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]

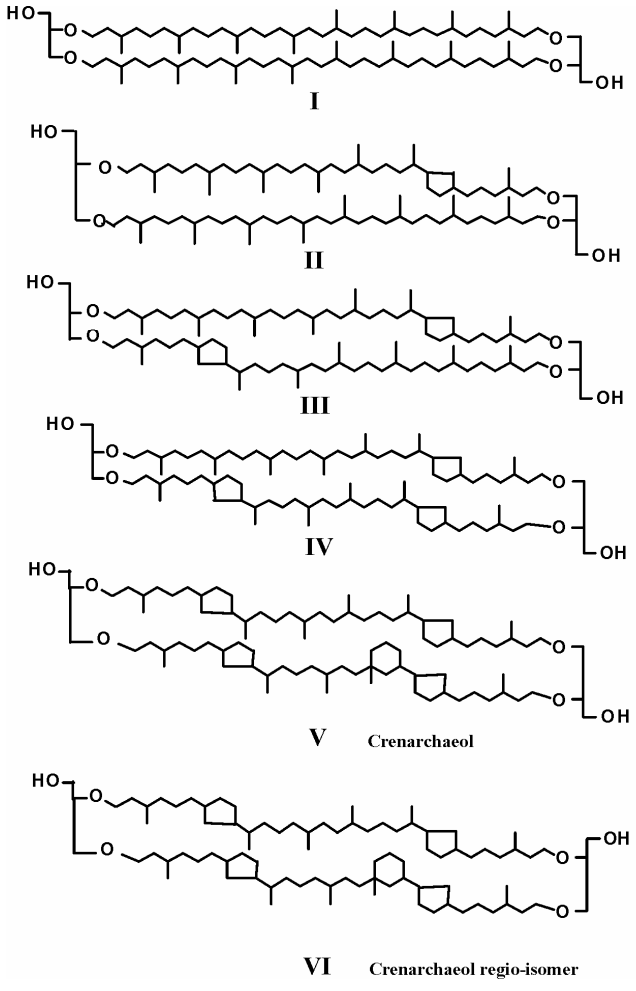

Figure 1. Structures of intact core tetraether membrane lipids of marine pelagic Crenarchaeota. The structure of the stereoisomer (VI) of crenarchaeol is likely a regio-isomer (Sinninghe Damsté et al., unpublished results).

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.

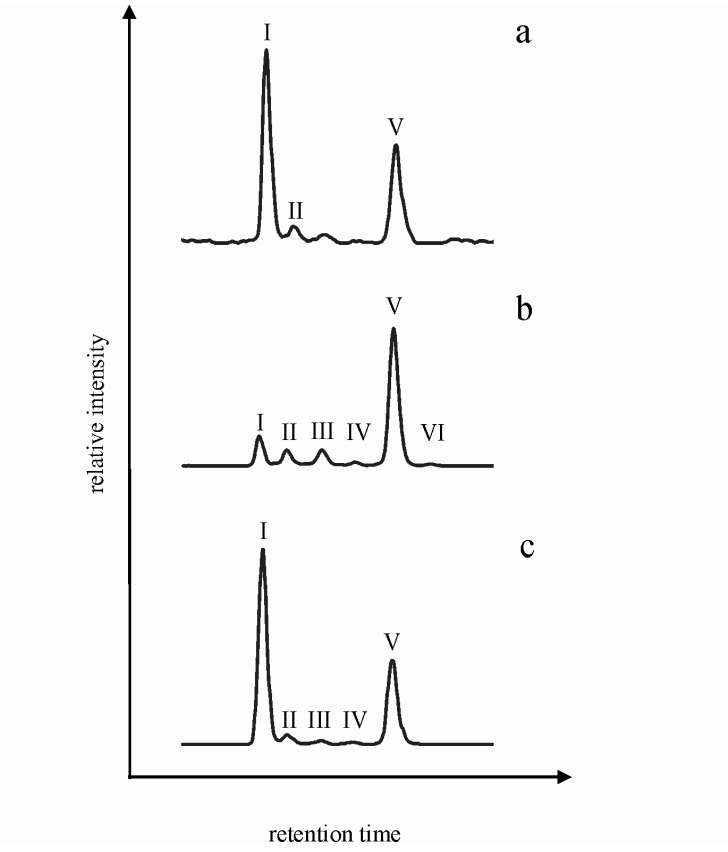

Figure 2. Partial HPLC-APCI/MS base peak chromatograms of particulate organic matter derived from the mesocosm tank experiments: (a) GDGT pattern of the initial water used for the mesocosm experiment, (b) final GDGT pattern of the water from mesocosm tank I incubated at 27ºC for three month and (c) final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

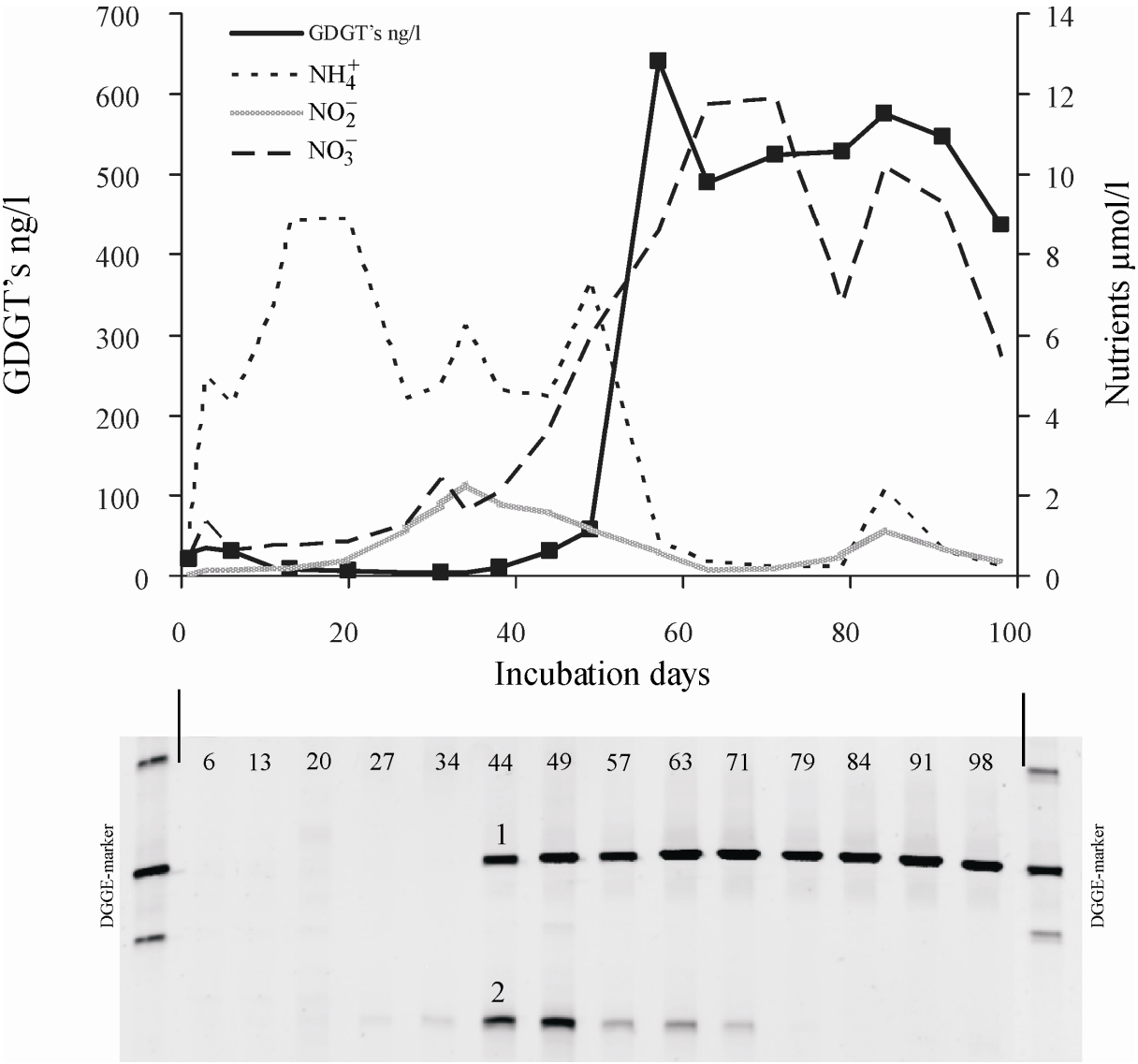

Figure 3. (top) Nutrient and GDGT concnetrations in mesocosm tank I incubated at 27°C with time. Results for mesocosm tank II incubated at 13ºC were similar (data not shown). Phosphate concentration in both mesocosm tanks remained constant and ranged between 0.1 µmol/l and 1.0 µmol/l during the incubation 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).

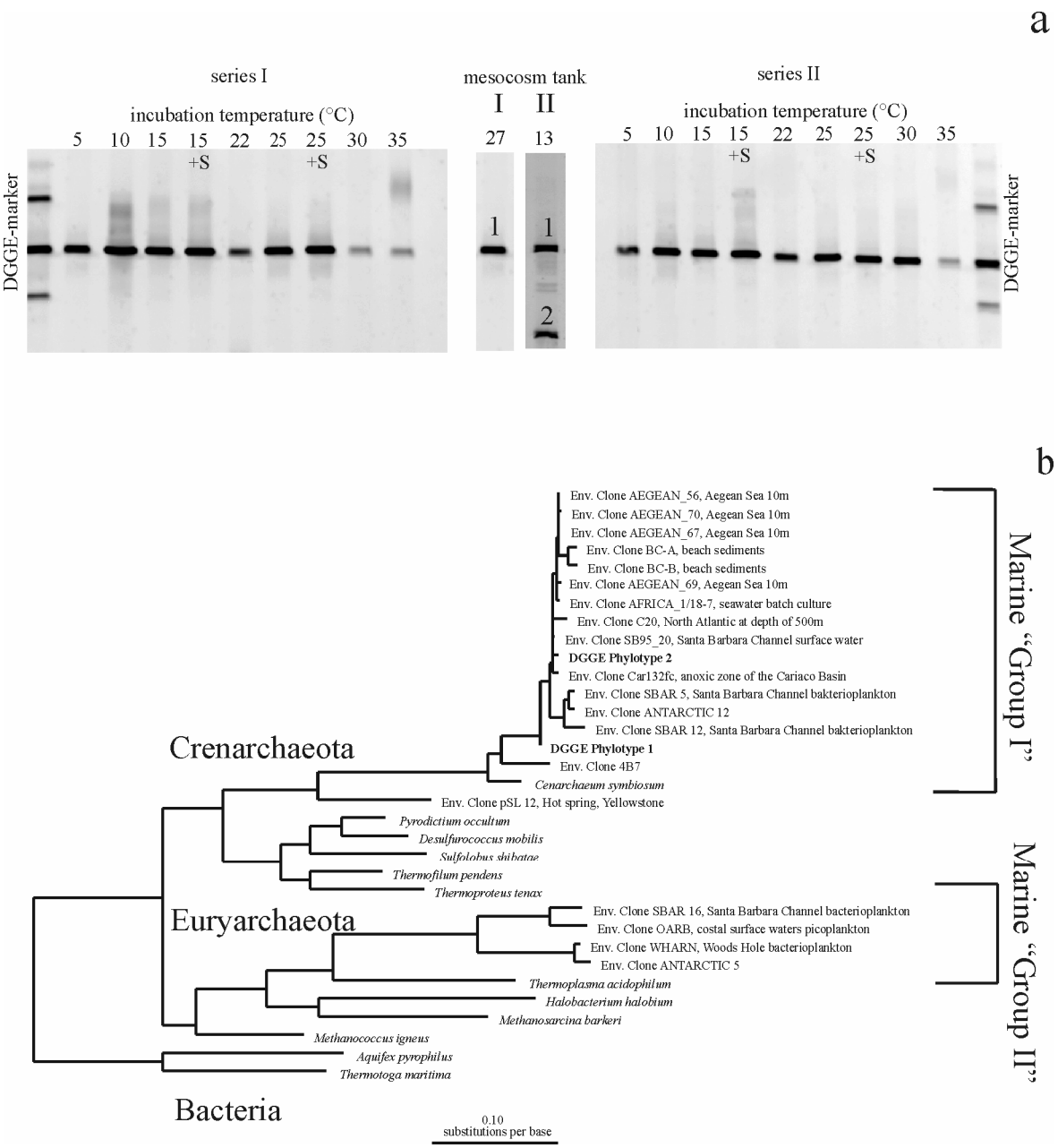

Figure 4. (a) DGGE analysis of PCR amplified 16S rRNA genes fragments of Archaea obtained from mesocosm tank I and II and incubation series I and II, all after three month incubation at the indicated temperature. Experiments performed at elevated salinity are denoted by +S. One dominant partial 16S rDNA 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.

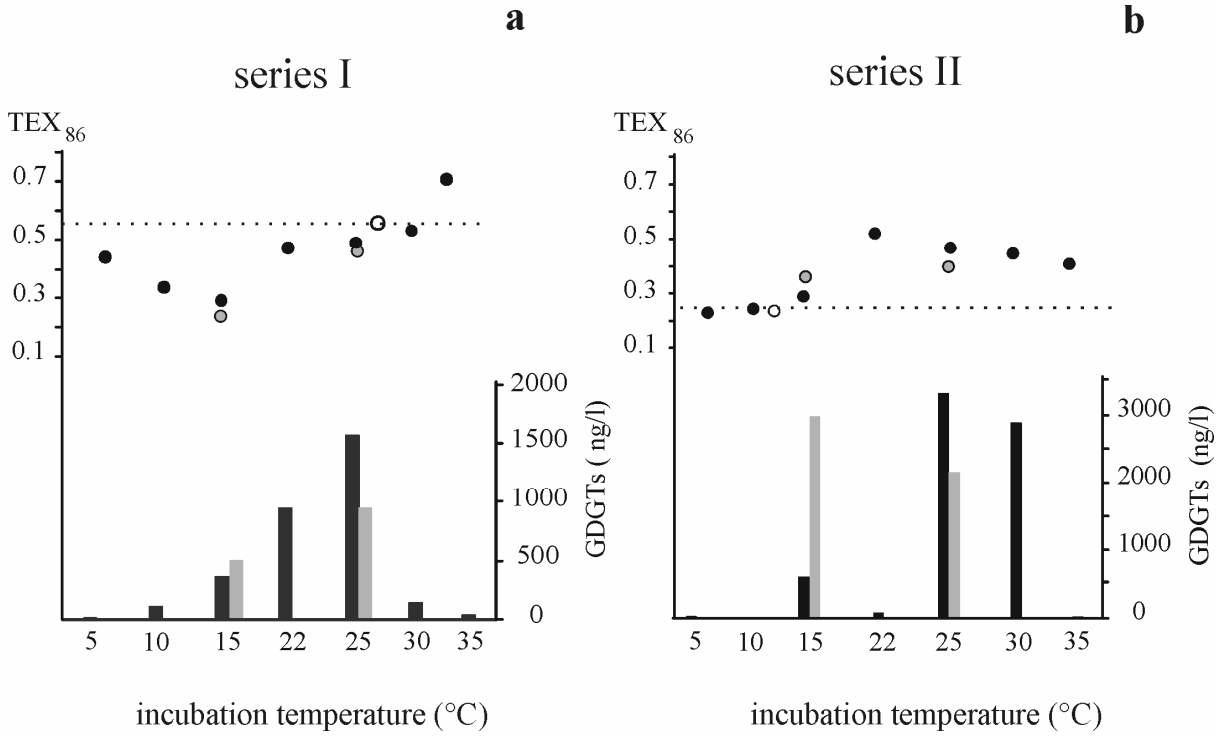

Figure 5. Concentration of newly produced GDGT's (i.e. corrected for the initial concentration) in tanks incubated at different temperatures and salinities for three months for (a) incubation series I and (b) incubation series II. In the upper parts of the graph TEX_{86} values calculated from the GDGT 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).

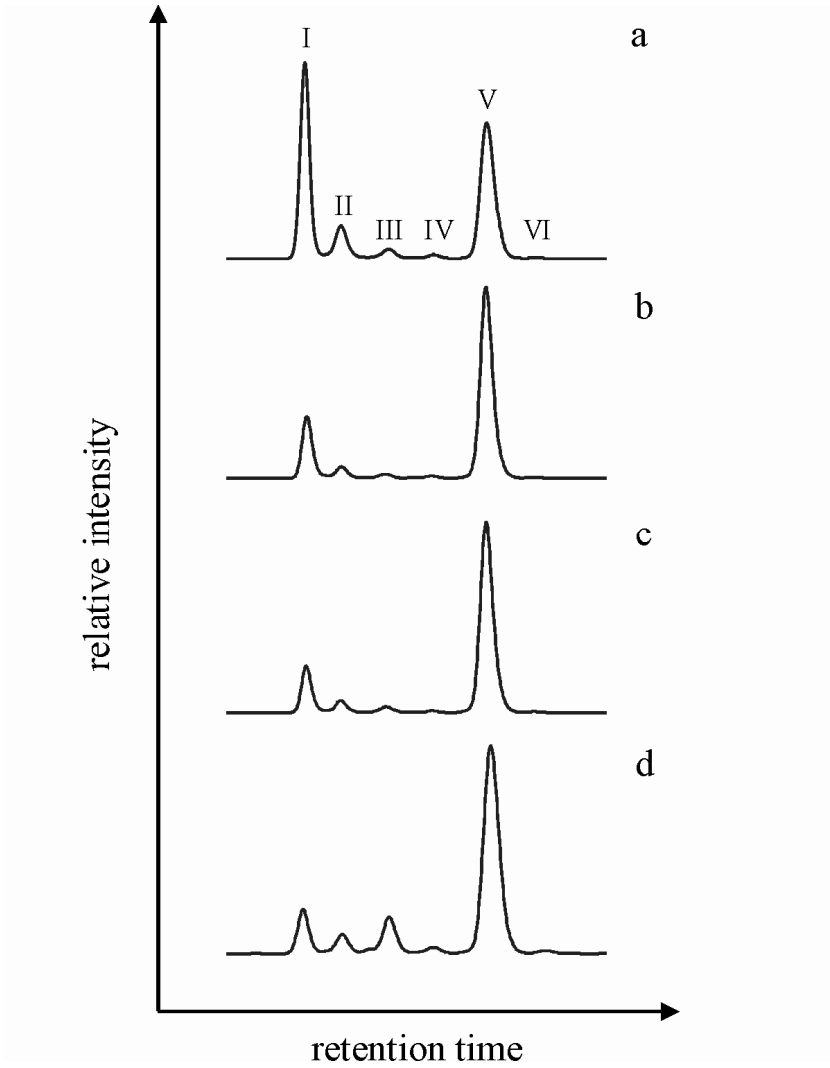

Figure 6. HPLC-APCI/MS base peak chromatograms of particulate organic matter from incubation series I at different temperatures and salinities, i.e. (a) 10ºC, 27‰; (b) 25ºC, 27‰; (c) 25ºC, 35‰; (d) 35ºC, 27‰. Numbers indicate structures drawn in Fig. 1.

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.

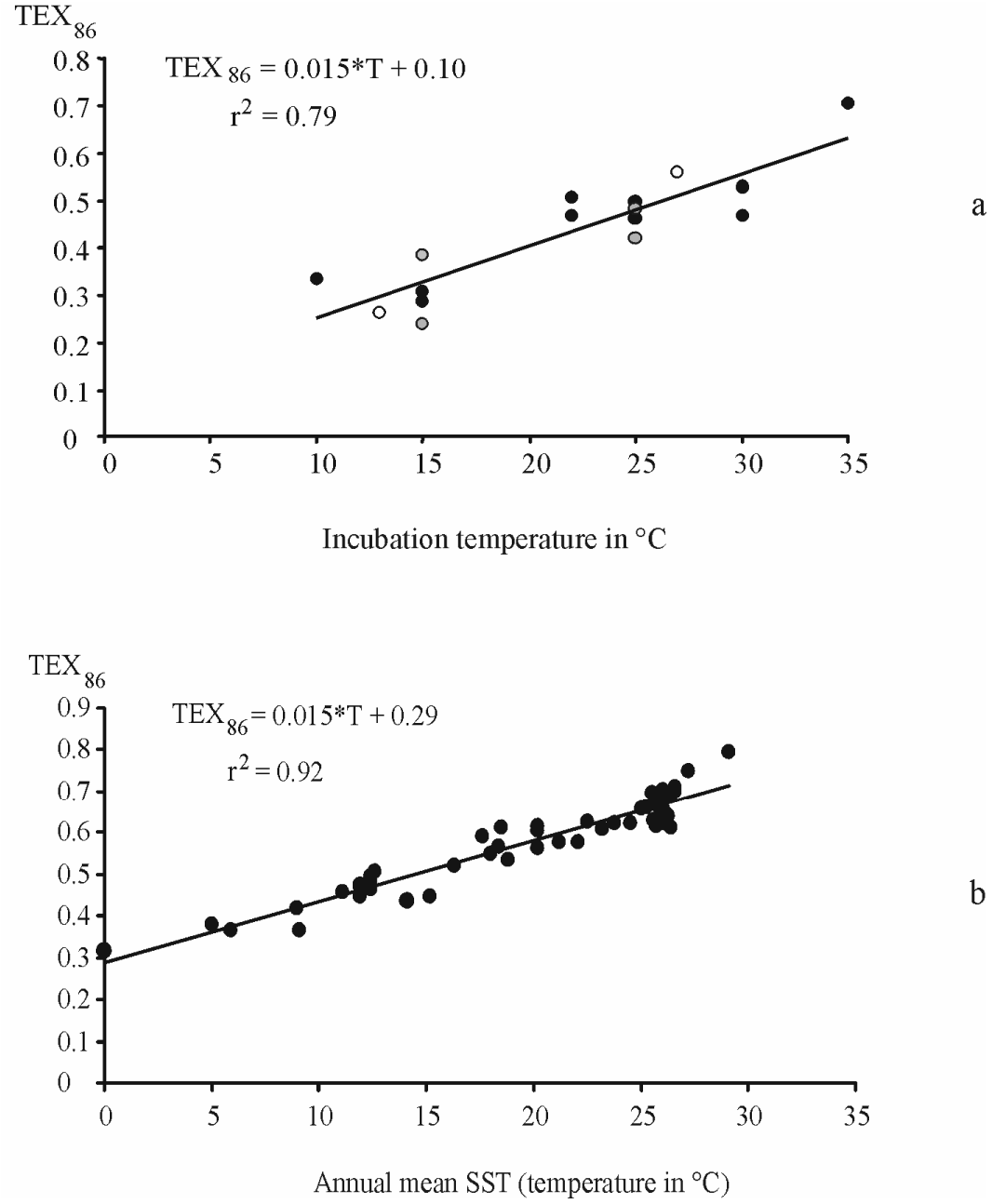

Figure 7. Correlation of the GDGT distribution with temperature. A: Correlation of the incubation series. Black dots represent TEX_{86} values from incubation at 27‰ salinity and grey dots represent TEX_{86} values from incubation at 35‰ salinity. Open dots represent TEX_{86} values from the initial 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.

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