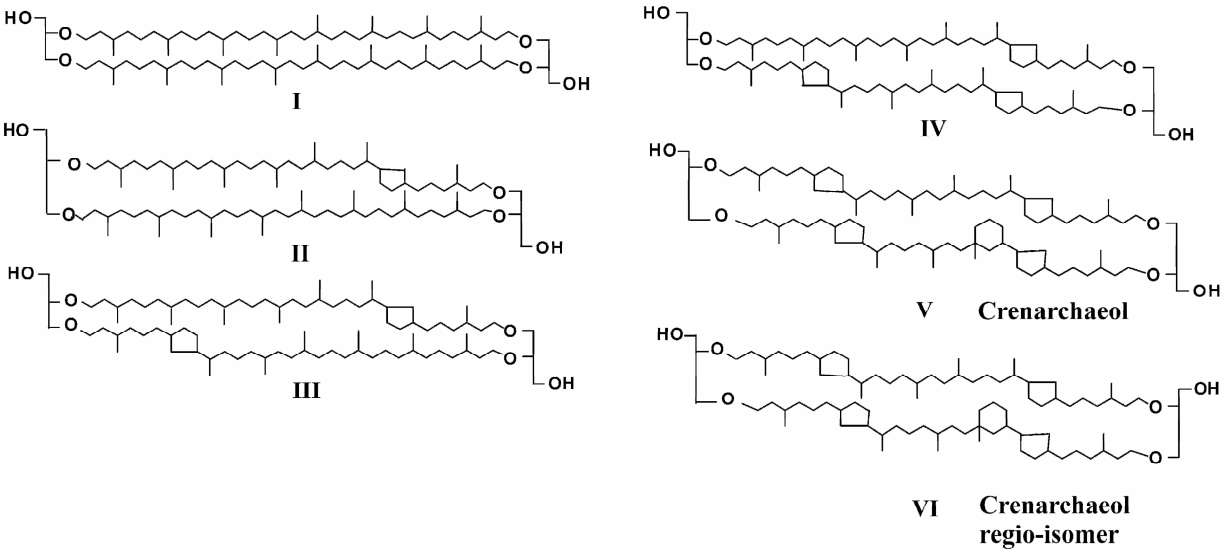

Figure 1. Structures of GDGT membrane lipids of marine pelagic Crenarchaeota.

rate [*Albers et al.,* 2000]. Recent mesocosm studies confirmed that the GDGT composition of marine Crenarchaeota is also determined by temperature [*Wuchter et al.,* 2004]. The membrane lipids of marine Crenarchaeota are relatively stable components, and are found in sediments up to 140 million years old [*Kuypers et al.,* 2001, *Carrillo-Hernandez et al.,* 2003].

Schouten et al. [2002] showed that the distribution of crenarchaeotal GDGTs in core top sediments derived from different geographic locations correlate with SST. In cold areas the GDGT distribution is dominated by GDGT I (see Fig. 1 for structures) and crenarchaeol (V). In warmer regions the GDGT distributions differ substantially as crenarchaeol is the most abundant GDGT and higher amounts of 1-3 cyclopentane-containing GDGTs (II-IV) and a regio-isomer of crenarchaeol (VI) are detected (Fig. 2). The relative GDGT distribution can be expressed as an index of GDGT isomers, which was named the TEX_{86} and is defined as follows:

$$TEX_{86} = (III+IV+VI) / (II+III+IV+VI) \quad [1]$$

This index is correlated with the annual mean SST [*Schouten et al.,* 2002]:

$$TEX_{86} = 0.015*T+0.28, (r^2=0.92) \text{ with } T = \text{annual mean SST (in ºC).} \quad [2]$$

The observed correlation of TEX_{86} with annual mean SST suggests that marine Crenarchaeota are mainly present in surface waters throughout the whole year. However, this seems in contradiction to ribosomal DNA work from *Massana et al* [1997] and *Murray et al.* [1998 and

1999], who observed a seasonal occurrence of marine Crenarchaeota in Santa Barbara Channel surface waters and coastal Antarctic surface waters. In addition, cell counts at the tropical Pacific revealed that marine crenarchaeotal cell numbers are relatively invariant throughout the water column during the annual cycle and cell numbers only drop below 250 m [*Karner et al.,* 2001]. In contrast, crenarchaeotal GDGT concentrations are actually higher below the photic zone in the Arabian Sea [*Sinninghe Damsté et al.,* 2002a].

These different observations show that it is as yet not clear when and at which depth in the water column the GDGT signal for TEX_{86} paleothermometry is produced. To answer this question, we investigated the GDGT composition of particulate organic matter (POM) from different oceanic regimes. We analyzed surface water time series and depth profiles to shed more light on the seasonal and spatial distribution of the marine crenarchaeotal GDGTs and how this affects TEX_{86} values.

6.2 Material and Methods

To study the seasonal GDGT abundance in surface waters we analyzed particulate organic matter (POM) from time series of two different locations, i.e. Bermuda Atlantic Time Series (BATS) and shallow coastal North Sea waters. The vertical GDGT distribution was studied by analyzing POM from depth profiles from three different locations, i.e. the Equatorial Pacific, eastern North Pacific (VERTEX 5) and Santa Monica Basin. In addition, TEX_{86} values were calculated for depth profiles from Arabian Sea and Cariaco Basin which were investigated previously [*Sinninghe Damsté et al.,* 2002a, *Wakeham et al.,* 2004]. Finally, a number of surface and depth samples from Peru upwelling region, Seychelles, Southern Ocean and Mediterranean Sea (collected at the French DYFAMED site) were analyzed. For further detailed information about the collection of the samples, see Figure 2 and Table 1.

6.2.1 Sampling and extraction

POM was collected by large volume filtration systems through extracted 50 µm Nitex screen and pre-combusted glass fiber filters (GF) with a nominal pore size of 0.7 µm. The filters were wrapped in clean aluminum foil and kept frozen at –20˚C until extraction. Filters were Soxhlet extracted with dichloromethane (DCM) and methanol (2:1 by volume) at Skidaway Institute of Oceanography.

Coastal North Sea surface waters were sampled for POM at about 1 m depth in weekly intervals from August 2002- July 2003 at the NIOZ jetty. 20 l of water collected at high tide were filtered through ashed 3 µm and 0.7 µm GF filters. The filters were wrapped in clean aluminum foil and stored at –20°C until further analyses. The GF filters were freeze-dried and ultrasonically extracted with methanol, methanol/DCM (1:1 by volume) and three times with DCM at NIOZ.

6.2.2 Lipid analysis

An aliquot of the total lipid extract was cleaned over an activated Al_2O_3 column by eluting with methanol/DCM (1:1 by volume). For analysis of intact GDGTs, the solvent was removed under a stream of nitrogen and the residue was dissolved by sonication (5 min) in hexane/propanol (99:1 by volume). 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 (HPLC) - atmospheric pressure positive ion chemical ionization mass spectrometry (APCI-MS) by applying conditions slightly 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 were 15 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, by volume) 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 temperature 200 °C, capillary voltage -3 kV, corona 5 µA (~ 3.2 kV). GDGTs were detected by single ion monitoring of their $[M+H]^+$ ions (dwell time 237 ms). For TEX_{86} calculation the peak areas of GDGTs I-VI were integrated and TEX_{86} values were calculated according to Equation I. For the temperature correlation only TEX_{86} values were used where all isomers of GDGTs could be detected and accurately quantified.

sampling site	area/station	sampling intervals	cruise/ cruise no.	sampling depth	sampling size (l)	number of samples	references
(1) Northeastern Pacific (VERTEX 5)	V5A: 33.3°N, 139.2°W; V5C: 36.1°N, 122.6°W.	June 1984	R/V *Wecoma*	10-1500m	900-5000	12	*Wakeham and Canuel,* 1988 *Silver and Gowing* 1991
(2) Santa Monica Basin	33°75'N, 119°08'W	March 1991-April 1992	R/V *Sea Watch* (6 cruises)	25-850m	250-4000	15	*Bidigare et al.,* 1997
(3) Equatorial Pacific	Station 6: 140°W, 2°N; Station 15: 140°W, 12°S.	February-March and August-September 1992	R/V *Thomas G. Thompson* TT007 and TT011	25-840m	2000-17000	32	*Murray et al.,* 1995 *Sheridan et al.,* 2002
(4) Peru upwelling region	Transect 12°S and 13.5°S	October, 1992	R/V *Seward Johnson*	surface	70-190	20	*Pancost et al.,* 1997
(5) Bermuda Atlantic Time Series (BATS)	32°10'N, 64°30'W (Hydrostation S)	November 1991 till January 1996	R/V *Weatherbird II* BATS 38-88	surface	1000-3000	50	*Steinberg et al.,* 2001, *Conte et al.,* 2001
(6) Cariaco Basin	10° 40'N, 65°36'W	February- March 1986	R/V *Iselin*	10-1150m	500-5000	10	*Wakeham et al.,* 1990 *Wakeham et al.,* 2004
(7) Shallow coastal North Sea waters	53°00'25"N, 4°78'27"E (NIOZ Jetty)	August 2002-July 2003	--------	surface	20	33	This manuscript
(8) MedFlux (DYFAMED)	43°20'N, 7°40'W	September 2002	R/V *Tethys II*	3-150m	550-800	6	http:// alphal.msrc.sunysb.edu/MedFlux/ *Liu et al., in press*
(9) Arabian Sea Process Study	Sta. 2: 15°58'N, 61°29'E; Sta. 4: 17°12'N, 59°35'E; Sta. 5: 17°24'N, 58°49'E; Sta. 6: 17°41'N, 57°50'E; Sta. 7: 17°40'N, 57°41'E; Sta.10: 17°44'N, 57°29'E; Sta.13: 19°13'N, 58°31'E.	May 1995	R/V *Thomas G. Thompson* TN047	30-1500m	2400-3000	19	*Smith et al.,* 1998 *Sinninghe Damsté et al.,* 2002
(10) Seychelles	6°12'S, 52°7'E	November 2003	R/V *Darwin*	surface	20	1	This manuscript
(11) Southern Ocean	Transect along 170°W	Nov-Dec.1996, Feb-March 1998	R/V *Nathaniel B. Palmer*; NBP 96-5, NBP 98-2.	surface	1000-1100	22	This manuscript

Table 1. Sampling information and related references of the POM samples investigated in this study.

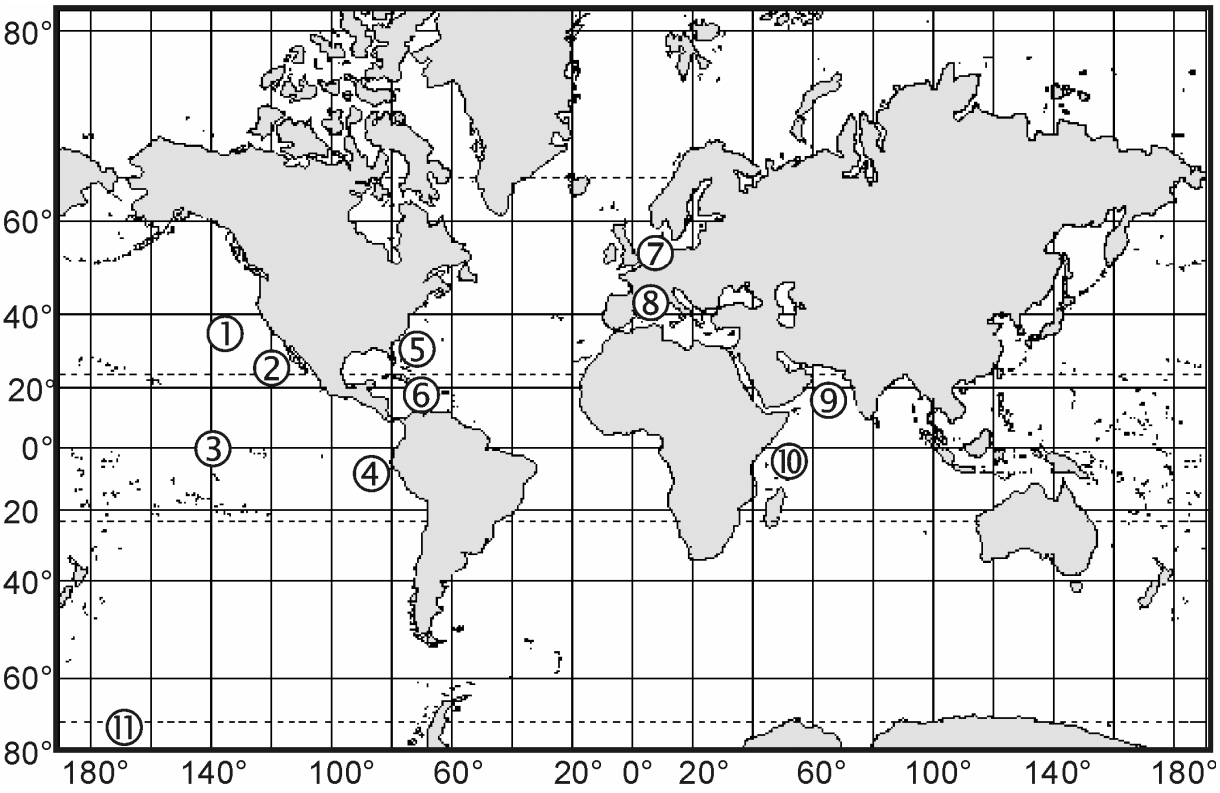

Figure 2. Sampling location of particulate organic matter (POM) analyzed in this study. 1: Northeastern Pacific (VERTEX 5); 2: Santa Monica Basin; 3: Equatorial Pacific; 4: Peru upwelling region; 5: Bermuda Atlantic Time Series (BATS); 6: Cariaco Basin; 7: Shallow Coastal North Sea waters; 8: Mediterranean Sea (DYFAMED); 9: Arabian Sea, 10: Seychelles and 11: Southern Ocean. See Table 1 for further sampling details.

6.3 Results

We analyzed 220 POM samples from different oceanic regimes to determine the GDGT occurrence and distribution with depth and with time. Of these, 118 POM samples contained sufficient amounts of GDGTs for reliable TEX_{86} calculation. The sampling locations ranged from shallow coastal North Sea waters to open ocean samples such as Arabian Sea, Equatorial Pacific, and the Southern Ocean (see Fig. 2).

6.3.1 Surface time series

Both time series, the Bermuda Atlantic Time series (BATS) and the shallow coastal North Sea water, showed a seasonal occurrence of GDGTs during the annual cycle. At the

shallow coastal North Sea water site the GDGTs were most abundant during the winter and early spring seasons (Fig. 3a). In late spring, summer and autumn the concentration of GDGTs was <10% of that in winter. The GDGT distribution was dominated by GDGT I and, to a lesser extent crenarchaeol (V) and a relatively low amount of cyclopentane-containing GDGTs (Fig. 4a). The TEX_{86} values ranged between 0.39 and 0.51 for shallow coastal North Sea waters during winter and spring (Fig. 3a).

At the BATS site, surface water POM samples were collected monthly [*Conte et al.,* 2001] but GDGTs were only detected in the winter and spring months (Fig. 3b). During the rest of the year the GDGT concentration was near the detection limit (ca. 1 pg/l). The GDGT distribution was dominated by crenarchaeol and relatively high amounts of cyclopentane-containing GDGTs (Fig. 4b). TEX_{86} values ranged between 0.56 and 0.73 (Fig. 3b).

6.3.2 Depth profiles

All three studied depth profiles had a similar GDGT distribution throughout the water column. POM from different depths of the Santa Monica Basin were obtained in August and October 1991 and in February 1992 at 4 depths ranging between 25 m and 850 m. Apart from the October samples, the GDGT abundance was highest between 100 to 850 m water depths (Fig. 5a). The GDGT distribution was dominated by GDGT I and to a lesser extent crenarchaeol with a relatively low amount of cyclopentane-containing GDGTs. The TEX_{86} values ranged between 0.42-0.53 (Fig. 5a).

Depth profiles were obtained from POM taken in February/March 1992 and August/September 1992 at two Equatorial Pacific sampling sites at 8 sampling depths ranging from 10 to 842 m in the water column. In all instances GDGTs were more abundant below 100 m (Fig. 5b) The GDGT distribution was dominated by crenarchaeol and relatively high amounts of cyclopentane-containing GDGTs and the obtained TEX_{86} values ranged between 0.63-0.85 (Fig. 5b).

The two depth profiles of the northeast Pacific setting were obtained from POM at 5 different depths ranging from 10 to 1500 m. The highest GDGT concentrations were again measured below 100 m (Fig. 5c). GDGT I and crenarchaeol were present in equal amounts and substantial amounts of cyclopentane-containing GDGTs were detected. The obtained TEX_{86} values ranged between 0.47-0.59 (Fig. 5c).

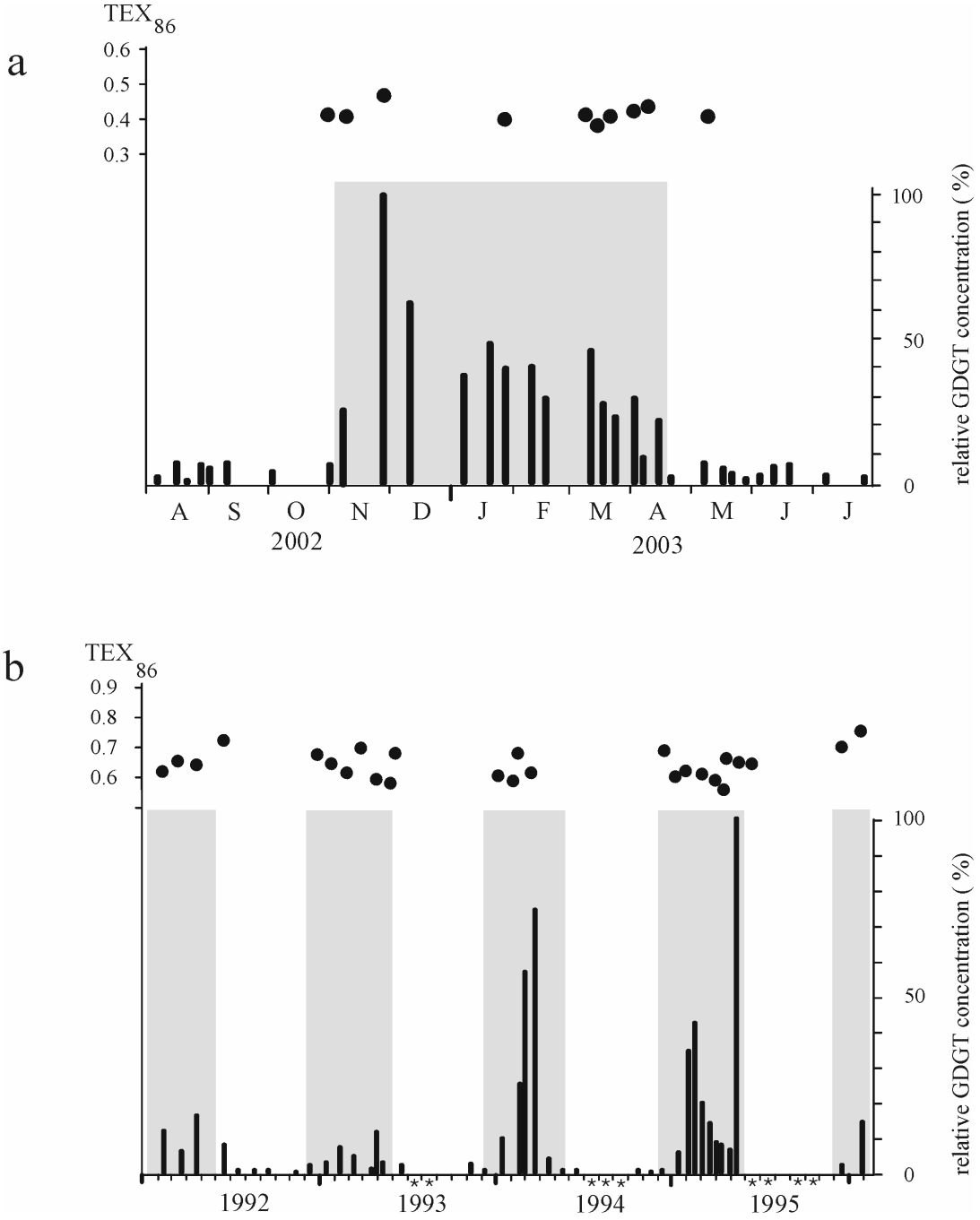

Figure 3. Relative GDGT concentrations and TEX_{86} values measured in POM of time series at two different settings. (a) Shallow coastal North Sea waters time series from August 2002 till July 2003 and (b) Bermuda Atlantic Time Series starting from winter 1991 till winter 1995. Grey shaded areas correspond to winter and spring seasons. Month marked with an asterisk indicate that water samples were analysed but GDGTs were not detected.

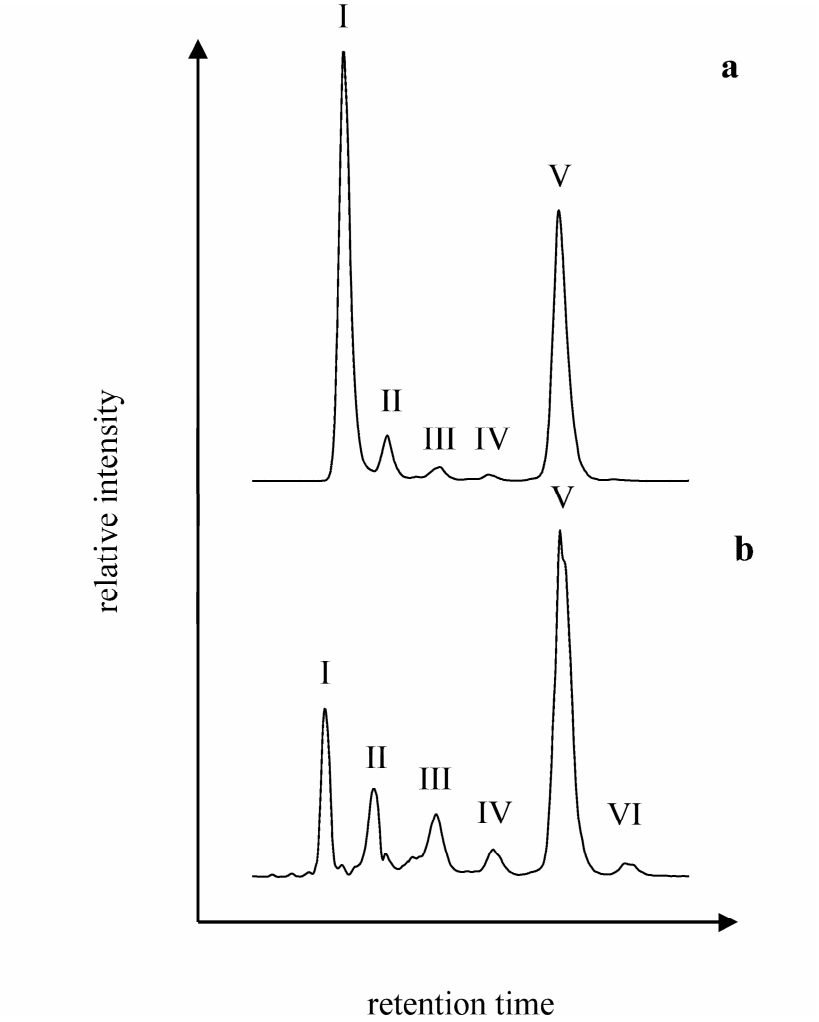

Figure 4. Partial HPLC-APCI/MS base peak chromatograms of POM derived from (a) shallow coastal North Sea waters and (b) Surface water sampled in the Bermuda Atlantic Time Series. Numbers indicate structures shown in Fig. 1.

6.4. Discussion

6.4.1 Seasonality of GDGT abundance

Our data show that there is a strong seasonal contrast in the abundance of GDGTs in surface waters at the BATS and the coastal North Sea sampling station (Fig. 3). GDGT concentrations peak during the spring and winter seasons, respectively. Marine Crenarchaeota are the dominant Archaea in coastal North Sea surface waters during winter (November-February) as revealed by 16S rDNA work [Wuchter et al., unpublished data]. In contrast, chlorophyll-*a* concentrations were high during late spring and early summer over the last 30 years in this area [*Cadée and Hegeman,* 2002, *Colijn and Cadée,* 2003]. This seasonal cycle

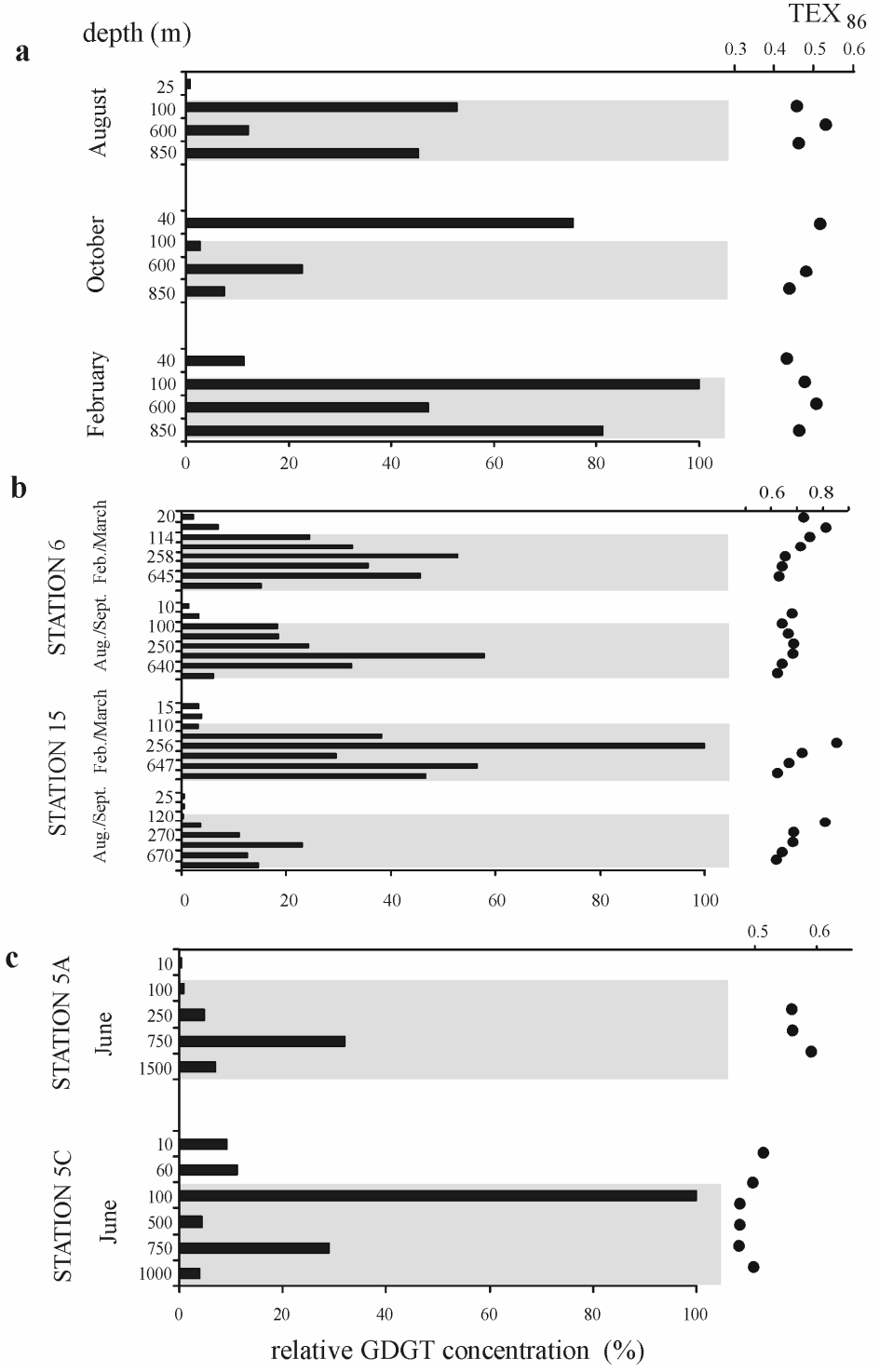

Figure 5. Relative GDGT concentrations (in %) and TEX_{86} values measured in POM of three depth profiles. (a) Santa Monica Basin; (b) Equatorial Pacific and (c) Northeastern Pacific (VERTEX 5). Grey shaded areas correspond to water depths below 100 m.

was also observed in 2002-2003. The GDGT and the chlorophyll-*a* concentration thus show a strong negative correlation during the annual cycle in coastal North Sea waters. This indicates that archaea are not abundant during periods of high primary production. This is consistent with previous 16S rDNA findings in the Antarctic Peninsula region and Santa Barbara Channel which showed that archaeal abundance in surface waters is negatively correlated with chlorophyll-*a* concentrations [*Murray et al.,* 1998 and 1999].

At the BATS site, however, a positive correlation between chlorophyll *a* and archaeal lipids in surface waters is observed. In this region the passage of cold fronts erodes the seasonal thermocline and forms subtropical water during the winter season at the BATS station [*Steinberg et al.,* 2001]. Convective mixing caused by the winds results in mixed layers at 150-300 m depth and associated nutrient enrichment of the surface layers [*Steinberg et al.,* 2001]. High chlorophyll-*a* concentrations in the late winter, early spring season in surface waters are associated with convective deep mixed water layers but are not sustained throughout the year [*Steinberg et al.,* 2001]. The nutrient enrichment in surface waters may also support growth of marine Crenarchaeota. Alternatively, the higher archaeal lipid abundance may be due to the water mixing when the GDGT signal originally derived from deeper waters is upwelled to the surface layer. Deeper waters generally contain higher concentrations of GDGTs (see discussion below).

6.4.2 Depth distribution of GDGTs

The GDGT depth distribution at the Equatorial Pacific, northeast Pacific and Santa Monica Basin sampling sites show a similar pattern (Fig.5). GDGT concentrations increase with depth and have maxima generally below 100 m. These findings are consistent with previous findings of GDGT depth distributions in the Arabian Sea [*Sinninghe Damsté et al.,* 2002a] where the authors reported higher GDGT concentrations below the photic zone and the Cariaco Basin [*Wakeham et al.,* 2004] with maximum GDGT concentrations at 250 m depth. However, there is a discrepancy between the lipid data and 16S rDNA studies. While fluorescence in situ hybridization (FISH) data show that the marine crenarchaeotal absolute abundance in the tropical Pacific Ocean is relatively invariant throughout the upper 250 m of the water column and decreases at lower depth [*Karner et al.,* 2001], our lipid data show maximum GDGT concentrations generally below 100 m at the sampling sites. The FISH

technique detects only living cells whereas our lipid analysis does not discriminate between living and dead crenarchaeotal cell material. Thus, a substantial percentage of the GDGTs encountered at greater depth may be derived from suspended or sinking dead cell debris. This is likely due to the generally more refractory nature of lipids compared to other cell material such as DNA. GDGTs are themselves not more refractory than other membrane lipids as studies have shown that they degrade, under oxic conditions, at similar rates as other phytoplanktonic lipids [*Sinninghe Damsté et al.,* 2002c, *Schouten et al.,* 2004].

6.4.3 TEX_{86} and temperature

As GDGTs are found throughout the water column, it may be expected that the TEX_{86} values would reflect a large range of temperatures from surface to deep waters. However, compared to the large range of in situ temperatures, the TEX_{86} values showed only minor variation with depth (e.g., the Equatorial Pacific and Cariaco Basin sites, Fig. 6). Indeed, when TEX_{86} values from all sampling locations are compared with in situ temperatures only a weak correlation is observed (Fig. 7a). The TEX_{86} in surface sediment correlates best with annual mean SST [*Schouten et al.,* 2002], and this suggests that the TEX_{86} in POM probably also reflects mainly temperatures of the photic zone. To investigate this hypothesis, we splitted the POM TEX_{86} data into two sets, those derived from the upper 100 m of the water column and those from below 100 m. The correlation of TEX_{86} for POM in the upper 100 m of the water column with in situ temperature was high, with an r^2 of 0.80 (Fig. 7b), and is nearly identical to the core top equation (Fig. 7f). In contrast, a weak correlation is found between TEX_{86} values of POM from below 100 m depth with in situ temperature (Fig. 7c). Instead, TEX_{86} values of POM below 100 m depth correlated well with surface temperatures at the time of sampling (Fig. 7d). When TEX_{86} values of all POM samples are compared with actual surface temperature, a correlation is obtained (Fig. 7e) that is strikingly similar to the TEX_{86} core top correlation (Fig. 7f). These findings indicate that the GDGT signal in POM at all depth is mainly reflecting the temperature of the upper 100 m of the water column. Growth of marine Crenarchaeota in deeper waters may influence the TEX_{86} in POM to a minor degree as observed, for example, in the Equatorial Pacific (Fig. 6) where the TEX_{86} values slightly decrease with depth. It is most likely that the TEX_{86} values of deep water POM reflect a mixed GDGT signal from living but slowly growing crenarchaeotal cells, since

microorganisms living in the deep ocean have a slow metabolic rate [*Tamburini et al.,* 2003], and sinking, dead crenarchaeotal cell material in which GDGTs may become enriched through their refractory nature. This is consistent with our depth profile data where GDGTs are enhanced in abundance in deeper water layers and are probably derived for a large part of dead cell material.

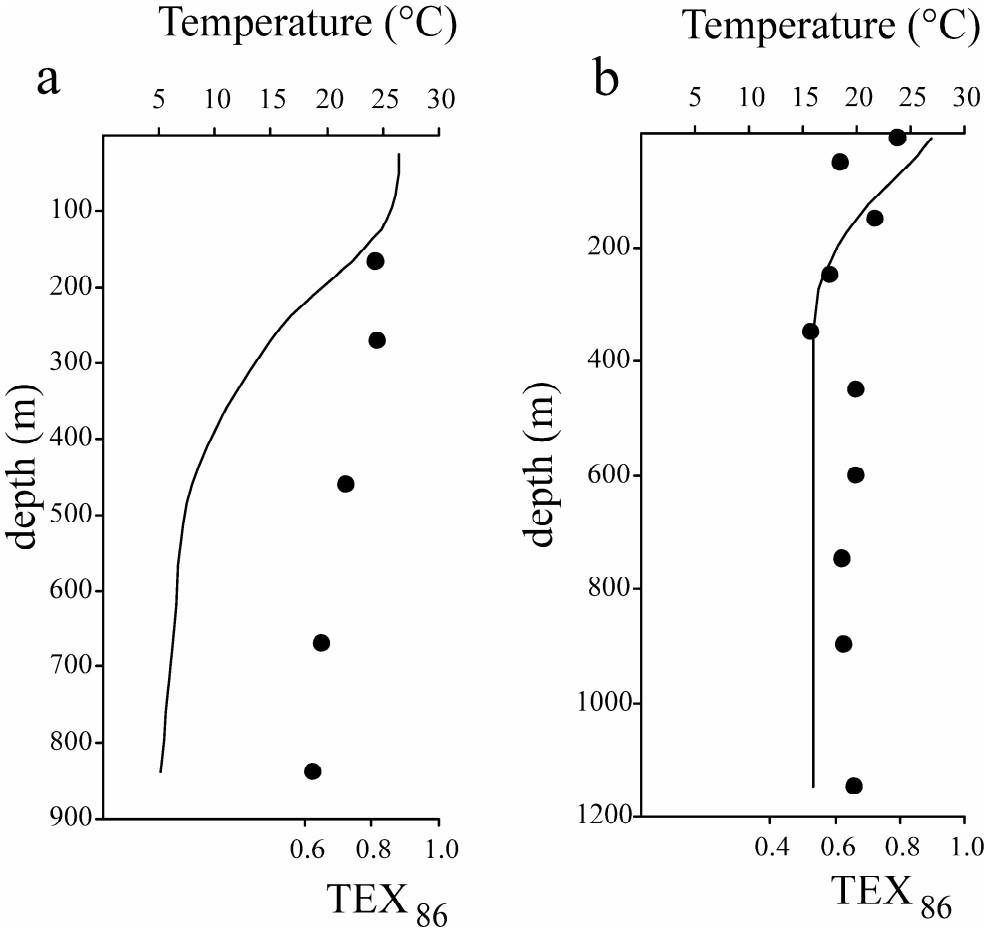

Figure 6. TEX_{86} values and in situ temperature versus depth for (a) Equatorial Pacific and (b) Cariaco Basin.

6.4.4 Origin of sedimentary GDGT signal

The similarity of the TEX_{86} POM and the TEX_{86} core top equations (cf. Figs. 7e and 7f) suggests that the temperature signal that is found in the sediment must be mainly derived from the upper 100 m of the water column. The GDGTs that are found in sediments thus seems to be mainly derived from crenarchaeotal cell material derived from the photic zone and

transported with fast sinking aggregates that reaches the sea floor. These findings are supported by previous work done on the Black Sea [*Wakeham et al.,* 2003]. We analyzed POM, sediment traps and surface sediment samples to investigate the GDGT distribution and isotopical signature of the biphytane skeletons contained in these GDGTs. We found high amounts of ^{13}C-depleted biphytanes in POM of the deep anoxic zone of the Black Sea (>1000 m) but could not find the corresponding ^{13}C-depleted biphytanes in the sediment traps or the underlying sediments. This led us to suggest that due to the absence of grazing in the deep anoxic zone of the Black Sea, the GDGTs from deeper waters may lack transport mechanisms (e.g. by fecal pellets) to the sea floor and that, therefore, the dominant flux of GDGTs to the surface sediments was derived from the upper part of the water column where particle packaging through grazing can occur. A distinct compositional difference between suspended and sinking particles was also observed for other lipid classes in the Black Sea [*Wakeham and Beier* 1991] and in oceanic settings like the eastern tropical North Pacific Ocean [*Wakeham and Canuel,* 1988, *Wakeham and Lee,* 1993]. Sediments are mainly derived from fast-sinking large particles [*McCave,* 1984] which are produced by biologically-mediated aggregation (e.g. grazing), and laboratory and field observations showed that fecal pellets and marine snow may sink at rates of tens to hundreds of meters per day [e.g. *Small et al 1979*]. Thus, due to these mechanisms the sedimentary signal is primarily derived from the upper part of the water column where an active food web exists.

Marine crenarchaeota are generally abundant in the surface water during times when the majority of the phytoplankton is not blooming [*Murray et al.,* 1998 and 1999, this study]. Therefore, the GDGT signal which is biosynthesized in the upper 100 m of the water column probably reflects the temperatures at this time interval in the seasonal cycle. The TEX_{86} temperature signal which is preserved in the sedimentary record may thus mainly reflect a seasonal signal, which may explain some of the scatter in the correlation of the TEX_{86} with annual mean SST in core tops. Indeed, for surface sediments of the eastern Southern Atlantic *Schouten et al.* [2002] observed that the best correlation of TEX_{86} is with austral winter SST.

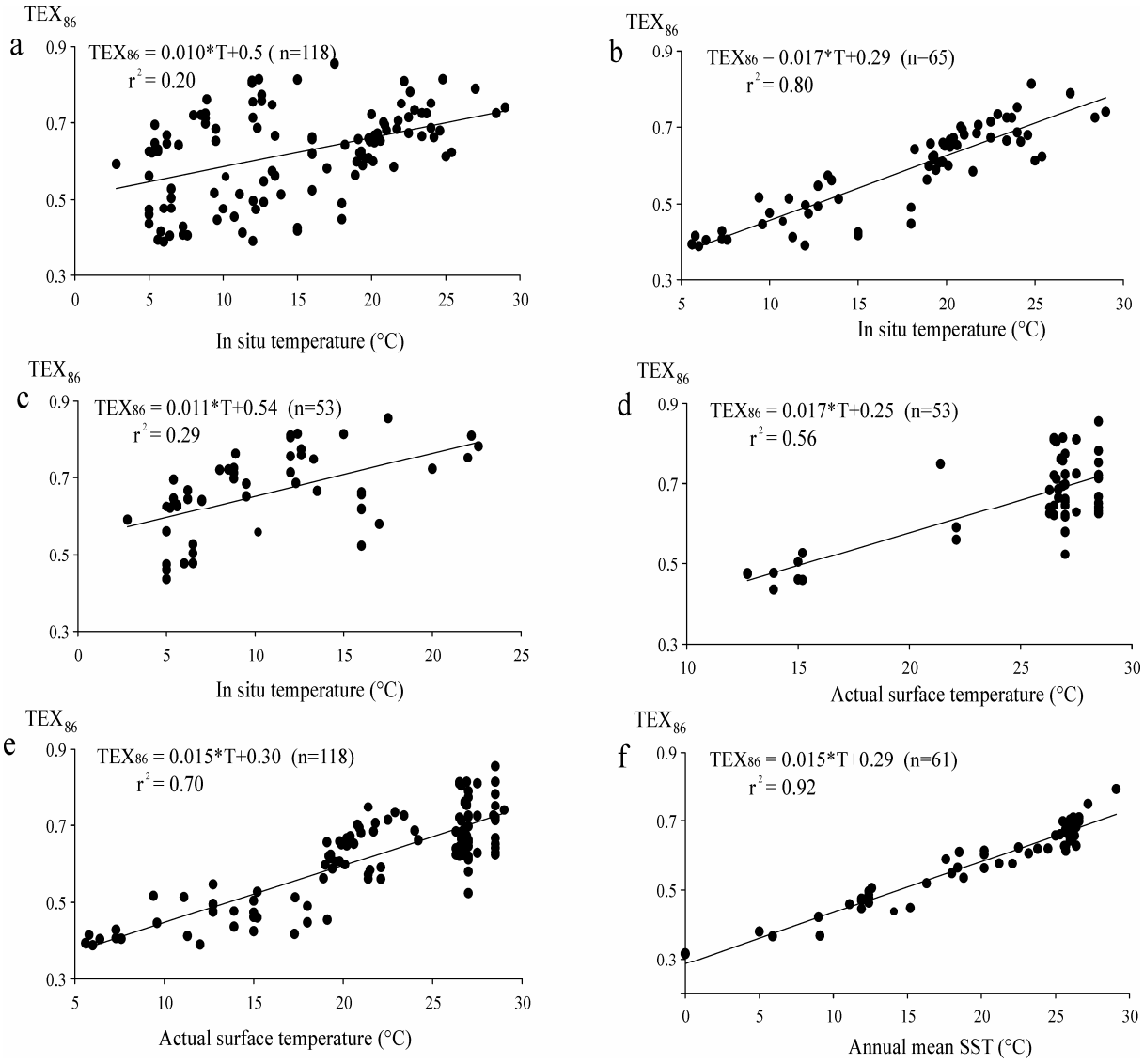

Figure 7. Cross plots of TEX_{86} and temperature for POM (a-e) and sediments (f). The best linear fit is shown. (a) TEX_{86} of POM from all depth versus the in situ temperature, (b) TEX_{86} of POM from the upper 100 m of the water column versus in situ temperature, (c) TEX_{86} of POM from below 100 m depth versus in situ temperature, (d) TEX_{86} of POM from below 100 m depth versus surface temperature, (e) TEX_{86} of POM from all depth versus surface temperature, and (f) Updated TEX_{86} correlation line from surface samples versus annual mean sea surface temperature [*Wuchter et al*, 2004].

6.5. Conclusion

Our data show that the TEX_{86} of POM from different oceanic regimes correlates well with temperature at depths shallower than 100 m. TEX_{86} values of POM from deeper waters (>100 m) do not correlate with in situ temperature but with surface temperature. Thus, these findings suggest that the GDGT signal which reaches the sediment must be mainly biosynthesized in the upper 100 m of the water column. Packaging of small GDGT-containing crenarchaeal cells into fecal pellets and marine snow aggregates probably results in rapid transportion from the photic zone to the sediment. Living suspended deep sea Crenarchaeota do not substantially influence the sedimentary GDGT signal probably due to the lack of aggregation processes and transport mechanisms to the sea floor. Marine Crenarchaeota appear seasonally in surface waters and the GDGT signal accumulating in the underlying sediment may mostly reflect these periods of elevated GDGT biosynthesis. The data presented here, in combination with previous studies on core tops [*Schouten et al.,* 2002], diagenetic stability [*Schouten et al.,* 2004], and mesocosm experiments [*Wuchter et al.,* 2004], show that the TEX_{86} may be used to reconstruct upper water column temperatures in ancient environments.

6.6. Acknowledgements

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6.7 Supplementary information

Table 1 TEX_{86} values and corresponding temperature used for the validation of the temperature proxy TEX_{86} in particulate organic matter. Grey shaded temperature values for some sampling locations are monthly mean temperatures obtained from the world ocean atlas (http://www.nodc.noaa.gov/OC5/WOA01F/tsearch.html). All other temperature data were measured at the time of sampling.

sampling location	sampling date	sampling depth (m)	in situ temp.(°C)	SST(°C)	TEX_{86}
Bermuda Atlantic Time Series					
cruise no. 41	12.02.92	surface	19.3	19.3	0.63
cruise no. 42	10.03.92	surface	19.8	19.8	0.66
cruise no. 43	21.04.92	surface	19.9	19.9	0.65
cruise no. 45	17.06.92	surface	23.4	23.4	0.73
cruise no. 52	12.01.93	surface	21	21	0.68
cruise no. 53	11.02.93	surface	20.2	20.2	0.65
cruise no. 54	10.03.93	surface	19.3	19.3	0.62
cruise no. 55	16.04.93	surface	20.8	20.8	0.70
cruise no. 55a	27.04.93	surface	20.1	20.1	0.60
cruise no. 56	10.05.93	surface	21.5	21.5	0.58
cruise no. 57	19.06.93	surface	24	24	0.69
cruise no. 65	18.02.94	surface	20.4	20.4	0.67
cruise no. 65a	28.02.94	surface	19.6	19.6	0.61
cruise no. 66	21.03.94	surface	19.4	19.4	0.59
cruise no. 67	19.04.94	surface	21.7	21.7	0.68
cruise no. 73	19.10.94	surface	24.2	24.2	0.66
cruise no. 74	15.11.94	surface	22.9	22.9	0.73
cruise no. 75	09.12.94	surface	22.5	22.5	0.71
cruise no. 76	11.01.95	surface	20.9	20.9	0.69
cruise no. 76a	01.02.95	surface	19.8	19.8	0.61
cruise no. 77	14.02.95	surface	19.2	19.2	0.62
cruise no. 77a	01.03.95	surface	19.3	19.3	0.61
cruise no. 78	16.03.95	surface	19	19	0.60
cruise no. 78a	29.03.95	surface	18.9	18.9	0.56
cruise no. 79	10.04.95	surface	20.2	20.2	0.67
cruise no. 79a	27.04.95	surface	20.6	20.6	0.65
cruise no. 80	10.05.95	surface	19.1	19.1	0.66
cruise no. 88	30.01.96	surface	21.8	21.8	0.71

North Sea Time Series					
	04.11.02	surface	11.1	11.1	0.51
	07.11.02	surface	11.3	11.3	0.41
	28.11.02	surface	7.6	7.6	0.41
	28.01.03	surface	5.6	5.6	0.39
	12.03.03	surface	5.8	5.8	0.42
	18.03.03	surface	6.0	6.0	0.39
	24.03.03	surface	6.4	6.4	0.40
	03.04.03	surface	7.3	7.3	0.41
	07.04.03	surface	7.3	7.3	0.43
	07.05.03	surface	12	12.0	0.39
MedFlux (DYFAMED)					
	Sep-02	50	13.5	21.4	0.56
	Sep-02	100	13.3	21.4	0.57
	Sep-02	150	13.3	21.4	0.75
Peru upwelling region					
12S, #109	Oct-92	surface	18	18	0.45
13.5S #014	Oct-92	surface	18	18	0.49
Southern Ocean					
NBP 98-02 #35	29.03.98	surface	9.6	9.6	0.45
NBP 98-02 #37	30.03.98	surface	9.4	9.4	0.52
Seychellen	13.11.03	surface	29	29	0.74
Cariaco basin					
	Mar-86	10	27	27	0.79
	Mar-86	50	25	27	0.61
	Mar-86	150	20	27	0.72
	Mar-86	250	17	27	0.58
	Mar-86	350	16	27	0.52
	Mar-86	450	16	27	0.66
	Mar-86	600	16	27	0.66
	Mar-86	750	16	27	0.62
	Mar-86	900	16	27	0.62
	Mar-86	1150	16	27	0.66
Equatorial Pacific					
station 6 survey 1	10.03.92	44	28.4	28.4	0.72
	10.03.92	114	24.8	28.5	0.81
	10.03.92	161	22	28.5	0.75
	10.03.92	258	12	28.5	0.71
	10.03.92	456	9.5	28.5	0.65
	10.03.92	645	7	28.5	0.64

	10.03.92	838	5.6	28.5	0.63
station 6 survey 2	26.08.92	35	24.6	26.7	0.68
	26.08.92	100	18.2	26.7	0.64
	26.08.92	150	13.5	26.7	0.67
	26.08.92	250	12.3	26.7	0.69
	26.08.92	450	9.5	26.3	0.68
	26.08.92	640	7	26.3	0.64
	26.08.92	840	5.6	26.3	0.63
station 15 survey 1	07.03.92	158	22.6	28.5	0.78
	07.03.92	256	17.5	28.5	0.85
	07.03.92	452	8.5	28.5	0.72
	07.03.92	647	6.2	28.5	0.67
	07.03.92	842	5	28.5	0.63
station 15 survey 2	14.09.92	168	22.2	26.5	0.81
	14.09.92	270	15	26.5	0.81
	14.09.92	460	8.0	26.5	0.72
	14.09.92	670	6.2	26.5	0.65
	14.09.92	840	5	26.5	0.62
Northeastern Pacific					
VERTEX 5A	Jun-84	250	10.2	22.1	0.56
	Jun-84	750	5	22.1	0.56
	Jun-84	1500	2.8	22.1	0.59
VERTEX 5C	Jun-84	10	12.7	12.7	0.55
	Jun-84	10	12.7	12.7	0.49
	Jun-84	60	12	12.7	0.50
	Jun-84	100	10	12.73	0.48
	Jun-84	500	6	12.7	0.48
	Jun-84	750	5	12.7	0.47
Santa Monica Basin					
cruise no 3	07.08.91	100	10.8	19.1	0.45
cruise no 3	07.08.91	600	6.5	15.2	0.53
cruise no 3	07.08.91	850	5	15.2	0.46
cruise no 4	31.10.91	40	13.9	17.3	0.51
cruise no 4	31.10.91	600	6.5	13.9	0.48
cruise no 4	31.10.91	850	5	13.9	0.44
cruise no 5	06.02.92	40	15	15	0.42
cruise no 5	06.02.92	100	12.2	15	0.47
cruise no 5	06.02.92	600	6.5	15	0.50
cruise no 5	06.02.92	850	5	15	0.46
cruise no 6	15.04.92	40	15	17.3	0.42
Arabian Sea Process Study					
station 2	May-95	500	12	27	0.81
	May-95	1000	8.8	27	0.72
	May-95	1500	5.4	27	0.63

station 4	May-95	70	23.7	27.5	0.72
	May-95	500	12.6	27.5	0.77
	May-95	1000	8.8	27.5	0.70
	May-95	1500	5.4	27.5	0.65
station 5	May-95	90	22.5	26.9	0.67
	May-95	500	12.4	26.9	0.81
	May-95	1500	5.4	26.9	0.69
station 6	May-95	500	12	26.6	0.80
	May-95	1000	8.8	26.6	0.71
station 7	May-95	85	23.4	26.6	0.67
station 10	May-95	80	24	26.9	0.75
	May-95	450	12	26.9	0.76
station 13	May-95	35	25.4	26.8	0.62
	May-95	500	12.6	26.8	0.76
	May-95	1000	8.9	26.8	0.76

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Chapter 7

Archaeal tetraether membrane lipid fluxes in the northeastern Pacific and the Arabian Sea: Implications for TEX_{86} paleothermometry

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Submitted to *Paleoceanography*

Abstract.

The newly introduced temperature proxy TEX_{86} is based on the number of cyclopentane moieties in the glycerol dialkyl glycerol tetraethers (GDGTs) lipids of marine Crenarchaeota which change as a function to temperature. The GDGT signal used for TEX_{86} paleothermometry found in sediments reflect sea surface temperature (SST). However, marine Crenarchaeota occur ubiquitously in the world oceans over a large depth range and until now only work done on suspended particulate organic matter (POM) confirmed that the TEX_{86} in depth correlates well with SST. We analysed the GDGT distribution in settling POM using sediment traps from the northeastern Pacific Ocean and the Arabian Sea to investigate the seasonal and spatial distribution of the fluxes of crenarchaeotal GDGTs and the TEX_{86} signal which is transported to the sediment. At both settings the obtained TEX_{86} at all trap deployment depth reflects SST. At the Arabian Sea time series the TEX_{86} in the shallow trap at 500 m followed the in situ SST with an offset of about 3 weeks due to the settling speed of the GDGTs. This revealed that the GDGT signal which reaches deeper water is derived from the upper water column and reflect SST. The GDGT temperature signal in the deep traps at 1500 m and 3000 m do not show seasonal cycle but reflected annual mean SST. This is probably due to lateral transport of particles and associated mixing.

7.1 Introduction

Recently a new organic geochemical sea surface temperature proxy, the TetraEtherIndex of lipids with 86 carbon atoms (TEX_{86}) was proposed [*Schouten et al.,* 2002]. This new proxy is based on the number of cyclopentane moieties in the tetraether membrane lipids of marine Crenarchaeota. Marine Crenarchaeota are prokaryotes belonging to the domain of the Archaea. They are ubiquitously distributed in the world's ocean [*Fuhrman et al.,* 1992; *DeLong,* 1992; *Hoefs et al.,* 1997; *Sinninghe Damsté et al.,* 2002a] and it was estimated that marine Crenarchaeota comprises ca. 20% of all picoplankton in marine environments [*Karner et al.,* 2001].

Crenarchaeota possess unique membrane lipids which consist of glycerol dibiphytanyl glycerol tetraethers (GDGTs) [*DeRosa and Gambacorta* 1988] (see Fig.1 for structures). *Schouten et al.* [2002] showed that the distribution of marine crenarchaeotal GDGTs in core top sediments derived from different geographic locations correlate well with SST (Table 1). In cold areas the GDGT distribution is dominated by GDGT I and crenarchaeol (V). In warmer regions the GDGT distributions differ substantially as crenarchaeol is the most abundant GDGT and higher amounts of 1-3 cyclopentane-containing GDGTs (II-IV) and a regio-isomer of crenarchaeol (VI) are detected.

The increase of cyclopentane moieties in the membrane lipids of thermophilic Crenarchaeota, close phylogenetic relatives of the marine Crenarchaeota, is considered to be a temperature adaptation mechanism of the cell membrane [*Gliozzi et al.,* 1983, *Uda et al.,* 2001]. Recent mesocosm studies confirmed that marine Crenarchaeota inherited a similar way of temperature adaptation from their hyperthermophilic ancestors [*Wuchter et al.,* 2004]. With increasing temperature an increase in the number of cyclopentane moieties was observed in the GDGT membrane lipids of marine Crenarchaeota. 16S rDNA analyses revealed that a single crenarchaeotal species, derived from North Sea waters, was present in the incubated mesocosm tanks and that the changing GDGT distribution reflected a physiological adaptation. The obtained TEX_{86} equation from the mesocosm studies had a similar slope but differed in the intersection to the y axes compared to the core top equation (Table 1). This offset in the slope of the equation was caused by a relatively low amount of the regio-isomer of crenarchaeol. This GDGT was less pronounced in the cold water crenarchaeotal species,

cultivated in the laboratory, compared to crenarchaeotal species which dwell in warmer environments.

The observed correlation of TEX_{86} in core top sediments with annual mean SST suggests that the marine crenarchaeotal lipid signal is predominantly biosynthesized in the upper part of the water column. This is supported by our study of GDGT distributions in suspended particulate organic matter (POM) from different oceanic regions [*Wuchter et al.*, 2005], which revealed that the TEX_{86} correlates well with the upper 100m water column temperature (Table 1). GDGTs from the photic zone are probably much more effectively transported to the sediment by grazing and repackaging in large particles than GDGTs from deeper waters. The obtained equation for suspended POM samples was similar to the core top equation although there was a larger amount of scatter apparent compared to the core top equation. This was probably caused by contributions of both living and dead cell material of marine Crenarchaeota [*Wuchter et al.*, 2005]. The POM samples represented a mixture of fine suspended material, with negligible settling velocities and long residence time in the water column, and rapid sinking particles with a high settling rate [*Wakeham and Canuel* 1988].

Our previous studies established that the TEX_{86} in suspended POM is strongly correlated with SST. However, it has not been demonstrated yet how and when the signal produced in the water column is transported to the sediment floor. For this the fluxes of GDGTs through the water column need to be determined using sediment traps. Sediment traps are used to sample the flux of particulate organic matter which is derived from the surface waters mostly at time of high productivity [*Wakeham and Lee* 1993]. In this study, we analyzed the GDGT distribution in settling particulate organic matter using sediment traps from the northeastern Pacific Ocean and the Arabian Sea to investigate the seasonal and spatial distribution of the fluxes of crenarchaeotal GDGT and TEX_{86} signal transported to the sediment.

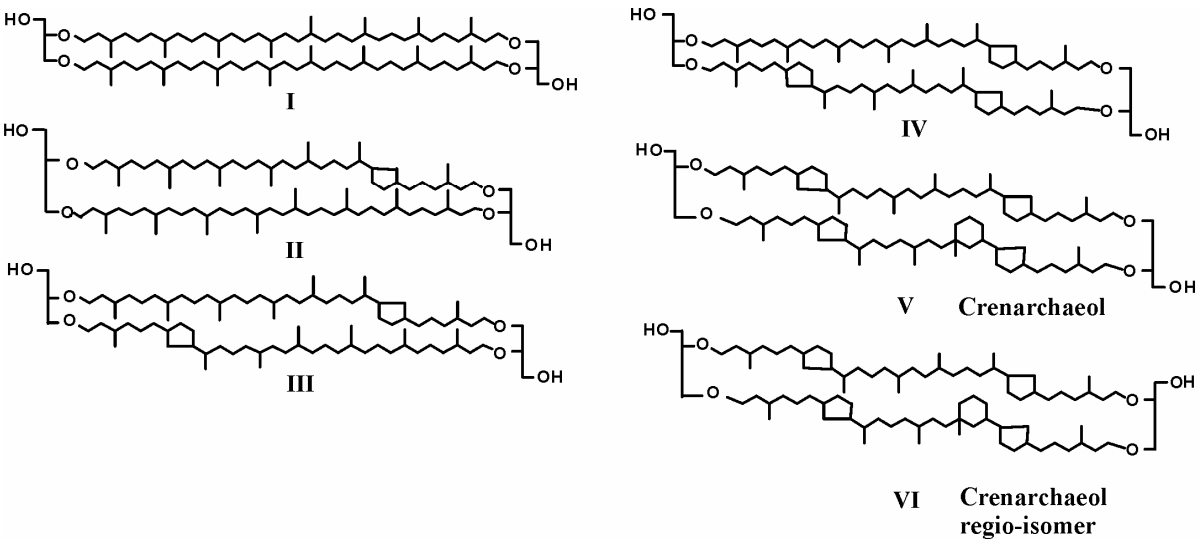

Figure 1. Structures of GDGT membrane lipids of marine pelagic Crenarchaeota.

origin	equation	r^2	sample amount
core tops	TEX_{86}= 0.015*T+0.29	0.92	n= 61
mesocosm experiment	TEX_{86}= 0.015*T+0.10	0.79	n= 16
POM from upper 100m	TEX_{86}= 0.017*T+0.29	0.80	n= 65

Table 1. Comparison of the TEX_{86} equations obtained from core top sediments [*Schouten et al.,* 2002], the mesocosm experiment [*Wuchter et al.,* 2004] and POM from different oceanic provinces [*Wuchter et al.,* 2005].

7.2 Material and Methods

7.2.1 Study sites and collection of samples

7.2.2 Northeastern Pacific

During June 1984 sediment traps were deployed in the northeastern Pacific at two stations. One station was located in the oligotrophic center of the northeastern Pacific Ocean at 33.3°N, 139.2°W [VERTEX 5A, *Silver and Gowing* 1991] and the other station was located in the upwelling area near the Californian coast at 36.1°N, 122.6°W [VERTEX 5C, *Silver and Gowing* 1991]. Free floating Soutar-type particle interceptor traps [for details see *Wakeham and Canuel* 1988], with a collecting area of 0.25 m^2, were used to collect rapidly sinking, large particles over four different depth intervals (100 m, 250 m, 750 m and 1500 m). The sinking particles were size fractioned in >300 µm and <300 µm. Trap deployment periods were 22 days for the oligotrophic setting and 13 days for the upwelling station. Mercury chloride was used to inhibit decomposition of the material in the traps.

7.2.3 Arabian Sea

The sampling site in the Arabian Sea was located approximately 350 km offshore of the Omani coast (MS-3: 17º12'N, 59º36'E) at a water depth of 3465 m. Three trap arrays at depth of ~500 m, ~1500 m and ~3000 m with varying module configurations (wide mouth traps with 0.37 m^2 collecting areas and narrow mouth traps with 0.018 m^2 collecting areas) were deployed in November 1994 (R/V *Thomas G. Thomson* cruise TN041), serviced and redeployed in May 1995 (cruise TN047) and recovered after a second time segment in January 1996 (cruise TN050). Carousels in the traps were programmed to collect sinking material over variable time intervals, ranging from 8.5 to 34 days. Shorter intervals were set for monsoon periods when higher fluxes were expected. In total for each depth 22 time-resolved samples were recovered. To inhibit decomposition mercuric chloride was used as a biocide (Lee et al 1992). For more detailed information about the Arabian Sea setting see *Wakeham et al.* [2002].

7.2.4 GDGT analysis

The sediment-trap samples were split and the lipid fraction was filtered over muffled glass fiber filters and Soxhlet extracted with dichloromethane (DCM) and methanol (2:1 by

volume) at Skidaway Institute of Oceanography (for details see *Wakeham et al*., 2002). An aliquot of the total lipid extract was cleaned over an activated Al_2O_3 column by eluting with methanol/DCM (1:1 by volume). For analysis of GDGTs, the solvent was removed under a stream of nitrogen and the residue was dissolved by sonication (5 min) in hexane/propanol (99:1 by volume). 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 (HPLC) - atmospheric pressure positive ion chemical ionization mass spectrometry (APCI-MS) by applying conditions slightly 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 were 15 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, by volume) 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 temperature 200 °C, capillary voltage -3 kV, corona 5 µA (~ 3.2 kV). GDGTs were detected by single ion monitoring of their $[M+H]^+$ ions (dwell time 237 ms) and quantified by integration of the peak areas and comparison with a standard curve of a Crenarchaeol standard.

For TEX_{86} calculation the peak areas of GDGTs I-VI were integrated and TEX_{86} values were calculated according to the index from *Schouten et al.,* [2002] which was defined as follows:

$TEX_{86} = (III+IV+VI) / (II+III+IV+VI)$. [1]

For the temperature correlation only TEX_{86} values were used where all isomers of GDGTs could be detected in sufficient abundances. TEX_{86} temperatures were calculated using the correlation obtained by *Schouten et al.* [2002]:

$TEX_{86} = 0.015*T+0.28$, ($r^2 = 0.92$) with T = annual mean SST (in °C). [2]

7.3 Results

The crenarchaeotal GDGT flux and distribution at different depths was analysed at two different settings in the northeastern Pacific during June 1984 and during the annual cycle at one station in the Arabian Sea in 1995. These data were used to investigate the variation with depth of the fluxes of crenarchaeotal GDGT and the TEX_{86} signal which is transported to the sediment. The station in the Arabian Sea also provided information on the seasonal flux of GDGTs and seasonality of the TEX_{86} signal.

7.3.1 GDGT flux and TEX_{86} signal in the northeastern Pacific

At the oligotrophic station in the northeastern Pacific GDGT fluxes between 0.2 and 4 µg GDGTs m^{-2} day^{-1} were measured. The maximum GDGT fluxes was measured at 750 m depth with about 4 µg GDGTs m^{-2} day^{-1} in the size fraction <300 µm (Fig. 3). Generally, higher GDGT fluxes were measured below 100 m depth and in the trap fraction <300µm (Fig. 2a). At 1500 m the GDGT flux was substantially lower with less than 0.5 µg GDGTs m^{-2} day^{-1} (Fig. 2a). At the 100m trap 22 % of the GDGT flux was derived from the >300µm fraction (Fig. 2a). With increasing depth the percentage of >300 µm fraction in the flux increased to 41 % at 250 m and 32 % at the 750 m trap. The TEX_{86} varied between 0.56 and 0.64 with highest TEX_{86} values in the 250 m traps (Table 2). TEX_{86} values between the different size fractions showed no substantial difference at the same depth (Table 2). The average TEX_{86} was 0.59 ± 0.04 at this station.

For the upwelling station GDGT fluxes varied between 5 µg and 4.3 mg GDGTs m^{-2} day^{-1}. The maximum GDGT flux was measured at 1500 m depth with about 4.3 mg GDGTs m^{-2} day^{-1} (Fig. 2b). The GDGT flux at the 100 m depth trap was ca. 60 µg GDGTs m^{-2} day^{-1} for the fraction <300 µm and comprised 93 % of the GDGT flux. The fraction >300 µm represented only 7% of the GDGT flux. The trap deployed at 250 m showed similar fluxes of ca. 40 µg GDGTs m^{-2} day^{-1} for both size fractions (Fig. 2b). At 750 m a higher flux was measured for the fraction <300 µm with about 100 µg GDGTs m^{-2} day^{-1} comprising 67 % of the GDGT flux (Fig. 2b). The fraction >300 µm at the same depth had a flux of about 60 µg GDGTs m^{-2} day^{-1} (Fig. 2b). The TEX_{86} varied between 0.45 and 0.54 and the average TEX_{86} was 0.50 ±0.03. Generally there was no substantial difference in the TEX_{86} from both size fractions of descending particles derived from the same depth (Table 2).

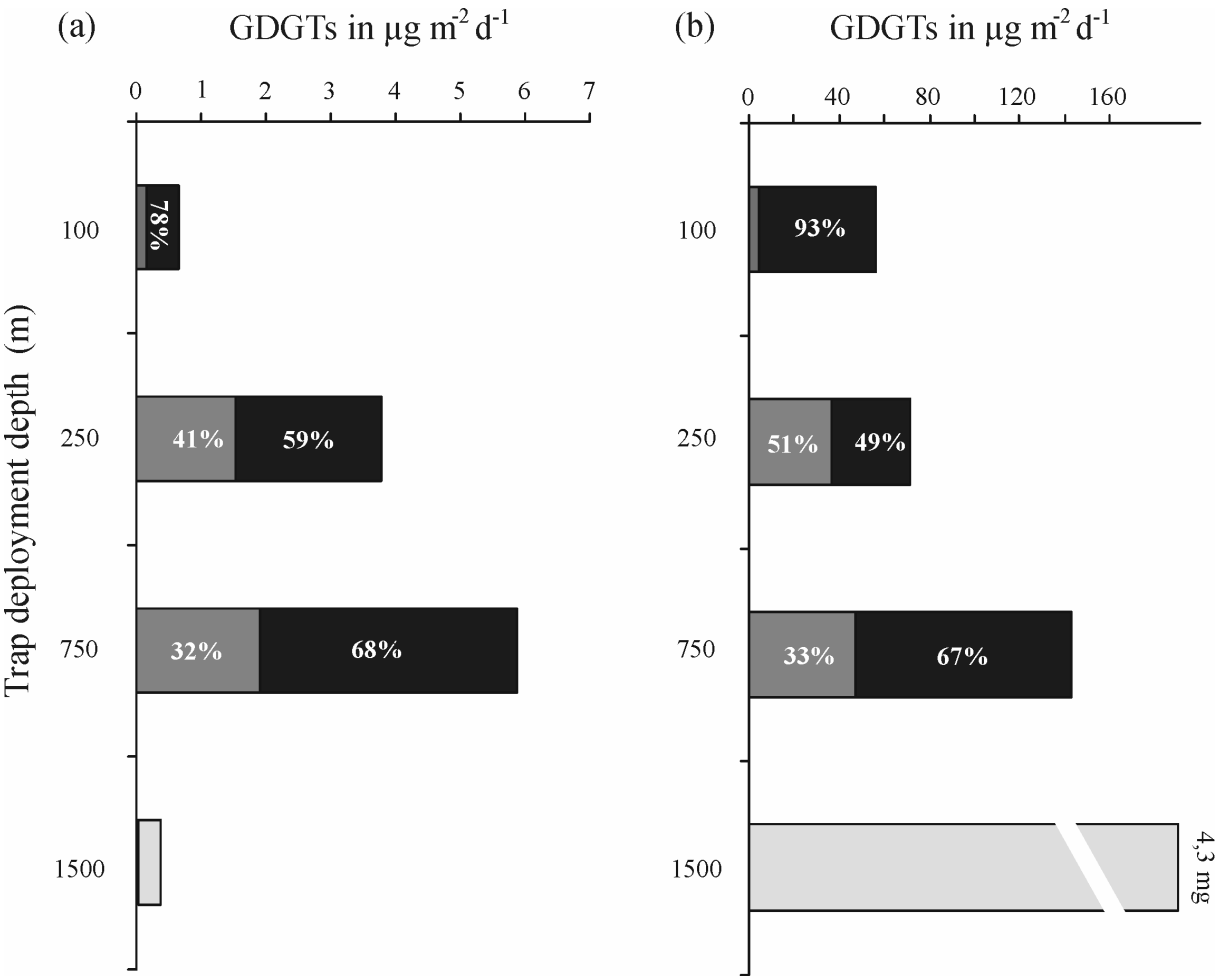

Figure 2. GDGT fluxes in different depth and size classes in the northeastern Pacific Ocean. Traps were deployed (a) in an oligotrophic setting and (b) at an upwelling site near the Californian Coast. Grey shaded bars represent the size fraction > 300 µm and black bars represent size fraction < 300 µm. White number in bars represent the percentage of the different size fractions of the total GDGT flux. White bars represent combined fractions at 1500 m deployment depth.

	depth (m)	TEX_{86} > 300um	TEX_{86} < 300um	TEX_{86} temperature (°C) > 300um	TEX_{86} temperature (°C) < 300um	mean *in situ* temp.(°C)	annual mean SST (°C)
oligotroph	100m	n.d	n.d	n.d	n.d	18	
	250m	0.64	0.60	23.3	20.7	10	
	750m	0.57	0.56	18.7	18.0	4.5	
	1500m		n.d		n.d	2.8	**20**
	average	**0.61**	**0.58**	**21.0**	**19.4**		
	Std.	**0.05**	**0.03**	**3.3**	**1.9**		
upwelling	100m	0.51	0.52	14.7	15.3	9	
	250m	0.54	0.49	16.7	13.3	7	
	750m	0.45	0.46	10.7	11.3	4.5	**14**
	1500m		0.52[a]		15.3[a]	2.8	
	average	**0.50**	**0.50**	**14.0**	**13.8**		
	Std.	**0.05**	**0.03**	**3.1**	**1.9**		

Not determined (n.d.) were samples with too low amount of GDGTs for reliable TEX_{86} calculation

(a) at the 1500 m trap both size fractions were combined

Table 2. TEX_{86} values and calculated TEX_{86} temperature compared to annual mean SST at the two sampling sites at the northeastern Pacific Ocean.

7.3.2 GDGT fluxes and TEX_{86} in the Arabian Sea

GDGT fluxes in the Arabian Sea showed a strong seasonality. At all three trap deployment depths (500, 1500, and 3000 m) the highest fluxes of GDGTs were measured during the monsoon periods with the highest fluxes during the south-west monsoon (SWM) (Fig. 3a). In the 500 m trap the maximum GDGT flux was about 20 µg GDGTs m^{-2} day^{-1} during the SWM and about 14 µg GDGTs m^{-2} day^{-1} during the north east monsoon (NEM) (Fig. 3a). During the summer inter-monsoon (SI) and fall inter-monsoon (FI) the fluxes were generally low with fluxes of 0.2 - 5 µg GDGTs m^{-2} day^{-1} during the SI (Fig.3a). In the trap deployed at 1500 m the GDGT fluxes were generally twice as high as in the 500 m trap. Maximum GDGT fluxes of ~40 µg GDGTs m^{-2} day^{-1} were found during the SWM and ca. 20 µg GDGTs m^{-2} day^{-1} during the NEM in this trap (Fig. 3a). During the SI and FI the fluxes were lower varying between 2-13 µg GDGTs m^{-2} day^{-1}. Compared to the other two traps, the GDGT flux was lowest in the trap deployed at 3000 m and varied between 2-9 µg GDGTs m^{-2}

day^{-1} with maximum fluxes in the SWM (Fig. 3a). The seasonal change in the flux of GDGTs was less pronounced in the 3000 m trap compared to the shallower traps.

The TEX_{86} varied between 0.67- 0.75 at the 500 m trap, at the 1500 m trap between 0.66-0.74 and between 0.66-0.71 at the 3000 m trap (Table 3). In the trap deployed at 500 m a pronounced seasonal variation in the TEX_{86} signal is observed (Fig. 4a). This seasonal variation in the TEX_{86} was less pronounced in the 1500 m depth trap (Fig. 4b) and the 3000 m trap (Fig. 4c).

7.4 Discussion

7.4.1 Variation in GDGT fluxes with depth in the water column

At both investigated sampling sites GDGT fluxes increased below 100 m depth and maximum GDGT fluxes were measured in the Arabian Sea in the mid- water trap at 1500 m in the Arabian Sea (Fig. 3) and in the Northeastern Pacific at 750 m trap at the oligotrophic site and at 1500 m trap in the upwelling area (Fig. 2).

Deep water flux maxima for particles and lipids were reported previously in other oceanic settings [see *Wakeham and Lee* 1993 for references] and several processes have been invoked to explain this distribution. The accumulation of sinking particles at density discontinuities within the water column could create this distinct distribution [*Karl et al.,* 1976]. Also the input of fecal matter from migrating zooplankton populations into deeper water layers [*Urrère and Knauer* 1981, *Karl and Knauer* 1984] could cause deep water flux maxima. In the northeast Pacific at both investigated sampling sites a similar increase in the ratio of GDGTs derived from the larger size fraction was observed in water below 100 m depth (Fig. 2).The GDGTs in the larger size fraction are probably derived from fecal pellets of vertically migrating zooplankter in deeper waters. This supports previous studies where high fecal pellet fluxes were reported in mesopelagic waters at both sampling sites of the northeastern Pacific [*Silver and Gowing* 1991].

In the Arabian Sea vertical migration of zooplankton even in the oxygen minimum zone was reported previously [*Smith et al.,* 1998, *Wishner et al.,* 1998]. This migration behavior could be responsible for fecal pellet transport into the mid- water trap, bypassing the trap at 500 m depth. Horizontal advective processes such as lateral transport probably also play an important role in the distribution of organic matter into deep waters [*Karl and Knauer*

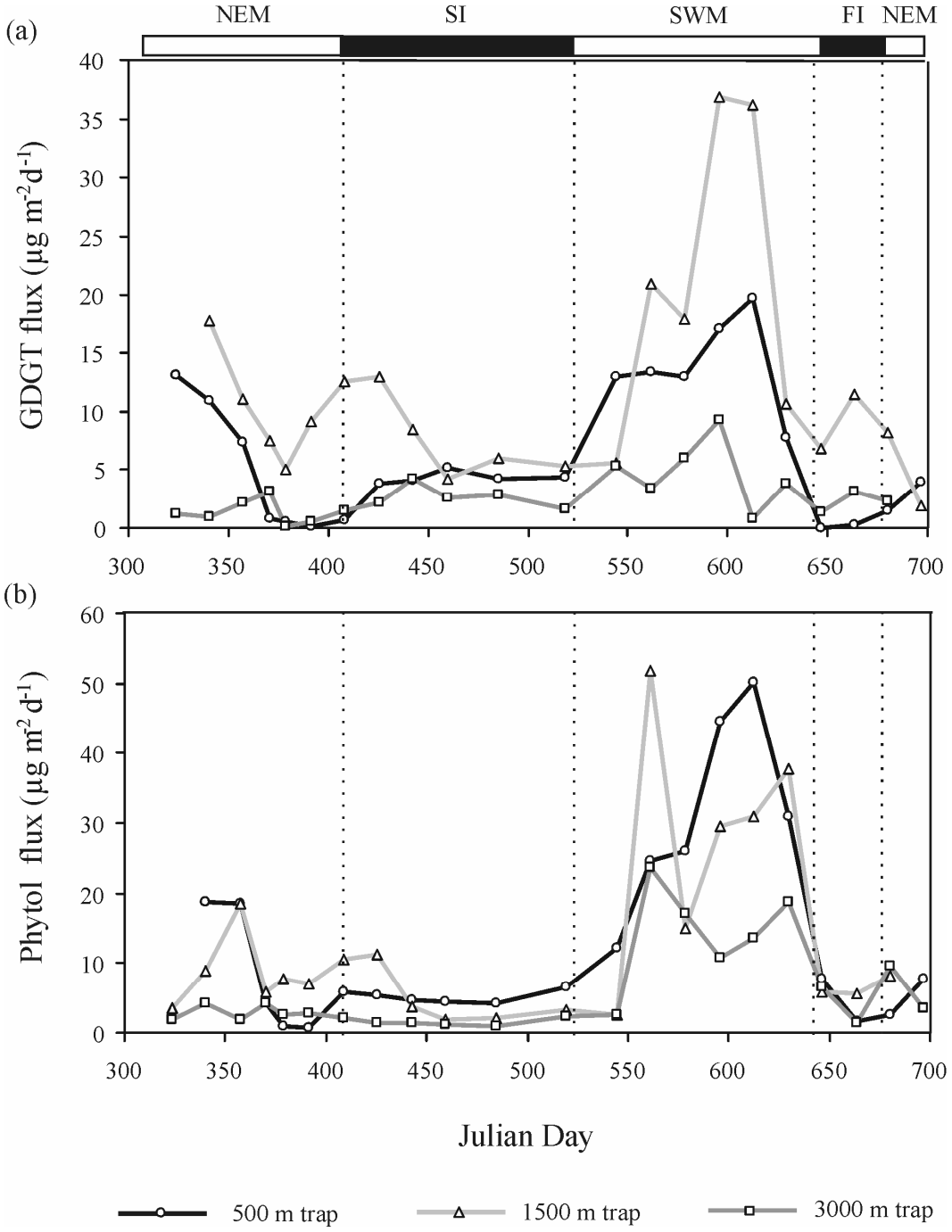

Figure 3. (a) GDGT and (b) phytol fluxes for the IRS-C traps at MS-3 in the Arabian Sea. Source data for phytol fluxes are obtained from http://usjgofs.whoi.edu/jg/dir/jgofs/arabian/. Bars at the top of the plot and dotted vertical lines indicate the monsoon periods based on *Weller et al.,*[1998]. NEM: North East monsoon, SI: Spring Inter-monsoon, SWM: South West monsoon, FI: Fall Inter-monsoon. Data points represent the center of collection intervals. The black line represent the shallow trap at ~500 m, the light grey correspond to the mid-trap at ~1500 m and the dark grey line represent the deep trap at ~3000 m depth.

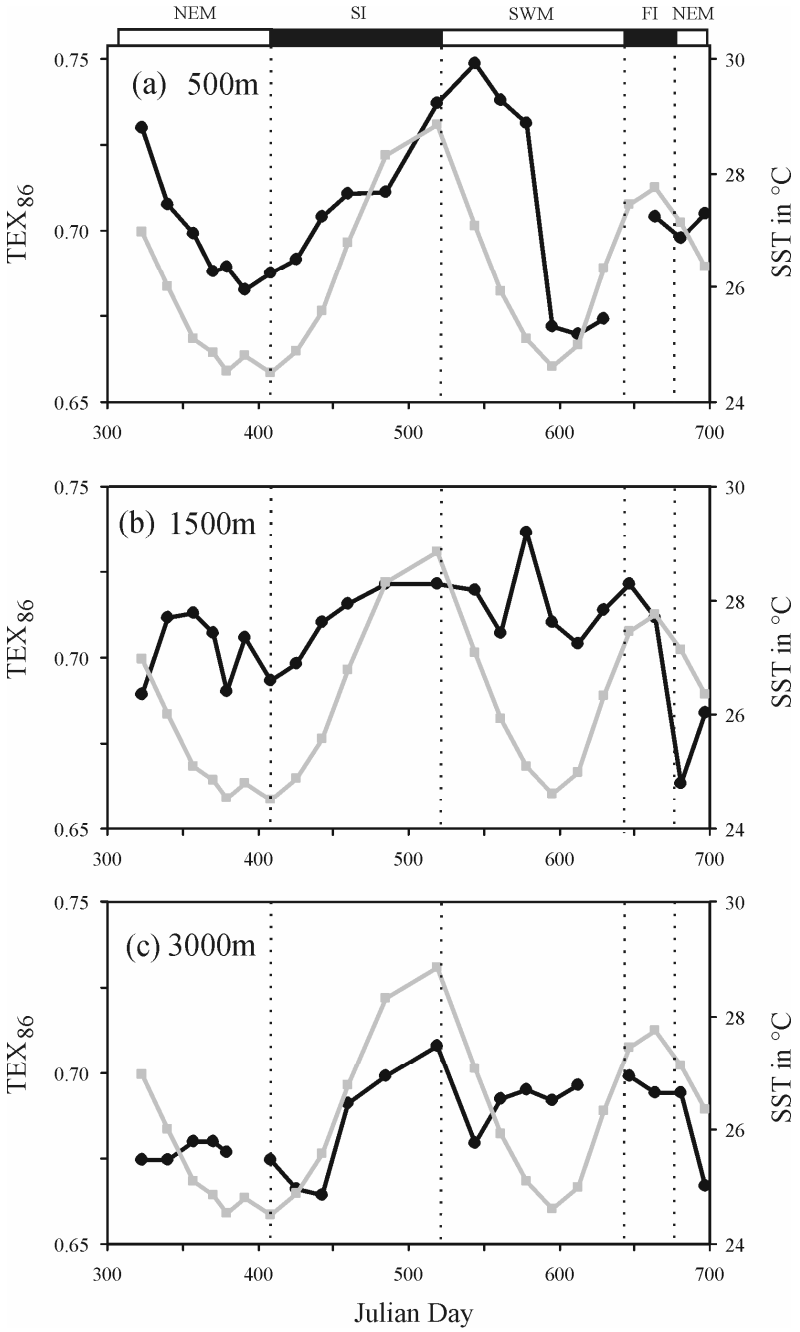

Figure 4. Seasonal variations in TEX_{86} and calculated SSTs in traps deployed at (a) 500 m, (b) 1500 m, (c) 3000 m depth at MS-3 station in the Arabian Sea. The black lines represents TEX_{86} values obtained at the different depths. Missing data points represent samples with insufficient amounts of GDGTs for TEX_{86} calculation. The grey lines represent integrated SST per deployment period. SST data were obtained from the pathfinder Advanced Very High Resolution Radiometer (AVHRR) data set at http: podaac.jpl.nasa.gov.

Julian Day	Average SST (°C)	500 m trap		1500 m trap		3000 m trap	
		TEX_{86}	TEX_{86} T (°C)	TEX_{86}	TEX_{86} T (°C)	TEX_{86}	TEX_{86} T (°C)
315 -332	27.0	0.73	29.3	0.69	26.6	0.67	25.6
332 -349	26.0	0.71	27.8	0.71	28.1	0.67	25.6
349 -366	25.1	0.70	27.3	0.71	28.2	0.68	26.0
366 -375	24.9	0.69	26.5	0.71	27.8	0.68	26.0
375 -383	24.5	0.69	26.6	0.69	26.7	0.68	25.8
383 -400	24.8	0.68	26.2	0.71	27.7	n.d.	n.d.
400 -417	24.5	0.69	26.5	0.69	26.9	0.67	25.6
417 -434	24.9	0.69	26.8	0.70	27.2	0.67	25.1
434 -451	25.6	0.70	27.6	0.71	28.0	0.66	24.9
451 -468	26.8	0.71	28.0	0.72	28.4	0.69	26.7
468 -502	28.3	0.71	28.1	0.72	28.8	0.70	27.3
502 -536	28.8	0.74	29.8	0.72	28.8	0.71	27.9
536 -553	27.1	0.75	30.6	0.72	28.6	0.68	26.0
553 -570	25.9	0.74	29.9	0.71	27.8	0.69	26.8
570 -587	25.1	0.73	29.4	0.74	29.8	0.70	27.0
587 -604	24.6	0.67	25.5	0.71	28.0	0.69	26.8
604 -621	25.0	0.67	25.3	0.70	27.6	0.70	27.1
621 -638	26.3	0.67	25.6	0.71	28.2	n.d.	n.d.
638 -655	27.5	n.d.	n.d.	0.72	28.8	0.70	27.3
655 -672	27.8	0.70	27.6	0.71	28.1	0.69	27.0
672 -689	27.1	0.70	27.2	0.66	24.9	0.69	26.9
689 -705	26.4	0.70	27.6	0.68	26.2	0.67	25.1
***In situ* annual mean (°C)**	**26.4**						
Standard deviation		**0.02**	**1.5**	**0.02**	**1.1**	**0.01**	**0.8**
Flux weighted mean		**0.71**	**27.8**	**0.71**	**27.9**	**0.69**	**26.6**

Not determined (n.d.) were samples with too low amount of GDGTs for reliable TEX_{86} calculation

Table 3. TEX_{86} values, TEX_{86} temperature and flux weighted mean TEX_{86} temperature at the three trap deployment depth compared to *in situ* annual mean SST at the MS-3 station in the Arabian Sea. The average SST for each trap deployment time interval was calculated satellite date of the pathfinder Advanced Very High Resolution Radiometer (AVHRR) data set at http: podaac.jpl.nasa.gov.

1984]. In the northeastern Pacific upwelling site strong lateral advection was observed in meso-and bathypelagic realm [*Silver and Gowing* 1991] which may explain the observed substantial increase in the GDGT flux at 1500 m depth. This increase is probably derived from re-suspended sedimentary material transported in the Californian coastal current.

In the Arabian Sea lateral advection via eddies and filaments and re-suspension of sediments off the shallow Oman shelf can decouple deep water trap collections from surface processes [*Siegel and Deuser* 1997] and could be responsible for the flux maxima in the mid-water traps [*Honjo et al.* 1999, Wakeham et al., 2002]. Descending particles sampled in deep

water traps probably represent a complex mixture of particles derived from large areas. For instance, in the Arabian Sea the particle catchment areas might be as large as 10^3-10^4 km^2 (e.g. van *Gyldenfeld et al.,* 2000). Also the lateral transport of suspended sediments off the Arabian shelf may have an important influence in particle fluxes [*Witte and Pfannkuche*, 2002].

7.4.2 Seasonal variation in GDGT fluxes

At all three deployment depths the highest GDGT fluxes in the Arabian Sea time series were measured during the monsoon periods, especially during the SWM (Fig. 3a). This is consistent with previous studies were maximum fluxes of organic carbon and biomarker lipids occurred during the SWM, regardless of trap deployment depth and location [*Wakeham et al.,* 2002]. Phytol is a general biomarker for photoautotrophic plankton and in the Arabian Sea the highest GDGT fluxes occur at the same time as the highest phytol fluxes (Fig. 3b). This GDGT flux pattern seems in contradiction with the ecology of marine Crenarchaeota. Marine Crenarchaeota appear to be chemolithoautotroph (*Wuchter et al.,* 2003, *Herndl et al.,* 2005, *Wuchter et al.,* submitted) competing with phytoplankton for nutrients and their abundance is negatively correlated with that of phytoplankton as reported previously in different oceanic regimes [*Murray et al.,* 1998, 1999, *Massana et al.,* 1997, *Wuchter et al.,* submitted]. However, higher fluxes of GDGTs during SWM do not necessary imply a higher abundance of marine Crenarchaeota during the SWM. The relative increase in the GDGT flux between SI and SWM at 500 m is substantially smaller in comparison to that of specific biomarker lipids derived from photoautotroph organisms (i.e. diatoms and haptophytes) and even of total particles and organic carbon (Table 4). The high organic carbon flux during the SWM is caused by blooming photoautotrophs as a response to the increased levels of nutrients in the photic zone derived from upwelling waters. Major particulate mass fluxes occur at times of high primary productivity [*Wakeham and Canuel* 1988] because at that time a highly active food-web exists in the upper water column and a lot of phytoplankton derived particles settle down in deeper waters as marine snow. Thus, the higher amounts of GDGTs which are transported at that time to the sea floor are likely caused by the much more active food-web during the SWM, resulting in more efficient scavenging of archaeal cells, and not because of higher cell abundances of the marine Crenarchaeota.

Compounts	Origin	average SI flux $m^{-2}d^{-1}$	average SWM flux $m^{-2}d^{-1}$	rel. increase %
Total particle s		60 mg	361 mg	602
Organic carbon		6 mg	28 mg	443
Phytol	Phototrophs	5 µg	28 µg	552
C25:3 HBI	Rhisozolenoid diatoms	0.3 µg	17 µg	5360
C30 alkane 1,14 diol	Proboscia diatoms	3 µg	25 µg	952
37:2 alkenones	Haptophyts	0.4 µg	5 µg	1425
GDGTs	marine Crenarchaeota	4 µg	12 µg	276

Table 4. Average fluxes of different components during (SI) Spring Inter-monsoon and (SWM) South West monsoon at the 500 m trap at MS-3 station in the Arabian Sea.

7.4.3 TEX_{86} and size fraction

At the northeastern Pacific at both sampling sites no substantial variation in the TEX_{86} was observed in the different size classes of sinking particles (Table 2). The size fraction of the sinking particles <300µm consists of small passively sinking particles derived from the upper 100m , whereas the size fraction >300µm is probably mostly comprised of freshly produced fecal pellets of vertically migrating zooplankter which probably actively grazing on sinking large marine snow particles and descending algae [*Silver and Gowing* 1991]. The GDGT distribution and thus the TEX_{86} stay relatively invariant in the two size fractions. In the larger fraction the GDGTs are probably derived from Crenarchaeota which have been grazed and digested by zooplankton. Thus, our data show that alteration of GDGTs caused by grazing and digesting by heterotrophs does not change substantially the GDGT distribution.

7.4.4 Variations in TEX_{86} with depth

At the Arabian Sea traps and the northeast Pacific traps the TEX_{86} stayed relative invariant throughout the water column (Table 2 and 3). At both sampling sites the calculated TEX_{86} temperatures did not reflect in situ temperature at the different trap deployment depth but correlated best with the annual mean SST at these settings (Table 2 and 3). Interestingly, marine Crenarchaeota occur over a large depth range in the ocean [*Karner et al* 2001, *Herndl et al* 2005] and especially the GDGT signal of living crenarcheotal cells derived from the meso- and bathypelagic realm could influence the TEX_{86}. However, sinking particles like fecal pellets and marine snow aggregates having a high settling rate [*Wakeham and Canuel* 1988] and are mostly responsible for the vertical transport of material from the upper ocean to

the sea floor [*Wakeham and Canuel* 1988]. The similar TEX_{86} from the sinking particle fraction show that the GDGT signal is mostly derived from the upper part of the ocean and that the GDGT signal derived from deep water living Crenarchaeota do not contribute substantially to the GDGT sedimentary fluxes. This is consistent with previous studies on GDGTs in suspended POM from different oceanic regimes [*Wuchter et al.,* 2005], which shows that the TEX_{86} over a large depth range correlates best with SST rather than in situ temperature [*Wuchter et al* 2005]. In addition, a study of GDGTs in suspended POM and sediment traps from the Black Sea [*Wakeham et al.,* 2003] showed that high amounts of ^{13}C-depleted GDGTs in suspended POM in the deep anoxic zone could not be found in sediment traps and the underlying sediments. The authors suggested that due to the absence of grazing in the anoxic zone of the Black Sea, GDGTs biosynthesized in deep waters lack transport mechanisms to the sea floor, and thus the dominant GDGT flux occurs from the upper water column were an active food-web exists [*Wakeham et al.,* 2003].

7.4.5 Seasonal variation in TEX_{86}

The TEX_{86} measured at the shallow 500 m trap in the Arabian Sea showed a pronounced trend that follows that of the *in situ* SST, obtained by satellite observations (Fig. 4a), with an apparent offset of about three weeks. The time a particle need to sink and be captured in the 500 m trap likely causes this time offset in the TEX_{86} signal. Fine particles move through the water column at rates of 1-3 m day^{-1}[*Krishnaswami et al* 1981, *Bacon and Anderson*, 1982], whereas fecal pellets and marine snow may sink at rates of tens-to hundreds of m day^{-1} [e.g. *Small et al*., 1979, *McCave* 1984, *Asper,* 1987]. The signal that reaches the trap at 500 m is an integrated signal of particles of different size classes and sinking speed. The offset of ca. three weeks in the 500 m trap indicates that the captured particles move on average 25 m day^{-1} through the water column, and this is well in the range with the above mentioned particle sinking speeds. In the mid-water trap at 1500 m and the deep trap at 3000 m the TEX_{86} temperature signal does not show this pronounced seasonality as observed at the 500 m trap (Figs. 4b and c). The mid-water and deep trap are probably more influenced by lateral transport of particles and the GDGT signal that is captured in these traps rather reflects an integrated mixed GDGT signal derived from a larger area. In contrast, the trap at 500 m shows for the first time that the TEX_{86} is following the seasonal SST cycle demonstrating that

this proxy is excellently capturing upper water column temperatures. The TEX_{86} SST signal measured at 500 m depth is on average ca. 1°C higher than the SST measured *in situ*. This temperature offset could be caused due to the calibration equation used, which is derived from core tops. This calibration line possibly slightly overestimates SSTs at high temperatures such as those in the Arabian Sea. Indeed, when the equation derived from POM is used (Table 2) the SST estimates are ~1.5°C lower than *in situ* SST. Further development of these calibration line is required to see if there is a systematic off set between sediment traps and core tops or whether the calibration line needs to be further improved.

The calculated flux-weighted mean TEX_{86} SST in the three traps of the Arabian Sea deployed at different depth reflect annual mean SST (Table 3). The calculated flux-weighted mean TEX_{86} SSTs for the 500 and the 1500 m trap are ca. 1ºC higher than for the 3000 m trap (Table 3). The calculated annual mean *in situ* SST during the trap deployment period is nearly the same as the calculated flux-weighted mean TEX_{86} SST signal from the 3000 m trap (Table 3). This shows that even if in the upper parts of the water column the TEX_{86} in descending particles reflects seasonal SST the GDGT signal that reaches deep water and sediment reflects an integrated signal and the corresponding TEX_{86} reflects annual mean SST. This integrated signal is probably caused by the previously mentioned lateral transport and mixing mechanisms of organic matter in the Arabian Sea. Our results may thus explain why TEX_{86} in core top sediments from different geographic locations which correlates well with annual mean SST [*Schouten et al* 2002].

7.5 Conclusion

Our data show that the TEX_{86} signal in GDGTs contained in descending particles reflects SST and confirms that the GDGT signal which reaches the sediment is mainly produced in the upper water layer. The TEX_{86} is relatively invariant in different size fractions of descending particles throughout the water column, suggesting that alteration due to grazing and re-packaging of sedimentary GDGTs by zooplankton does not appear to substantially influence the GDGT distribution and thus the TEX_{86}. In the Arabian Sea time series we observed a strong correlation of the TEX_{86} in the upper 500 m trap with seasonal SST. The TEX_{86} signal which reaches the deep trap during the annual cycle was relatively invariant and reflected annual mean SST. This deep water temperature signal is probably strongly influenced from

lateral transport of particles and, therefore, the TEX_{86} in deeper waters or in sediment rather reflects an integrated annual mean SST rather than a seasonal SST signal. Our data presented here, in combination with previous studies on GDGT distributions in core top sediments [*Schouten et al.,* 2002], in suspended POM [*Wuchter et al*, 2005] and in mesocosms [*Wuchter et al.,* 2004], show the potential to use the TEX_{86} for reconstruction of upper water column temperatures in ancient environments.

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Summary

Archaea form one of the three domains of life on Earth and together with the bacteria form the prokaryotes. The application of molecular techniques such as ribosomal DNA or lipid analyses revealed that Archaea appear widespread and abundant. In the marine environment planktonic Archaea consist of two major groups, the Crenarchaeota and the Euryarchaeota of which the former appears to be the most abundant and may account for ca. 20% of all prokaryotic cells in the global ocean. Despite the fact that marine Crenarchaeota constitute a substantial fraction of the picoplankton in the world oceans, little is known about their basic physiology. Results of stable isotope and radiocarbon analyses of the marine crenarchaeotal membrane lipids suggested that marine Crenarchaeota may utilize bicarbonate as their carbon source. Microautoradiography investigations performed in the neritic waters of the Mediterranean Sea, the Californian Coast and the deep Atlantic Ocean showed that some marine Archaea are capable to take up amino acids and some deep sea marine Crenarchaeota and Euryarchaeota are capable to utilize bicarbonate or CO_2 as their carbon source. Field studies in the Santa Barbara Channel and the Arabian Sea showed a positive correlation of the abundance of Crenarchaeota and nitrite concentrations. In Arctic waters a positive correlation of marine Crenarchaeota with particulate organic nitrogen was reported. This suggested that marine Crenarchaeota may be involved in the marine nitrogen cycle but the metabolic requirements of pelagic Archaea remain still enigmatic.

The membrane lipids of Crenarchaeota are unique and consist of glycerol dibiphytanyl glycerol tetraethers (GDGTs). Recent studies done on core top sediments showed that the distribution of sedimentary crenarchaeotal GDGTs from different geographic locations varies with sea surface temperature (SST). The change of the GDGT distribution of marine Crenarchaeota was expressed in an index of GDGT isomers, which was named the Tetraether Index of lipids with 86 carbon atoms, the TEX_{86}. GDGTs are preserved upon sedimentation and were found in sediments up to 140 million years old. Therefore this new index was considered as a new temperature proxy for reconstruction of SST in paleoenvironments. However, the observed correlation of the GDGT distribution in core top sediments with SST does not provide direct evidence that marine Crenarchaeota adjust their membrane lipids to

temperature or that temperature is the only controlling factor. Salinity and nutrient concentrations can vary to a substantial degree in marine systems and may also influence the GDGT distribution. Furthermore, marine Crenarchaeota occur highly seasonal in surface waters and are generally relatively more abundant in the deeper zones of the ocean. However, the new temperature proxy TEX_{86} correlates best with annual mean SST. Therefore, it is as yet not clear during which season and at which depth in the water column the lipid signal used for TEX_{86} paleothermometry is biosynthesized.

In this thesis we focused on two major objectives. The first objective was to shed more light on the basic physiology of marine Crenarchaeota and addreses the ecological and physiological aspects of the marine Crenarcheota (**Chapter 2**, **3** and **4**). The second objective was to validate the newly introduced temperature proxy TEX_{86} (**Chapters 5**, **6** and **7**). This part deals with factors which determine the relative GDGT distribution in marine Crenarchaeota and the GDGT signal found in the water column and surface sediment.

In **Chapter 2** an *in situ* ^{13}C bicarbonate labeling experiment to study the carbon acquisition mechanism of marine Crenarchaeota is described. Fully labeled $H_2{}^{13}CO_3$ was added to a mesocosm tank filled with coastal North Sea Water and incubated for a week. The experiment was performed in the dark to eliminate photosynthetic activity. The distribution of the label in the various lipids was studies and revealed that very low ^{13}C incorporation was detected in lipids derived from bacterial and plant material. Especially the *anteiso*-C_{15} fatty acid, a compound exclusively attributed to bacteria, showed minor ^{13}C enrichment with $\Delta\delta^{13}C$ values of 10‰. In contrast, the crenarchaeotal membrane lipids were heavily labeled in ^{13}C with $\Delta\delta^{13}C$ values between 400-440‰ for the crenarchaeotal biphytanes. About 70% of the detected ^{13}C incorporation into lipids is accounted for by the crenarchaeotal biphytane membrane lipids. This showed that marine Crenarcheota are actively incorporating $H_2{}^{13}CO_3$ or $^{13}CO_2$ derived thereof and that at least some pelagic marine Crenarchaeota are thus autotrophic organisms.

In **Chapter 3** a combined study on an enrichment culture of a marine Crenarchaeota and a field time series observation was conducted to gain more information about the energy source of marine Crenarcheota and their possible involvement in the marine nitrogen cycle. In the enrichment culture an increase in crenarchaeotal abundance was observed when the ammonia level dropped and the nitrite concentration strongly increased. This data suggested

that the marine crenarchaeotal species which proliferated under laboratory conditions is capable of ammonia oxidation. Further evidence for archaeal nitrification was obtained by quantification of an archaeal *amoA*-like gene with quantitative polymerase chain reaction (Q-PCR). A strong correlation (r=0.99) between the crenarchaeotal abundance and the archaeal *amoA*-like gene copy number abundance was observed during the course of the experiment. The involvement of Crenarcheota in nitrification processes was further investigated in coastal North Sea waters. In the winter season the marine Crenarchaeota dominate the archaeal community and show a strong positive correlation with nitrite concentrations. In contrast, the cell abundances of beta-and gamma-proteobacteria, the known nitrifying bacteria, was invariant and did not show a correlation with the generation of nitrite. Thus, the data obtained from the enrichment experiment and from the field studies indicate that marine Crenarchaeota are active in the marine nitrogen cycle as nitrifiers.

In **Chapter 4** the seasonal dynamics of marine Archaea in costal North Sea surface waters during a time series is described by using a wide array of molecular approaches. The identity of the marine Crenarchaeota was determined by PCR using a general archaeal primer par followed by denaturing gradient gel electrophoresis (DGGE) in combination with sequencing. Three different quantification methods were used, catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH), Q-PCR and GDGT lipid analyses, to determine the abundance of the marine Archaea and to compare the relative robustness of the methods. The abundance pattern of CARD-FISH and Q-PCR were similar and indicated that one archaeal cell contains on average 0.9 ± (0.6) copy numbers of 16S rRNA genes. GDGT concentrations and crenarchaeotal cell abundances determined by CARD-FISH indicated the source organisms for GDGT membrane lipids in oxygenated waters are indeed restricted to marine Crenarchaeota. Comparison of the cell counts and the lipid analyses revealed that one crenarchaeotal cell contains on average 3 (± 0.5) pg GDGTs. A distinct seasonal distribution pattern of pelagic marine Crenrchaeota was observed with the CARD-FISH, GDGT and DGGE approach. In the winter season, when nutrient levels are elevated in the water, the marine Crenarchaeota dominate the archaeal community whereas the marine Euryarchaeota are more abundant during summer and fall when nutrient levels are lower. Different metabolic requirements of both archaeal groups probably cause this seasonal succession. The data

suggests that at least some marine pelagic Crenarchaeota are chemoautotrophic while some pelagic Euryarchaeota are probably heterotrophic organisms.

Chapter 5 describes a study which shows that temperature is the major controlling factor in the marine crenarchaeotal GDGT distribution. Mesocosm studies with marine Crenarchaeota incubated at temperature ranging from 5 to 35ºC and salinities of 27‰ and 35‰ were performed to test the validity of the TEX_{86} proxy. During the course of the experiment an increase in the concentration of GDGTs was measured with amounts up to 3.4 $\mu g\ l^{-1}$, indicating substantial growth of marine Crenarchaeota. With increasing temperature an increase of cyclopentane-ring containing GDGTs was observed. Different salinities did not have an effect on the GDGT distribution. The obtained TEX_{86} values of the different incubation temperatures showed a linear correlation which had a similar slope to the core top correlation, but the intersection of the correlation line with the Y-axis was different. This was probably caused by the differences between the crenarchaeotal species enriched under laboratory conditions and the more complex crenarchaeotal communities in the field. These mesocosm experiments indicated that water temperature is indeed the major controlling factor for the GDGT distribution of marine Crenarchaeota, and confirms that the TEX_{86} reflects temperature.

In **Chapter 6** suspended particulate organic matter (POM) samples from surface and deep waters at different oceanic settings were analyzed for their GDGT distribution to find out at which season and at which depth the GDGT signal for the TEX_{86} is produced. This investigation showed that GDGTs occur in higher abundance during the winter and early spring in surface waters. The depth distribution showed that GDGTs are generally more abundant below 100 m depth. A weak correlation of the TEX_{86} from below the photic zone with in situ temperature was observed. However, the TEX_{86} from deeper parts of the water column correlated best with SST. The TEX_{86} from POM in the upper 100m of the water column showed a linear correlation with in situ temperatures which was nearly identical to the core top equation. All POM samples correlated best with SST and were similar to the core top equation but with a lower correlation coefficient probably due to scatter of a mixed signal from living suspended Crenarchaeota and fossil sedimenting GDGTs. These data suggest that the GDGT signal which is transported to the sediment mainly reflects temperatures of the upper 100 m. In the upper water column an active food-web exists and therefore the GDGTs

from the upper parts of the ocean are probably more effectively transported to the sediment by grazing and repackaging

In **Chapter 7** a study on sedimenting particles from the northeast Pacific and the Arabian Sea was performed to analyze the seasonal and spatial distribution of the GDGT fluxes and the TEX_{86} signal transported to the sediment. At the northeastern Pacific studies were done on GDGT fluxes in different depth and size classes and reveled that the TEX_{86} stays relatively invariant in different size fractions of descending particles. This suggests that alteration due to grazing and re-packaging of sedimentary GDGTs by zooplankter do not substantially influence the GDGT distribution and thus the TEX_{86}. In both settings the TEX_{86} signal in the sediment traps at all depths reflect SST. In the shallow trap at 500 m in the Arabian See time series a strong correlation of the TEX_{86} with seasonal SST was observed. The TEX_{86} signal in the deeper traps was relatively invariant during the annual cycle and reflects annual mean SST. In the deeper traps the TEX_{86} signal is probably strongly influenced by lateral transport of particles. This suggests that the TEX_{86} in deeper waters or sediments probably reflects an integrated annual SST rather than a seasonal temperature signal.

The experimental work and the field studies presented in this thesis brought new insights in the physiology of marine Crenarchaeota and the new paleothermometer TEX_{86}. With the ^{13}C label experiment, the enrichment culture and the field observation in the North Sea it has been shown that at least some marine Crenarchaeota are chemolithoautotrophic organisms involved in nitrification. Since marine Crenarchaeota are one of the most abundant classes of picoplankton in the world ocean and occur over a large depth range, marine crenarcheotal chemolithoautotrophy may be a general and quantitatively important oceanic process. Marine crenarchaeotal nitrification may play an important role in the biochemical cycling of nitrogen in the ocean. Furthermore, marine Crenarchaeota are probably a significant global sink for inorganic carbon and may represent an important but as yet unrecognized component of the global carbon cycle.

In case of the TEX_{86}, the experimental work and the field studies validate the newly introduced temperature proxy. The mesocosm experiments demonstrated that temperature is the major controlling factor in the crenarchaeotal GDGT distribution, thus the TEX_{86} reflects temperature. The field studies done on POM and trap samples clearly showed that the GDGT signal which reaches deep waters or the sediment reflects SST or temperature from the upper

100 m of the water column due to grazing and repackaging processes. Therefore it should be possible to use the newly introduced paleothermometer TEX_{86} for reconstruction of the upper water column temperatures in ancient environments. Since GDGTs are found in sediments up to a 140 million years old this new temperature proxy may have a great potential as a tool for ancient climate reconstruction.

Samenvatting

Archaea vormen één van de drie domeinen van het leven op Aarde en vormen tezamen met de bacteriën de prokaryoten. De toepassing van moleculaire technieken zoals de analyse van ribosomaal DNA en lipiden hebben laten zien dat Archaea wijd verspreid en in grote hoeveelheden in het milieu voorkomen. In het mariene milieu vormen de planktonische Archaea twee groepen: de Crenarchaeota en de Euryarchaeota. De Crenarchaeota zijn de meest voorkomende groep en vormen ca. 20% van alle prokaryotische cellen in de oceaan. Ondanks dat mariene Crenarchaeota zo belangrijk zijn is er maar weinig bekend over hun fysiologische eigenschappen. Stabiele and radioactieve koolstofisotopenanalyse van lipiden van mariene Crenarchaeota suggereren dat ze bicarbonaat gebruiken als koolstofbron. Studies van water uit de Middellandse Zee, de Californische kust en de diepe Atlantische Oceaan met behulp van microautoradiografie hebben laten zien dat mariene Archaea aminozuren kunnen opnemen en dat sommige Crenarchaeota en Euryarchaeota in de diepzee in staat zijn om bicarbonaat of CO_2 te gebruiken als koolstofbron. Veldstudies in het Santa Barbara kanaal en de Arabische Zee laten zien dat er een positieve correlatie is tussen de aantallen Crenarchaeota en nitrietconcentraties. In Arctische wateren is er een positieve correlatie tussen de aantallen Crenarchaeota en particulair organisch stikstof gerapporteerd. Dit suggereert dat mariene Crenarchaeota een rol spelen in de mariene stikstofcyclus maar in welk opzicht is nog onduidelijk.

Crenarchaeota bevatten unieke membraanlipiden die bestaan uit glycerol dibifytanyl glycerol tetraethers (GDGTs). Recente studies aan oppervlaktesedimenten van verschillende locaties in de oceanen laten zien dat samenstelling van sedimentaire GDGTs afkomstig van Crenarchaeota varieert met oppervlaktezeewatertemperaturen (OZT). De verandering in de relatieve samenstelling van GDGTs wordt uitgedrukt in een index bepaald door de relatieve concentratie van verschillende GDGTs, genoemd de Tetraether Index van lipiden met 86 koolstofatomen, de TEX_{86}. Deze GDGTs worden gepreserveerd gedurende sedimentatie en worden gevonden in sedimenten tot 140 miljoen jaar oud. Deze index is dus een nieuwe proxy

voor OZT en kan gebruikt worden om OZT in vroegere milieu's te reconstrueren. De correlatie tussen GDGT distributie en OZT is echter geen direct bewijs dat mariene Crenarchaeota de samenstelling van hun membraanlipiden daadwerkelijk aanpassen aan temperatuur of dat temperatuur de enige factor is. Het zoutgehalte en concentraties van verschillende nutriënten variëren ook substantieel in mariene systemen en zouden ook de relatieve verdeling van GDGT isomeren kunnen bepalen. Bovendien zijn mariene Crenarchaeota in sterk variërende aantallen aanwezig gedurende de verschillende seizoenen en zijn zij ook in grote aantallen aanwezig in het diepere gedeelte van de oceaan. Het is dus niet duidelijk in welk seizoen en op welke diepte in de waterkolom het lipidensignaal, dat gebruikt voor TEX_{86} paleothermometrie, wordt gebiosynthetiseerd.

Het onderzoek beschreven in dit proefschrift had twee belangrijke doelstellingen. Het eerste doel was om meer te weten te komen over de fysiologie van mariene Crenarchaeota en het eerste gedeelte van dit proefschrift richt zich dan ook op ecologische en fysiologische aspecten van mariene Crenarchaeota (**Hoofdstukken 2**, **3** en **4**). Het tweede doel was de validatie van de onlangs geïntroduceerde OZT-proxy, de TEX_{86}. Het tweede gedeelte van dit proefschrift behandelt dan ook de factoren die de distributie van GDGTs van mariene Crenarchaeota bepalen en uiteindelijk het GDGT signaal in de waterkolom van de oceaan en de onderliggende sedimenten (**Hoofdstukken 5**, **6** en **7**).

In **Hoofdstuk 2** wordt de studie van het koolstofacquisitiemechanisme van mariene Crenarchaeota met behulp van een *in situ* ^{13}C-bicarbonaat labelexperiment beschreven. Gelabeld $H_2{}^{13}CO_3$ werd toegevoegd aan een mesocosmtank gevuld met kustwater van de Noordzee en een week lang geïncubeerd. Het experiment werd uitgevoerd in het donker om fotosynthetische activiteiten te elimineren. De verdeling van het ^{13}C-label in de verschillende lipiden werd bestudeerd en liet zien dan er weinig tot geen ^{13}C-inbouw kon worden gedetecteerd in de lipiden afkomstig van bacteriën en planten. De bacteriële *anteiso*-C_{15} vetzuur vertoonde bijvoorbeeld weinig verrijking in ^{13}C met een $\Delta\delta^{13}C$-waarde van +10‰. Echter, de membraanlipiden van Crenarchaeota waren sterk verrijkt in ^{13}C met $\Delta\delta^{13}C$-waarden van +400 tot +440‰ voor de bifytanen afkomstig van GDGTs van Crenarchaeota. Ongeveer 70% van de vastgestelde inbouw van ^{13}C in lipiden tijdens het experiment was toe te schrijven aan inbouw in de membraanlipiden van Crenarchaeota. Dit liet zien dat mariene

Crenarcheota actief $H_2{}^{13}CO_3$ of $^{13}CO_2$ opnemen en dat tenminste sommige pelagische mariene Crenarchaeota autotrofe organismen zijn.

In **Hoofdstuk 3** worden de uitkomsten van een studie naar de energiebron van mariene Crenarchaeota en hun mogelijke betrokkenheid in de mariene stikstofcyclus met behulp van een verrijkingscultuur van een marine Crenarchaeote en veldobservaties gerapporteerd. Een sterke toename in de celaantallen in een verrijkingscultuur van Crenarchaeota werd waargenomen toen ammonium concentraties daalden en nitriet concentraties toenamen. Dit suggereerde dat deze Crenarchaeote onder laboratorium condities in staat was om ammonia te oxideren. Verder bewijs voor archaeale nitrificatie werd verkregen door de identificatie en kwantificering met behulp van kwantitatieve PCR (Q-PCR) van een archaeaal *amoA* gen, het gen dat codeert voor een enzym dat een sleutelreactie in de oxidatie van ammonium katalyseert. Een sterke correlatie (r=0.99) tussen de celaantallen van de Crenarchaeote en de concentratie aan het archaeale *amoA*-gen werd waargenomen. De betrokkenheid van Crenarchaeota in nitrificatie werd verder bestudeerd met behulp van onderzoek aan een tijdserie van kustwater van de Noordzee. In de winter waren de Crenarchaeota de dominerende groep in de archaeale gemeenschap en hun celaantallen correleerde sterk met de concentratie van nitriet, het product van de nitrificatiereactie. De celaantallen van beta- en gammaproteobacteriën, de twee groepen waarin zich de bekende bacteriële nitrificeerders bevinden, waren daarentegen constant en correleerde niet met de vorming van nitriet. Deze data laten zien dat mariene Crenarchaeota actief zijn in de mariene stikstofcyclus als nitrificeerders.

In **Hoofdstuk 4** wordt de successie van verschillende soorten mariene Archaea in Noordzeekustwater beschreven met gebruik van verschillende moleculaire benaderingen. De Crenarchaeota werden geïdentificeerd met behulp van PCR met een algemene archaeale primer gevolgd door denaturatiegradiënt gelelectroforese (DGGE) in combinatie met sequencing. Drie verschillende kwantificeringsmethoden werden gebruikt, Catalyzed Reporter Deposition Fluorescentie in situ Hybridisatie (CARD-FISH), Q-PCR en GDGT analyse, om de aantallen van mariene Archaea te bepalen en om de relatieve robuustheid van de verschillende methoden te vergelijken. De variaties in aantallen bepaald met CARD-FISH en Q-PCR vertoonden sterke overeenkomsten en suggereerde dat een archaeale cel gemiddeld 0.9±(0.6) kopieën van het 16S rRNA gen bevat. GDGT concentraties en celhoeveelheden van

Crenarchaeota bepaald met CARD-FISH lieten zien dat de bron voor GDGTs in zuurstofrijke water inderdaad mariene Crenarchaeota zijn. Vergelijking van celaantallen en GDGT concentraties lieten zien dat een cel van een Crenarchaeote gemiddeld 3±0.5 pg GDGTs bevat. Een duidelijke seizoenscyclus in het voorkomen van Crenarchaetoa werd geobserveerd met zowel CARD-FISH, GDGT analyse als DGGE. In de winter wanneer nutriëntconcentraties hoog zijn, domineerden de Crenarchaeota de archaeale gemeenschap terwijl de Euryarchaeota in hogere aantallen voorkwamen in de zomer en herfst wanneer nutriëntconcentraties relatief laag zijn. De verschillenen in fysiologie van deze archaeale groepen zijn waarschijnlijk de oorzaak van deze successie gedurende de jaarcyclus. De data suggereren dat tenminste sommige mariene pelagische Crenarchaeota chemoautotrofen zijn terwijl pelagische Euryarchaeota waarschijnlijk heterotrofe organismen zijn.

Hoofdstuk 5 laat zien dat temperatuur de belangrijkste factor is die de verdeling van GDGTs bepaald. Studies met behulp van mesocosms bestaande uit tanks gevuld met zeewater met een zoutgehalte van 27‰ en 35‰ geënt met mariene Crenarchaeota en in het donker geïncubeerd bij temperaturen variërend van 5 tot 35ºC werden ondernomen om de TEX_{86} proxy te testen. Gedurende de experimenten was er een toename in concentraties van GDGTs tot 3.4 μg l^{-1}, hetgeen duidde op een substantiële groei van mariene Crenarchaeota. Met toenemende temperatuur was er een toename in de relatieve hoeveelheid van cyclopentaan-bevattende GDGTs te zien. De verschillende zoutgehaltes hadden geen effect op de verdeling van GDGT isomeren. De berekende TEX_{86} waarden waren lineair gecorreleerd met de temperatuur tijdens de mesocosmexperimenten. De helling van de correlatielijn was identiek aan die van de correlatielijn beschreven voor oppervlaktesedimenten maar de afsnijding met de Y-as was wel verschillend. Dit komt waarschijnlijk omdat de Crenarchaeotesoorten verrijkt onder labcondities anders zijn dan de meer complexe archaeale gemeenschappen in het veld. De mesocosmosexperimenten suggereren dat temperatuur inderdaad de belangrijkste factor is die de samenstelling van GDGT isomeren bepaald en bevestigt dat de TEX_{86} een proxy is voor temperatuur.

Hoofdstuk 6 behandelt de analyse van GDGTs in gesuspendeerd particulair organisch materiaal (POM) in oppervlakte en dieper water om uit te vinden in welk seizoen en op welke diepte de GDGTs gebruikt in de TEX_{86} proxy worden geproduceerd. Dit liet zien dat GDGTs in grotere concentraties voorkomen in de winter en in het vroege voorjaar in

oppervlaktewateren. De verdeling met de diepte liet voor een aantal locaties zien dat GDGTs in het algemeen in hogere hoeveelheden voorkomen op diepten beneden de 100 m. Een zwakke correlatie van de TEX_{86}-waarden van beneden de fotische zone met de in situ temperaturen werd geobserveerd. De TEX_{86} van POM in de bovenste 100 m van de waterkolom was lineair gecorreleerd met in situ temperaturen en de correlatie was bijna identiek aan de temperatuur-TEX_{86} correlatie geobserveerd voor oppervlaktesedimenten. De TEX_{86} van alle POM monsters correleerde het beste met OZT en de correlatie was ongeveer hetzelfde als die gerapporteerd voor oppervlaktesedimenten. De correlatiecoëfficiënt was echter een stuk lager wat mogelijk verklaard kan worden door de menging van GDGTs van levende Crenarchaeota met die van fossiel sedimenterende GDGTs. De data suggereren dat het GDGT signaal dat naar het sediment wordt getransporteerd voornamelijk de temperatuur van de bovenste 100 m van de oceaan reflecteert. In het bovenste gedeelte van de waterkolom is er een actieve voedselweb en dus zullen GDGTs in dat gedeelte van de oceaan veel effectiever worden getransporteerd naar het sediment door begrazing en productie van faecale deeltjes ("verpakking").

In **Hoofdstuk 7** wordt de analyse van sedimenterende deeltjes afkomstig van de noordoostelijke Stille Oceaan en de Arabische Zee om de seizoenscyclus en ruimtelijke verdeling van de GDGT-fluxen en het TEX_{86} signaal te bestuderen gerapporteerd. Aan materiaal afkomstig van sedimentvallen op verschillende diepten in de noordoostelijke Stille Oceaan werd de TEX_{86} bepaald in verschillende groottefracties. Deze analyses lieten zien dat de TEX_{86} hetzelfde was in de verschillende fracties. Dit suggereert dat begrazing en verpakking van sedimenterende GDGTs geen invloed heeft op het TEX_{86} signaal. In beide gebieden was het TEX_{86} signaal in de verschillende sedimentvallen gerelateerd aan OZT. In de meest ondiepe sedimentval op 500 m diepte in de Arabische Zee werd er een sterke correlatie van de TEX_{86} met de seizonale variatie in de OZT gevonden. In de diepere sedimentvallen was de TEX_{86} relatief meer constant gedurende de seizoenscyclus en reflecteerde de jaargemiddelde OZT. In deze vallen is het TEX_{86} signaal waarschijnlijk sterk beïnvloed door lateraal transport van sedimentdeeltjes. Dit suggereert dat de TEX_{86} in dieper wateren en in sedimenten waarschijnlijk een geïntegreerd en jaargemiddelde OZT reflecteert en geen seizoenssignaal.

Het experimentele werk en de veldobservaties in dit proefschrift hebben nieuwe inzichten verschaft in de fysiologie van mariene Crenarchaeota en de nieuwe paleothermometer TEX_{86}. Met het ^{13}C-label experiment, de verrijkingscultuur en de veldobservaties in de Noordzee is aangetoond dat tenminste sommige mariene Crenarchaeota chemolithoautotroof zijn en nitrificeerders. Aangezien mariene Crenarchaeota één van de meest voorkomende klassen van picoplankton zijn in de oceanen en voorkomen over een grote dieptebereik is het waarschijnlijk dat archaeale chemolithoautotrofie een belangrijke rol speelt in de biochemische cyclus van stikstof en koolstof in de oceaan. Bovendien zijn Crenarchaeota waarschijnlijk een belangrijke "sink" voor organische koolstof en representeren zij een belangrijk maar nog niet eerder bekende component van de mondiale koolstofcyclus.

In het geval van de TEX_{86} heeft het experimentele werk en de veldobservaties deze nieuwe proxy gevalideerd. De mesocosmosexperimenten lieten zien dat temperatuur de belangrijkste factor is die de samenstelling van de GDGTs van Crenarchaeota bepaald en dus reflecteert de TEX_{86} temperatuur. De veldstudies aan POM en sedimentvallen lieten duidelijk zien dat het GDGT signaal dat het diepere water en het sediment bereikt de temperatuur van de bovenste 100 m van de waterkolom reflecteert, waarschijnlijk door processen als begrazing en verpakking. De nieuwe paleothermometer TEX_{86} kan dus gebruikt worden om temperaturen te bepalen van het bovenste gedeelte van de oceaan in vroeger tijden. Omdat GDGTs worden gevonden in sedimenten tot 140 miljoen jaar oud heeft deze temperatuurproxy een grote potentie om gebruikt te gaan worden in reconstructies van vroegere klimaten.

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Curriculum Vitae

Cornelia Wuchter was born at the 8th of August in 1971 in Stuttgart, Germany. After finishing high school she started to study Biology in September 1993 at the Carl von Ossietzky University of Oldenburg in Germany. She finished her master degree in April 2000. From August 2000 till February 2001 she participated an expedition to Antarctica as a research assistant. From April 2001 till April 2005 she worked as a PhD student at the Royal NIOZ in the Netherlands. Currently she is living in Falmouth, MA in the USA.