Biogeochemical controls on glycerol dialkyl glycerol tetraether lipid distributions in sediments characterized by diffusive methane flux

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[1] The TEX_{86} (TetraEther indeX of tetraethers consisting of 86 carbon atoms) is a proxy for sea surface temperature (SST) based on the distribution of isoprenoidal glycerol dialkyl glycerol tetraether (GDGT) membrane lipids synthesized by marine pelagic Thaumarchaeota. One of the caveats of this proxy is the production of additional GDGTs by sedimentary Euryarchaeota involved in anaerobic oxidation of methane (AOM) that occurs at deep-sea methane seeps but is also widespread in many continental shelf settings. Here, GDGT distributions are investigated through the sulfate-methane transition zone (SMTZ) in Aarhus Bay, Denmark, to examine the extent the TEX_{86} proxy is compromised in such a continental shelf setting where AOM is characterized by a diffusive rather than rapid advective methane flux. Both free extractable and non-extractable lipid fractions were analyzed as it was expected that pelagic-derived and sediment-derived GDGTs could become incorporated into the molecular and sedimentary matrix to a different extent. The results show a large change of TEX_{86} values mainly due to the relative high amounts of GDGT-2 produced by AOM-related Archaea, both in the free and non-extractable lipid fractions. This additional GDGT input renders calculation of SSTs based on TEX_{86} inappropriate at the SMTZ. The AOM-related GDGT signature, however, did not persist into deeper sediments, perhaps reflecting rapid

remineralization of the GDGTs at the SMTZ. Although the process of AOM at Aarhus Bay might not be representative for all continental margin settings, it illustrates that AOM in a variety of settings, not just cold seeps, can influence sedimentary GDGT distributions.

1. Introduction

[2] Glycerol dialkyl glycerol tetraethers (GDGTs) are membrane spanning lipids synthesized by a wide range of Archaea (isoprenoid GDGTs [e.g., *Koga et al.*, 1993]) and a group of yet unknown soil bacteria (branched GDGTs [e.g., *Weijers et al.*, 2006a]). Marine pelagic Thaumarchaeota (previously known as Marine Group I Crenarchaeota [see *Brochier-Armanet et al.*, 2008]) are among the most dominant prokaryotes living in today's oceans [e.g., *Karner et al.*, 2001]. They synthesize isoprenoid GDGTs containing 0–3 cyclopentane moieties and crenarchaeol, which contains four cyclopentane moieties plus an additional cyclohexane moiety [*Schouten et al.*, 2008; *Sinninghe Damsté et al.*, 2002]. The relative amount of cyclopentane moieties in the membrane lipids of these mesophilic Thaumarchaeota correlates to growth temperature, i.e., more cyclopentane rings occur in membrane lipids of organisms growing at higher temperatures and vice versa [*Schouten et al.*, 2002; *Wuchter et al.*, 2004]. Upon grazing, the isoprenoid GDGT lipids can be transported from the marine water column to the sediments [e.g., *Huguet et al.*, 2006a]. Based on a survey of isoprenoid GDGTs in marine sediments it was found that their relative distribution, expressed in the TEX₈₆ (TetraEther indeX of tetraethers consisting of 86 carbon atoms), correlates well with temperatures of the overlying surface waters [*Kim et al.*, 2010; *Schouten et al.*, 2002] and can be used to reconstruct past sea surface temperature (SST).

[3] Since its first proposal, a number of caveats to the application of the TEX₈₆ proxy have been identified. For example, a similar suite of isoprenoidal GDGT compounds is produced by Thaumarchaeota in soils. Upon soil erosion and fluvial transport these also become part of the marine sedimentary archive mixing with GDGTs derived from pelagic Thaumarchaeota and thus influencing the TEX₈₆ [*Weijers et al.*, 2006b]. In methanogenic settings, isoprenoidal GDGTs derived from methane-producing Euryarchaeota and containing 0 to 2 cyclopentyl moieties render the TEX₈₆ ineffective as a temperature proxy. This is, for example, often the case in lake systems [*Blaga et al.*, 2009]. Similarly, methane-oxidizing Euryarchaeota also produce GDGTs with 0 to 4 cyclopentyl moieties [e.g., *Pancost et al.*, 2001; *Wakeham et al.*, 2003], such that sediments characterized by high rates of anaerobic oxidation of methane (AOM) are also unsuitable for the application of the TEX₈₆ proxy [*Schouten et al.*, 2002]. Examples of such AOM sites are methane seeps that occur at mud volcanoes along regional-scale fault planes in the Mediterranean Sea [*Limonov et al.*, 1996] and in the Black Sea [*Ivanov et al.*, 1996], where archaeal GDGT distributions in the sediments and carbonate crusts often are dominated by the cyclopentyl bearing GDGTs produced by these methane oxidizing archaea [e.g., *Pancost et al.*, 2001; *Stadnitskaia et al.*, 2005]. These are, however, highly localized features and short distances away from the seep or the crust, both laterally and vertically, isoprenoid GDGT distributions reflect those of pelagic Thaumarchaeotal inputs, i.e., dominated by GDGT-0 and crenarchaeol [e.g., *Stadnitskaia et al.*, 2005]. AOM in the (deeper) water column rather than in the sediments seems to have less effect on the isoprenoid GDGT distribution in the underlying sediments, likely because of a lack of mechanisms to transport the lipids to the sediments [*Wakeham et al.*, 2003].

[4] The highly localized methane seeps associated with mud volcanoes are well investigated and the
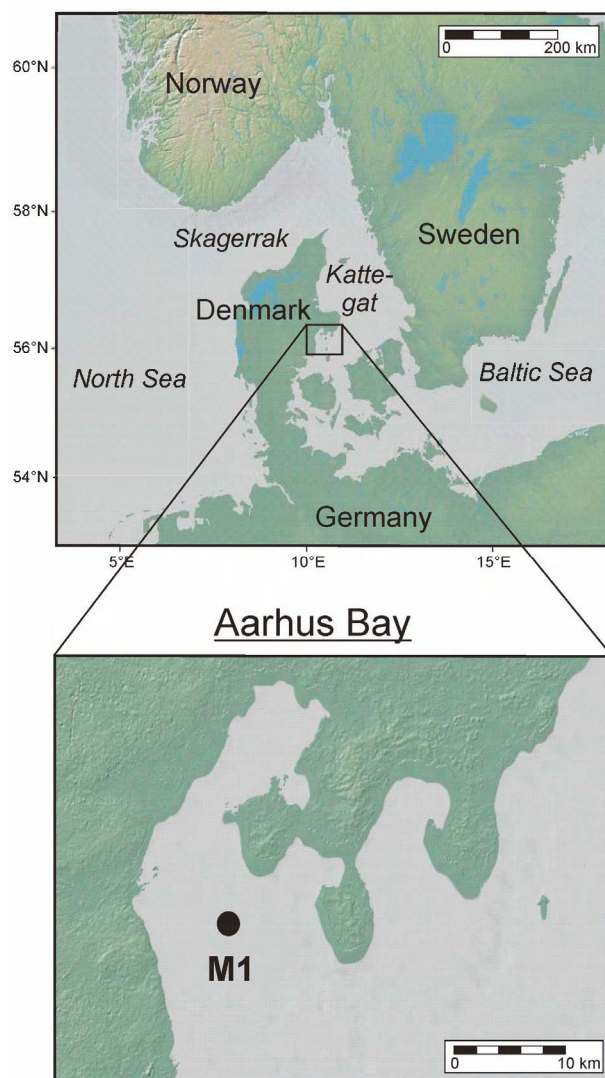
Figure 1. Location map of core 174GC from station M1 at Aarhus Bay, Denmark. Map created using GeoMapApp (www.geomapapp.org).

correlation of biomarker occurrence and their isotopic signatures with the AOM process is well established [*Hinrichs et al.*, 1999; *Pancost et al.*, 2001; *Stadnitskaia et al.*, 2005]. In addition to such methane hot spots, many shelf areas experience diffusive seepage of methane toward the sediment-water interface and most of that methane is anaerobically oxidized as well [*Hinrichs and Boetius*, 2002 and references therein; *Valentine*, 2002]. The biomarker approach has been applied to only a few of these settings, e.g., the Kattegat between Denmark and Sweden [*Bian et al.*, 2001] and the Skagerrak, Denmark [*Parkes et al.*, 2007], partly because a large input of allochthonous material, including soil derived GDGTs, relative to rather low AOM rates, might obscure the AOM biomarker signal. A recent study by *Aquilina et al.*

[2010] using detailed lipid biomarker, pore water chemistry and microbial biology approaches combined with rate measurements of AOM and sulfate reduction clearly showed the occurrence of AOM and an associated sulfate-methane transition zone (SMTZ) in the shallow subsurface of Aarhus Bay, Denmark (Figures 1 and 2). However, it remains unclear whether at this, or other diffusive sites, the production of GDGTs by AOM-associated archaea is sufficient to affect the TEX$_{86}$ signal.

[5] In this study we investigated a marine sediment core that spans the SMTZ from Aarhus Bay, Denmark. We determined concentrations as well as ratios of branched and isoprenoid GDGTs in order to determine whether or not AOM at the SMTZ has resulted in the production of additional GDGTs
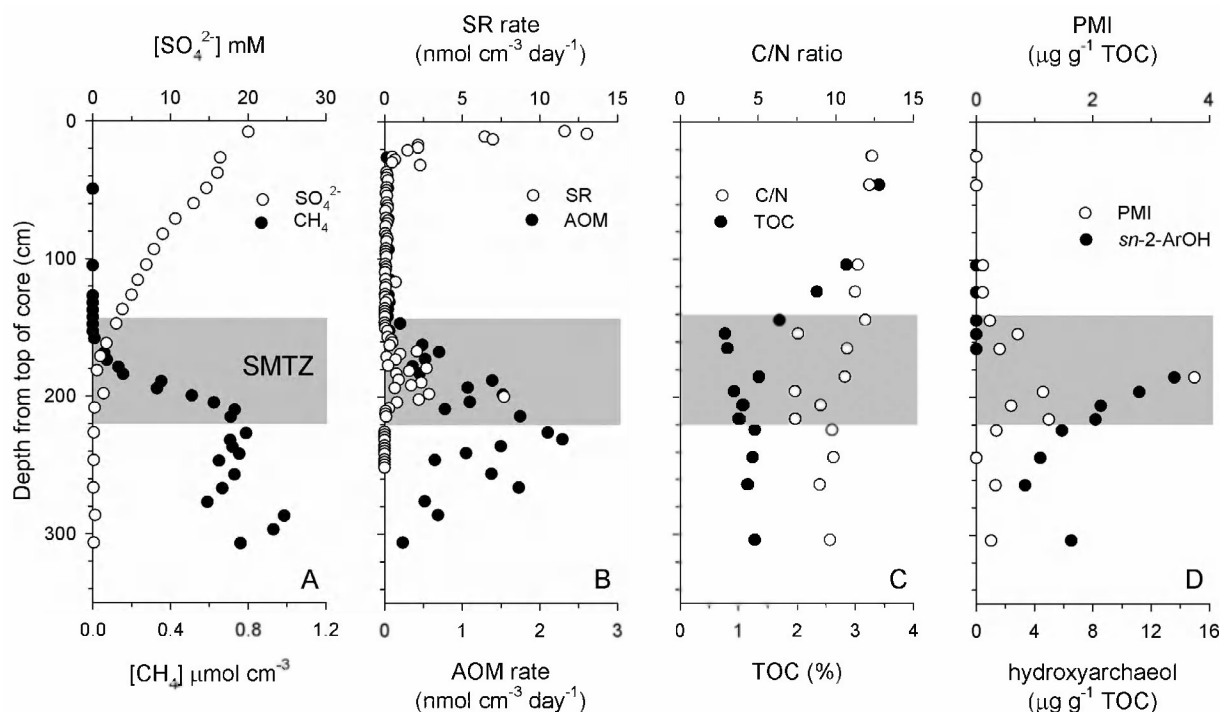
Figure 2. Biogeochemical data in sediment cores at station M1 at Aarhus Bay. (a) pore water concentrations of SO$_4^{2-}$ and CH$_4$, (b) rate measurements of sulfate reduction (SR) and the anaerobic oxidation of methane (AOM), (c) C/N ratio (molar) and TOC, and (d) 2, 6, 10, 15, 19-pentamethylicosane (PMI) and hydroxyarchaeol concentrations. Pore water concentrations and reaction rate measurements are obtained from core 173GC, taken next to core 174GC. Data in Figures 2a, 2b, and 2d were obtained from *Aquilina et al.* [2010].

above the background pelagic input and to ascertain its impact on the TEX$_{86}$ proxy. We also explored if any observed effects are prevalent in the free extractable lipid fraction, the non-extractable lipid fraction or both, as previous studies suggested that sediment-derived GDGTs are less likely to become incorporated in the macromolecular matrix than pelagic-derived GDGTs [*Pancost et al.*, 2008] and that, at least in soils, isoprenoidal GDGTs tend to become part of the non-extractable fraction to a larger extent than branched GDGTs [*Huguet et al.*, 2010a].

2. Material and Methods

2.1. Site and Core Description

[6] A ca. 3 m long core, core 174GC, was retrieved with a gravity corer aboard the *r/v* St.F. Henry as part of the METROL project (METhane flux contROL in ocean margin sediments) from station M1, Aarhus Bay (56°07.071′N, 10°20.819′E; 15 m water depth; Figure 1) in December 2004. Station M1 is positioned in the central part of Aarhus Bay, a large flat area where the sediments are dominated by clayish mud of Holocene age [*Jensen and Laier*, 2011] and

have rather high total organic carbon (TOC) content (Figure 2, ca. 1–3%) [*Aquilina et al.*, 2010]. A previous seismic survey had revealed the presence of free methane gas at 3.5–4 m below seafloor, consistently over an area of hundreds of square meters [*Jensen and Laier*, 2011]. The cores were sliced in 10 cm increments, sub-sampled, and samples were stored at −20°C until analysis. Pore water CH$_4$ and SO$_4^{2-}$ measurements showed the presence of the SMTZ, characterized by downward decreasing SO$_4^{2-}$ concentrations and a concomitant increase of CH$_4$ concentrations [*Aquilina et al.*, 2010; *Leloup et al.*, 2009]. Based on these data, AOM rate measurements and lipid analysis, the SMTZ is defined as the interval between 140 and 220 cm depth in the core [*Aquilina et al.*, 2010] (Figure 2).

2.2. Elemental Analysis

[7] Elemental carbon and nitrogen were measured using a Carlo Erba EA1108 Elemental Analyzer. The samples were weighed into tin capsules and via the auto-sampler introduced into the combustion furnace (1020°C), which was flushed with oxygen, such that the combustion temperature

reached ca. 1800°C. The combustion products passed over chromium oxide and silvered cobalt oxide catalysts to remove any sulfur and over a Cu reduction tube to reduce NO_x to N_2. The gases (CO_2, H_2O and N_2) were separated over a GC column (Porapak Q) and detected using a thermal conductivity detector and subsequently quantified.

[8] Inorganic carbon (IC) determinations were carried out on a modified Coulomat 702 analyzer. A known mass of sample was reacted with ortho-phosphoric acid to liberate CO_2 from the inorganic carbon. The liberated CO_2 was then flushed with nitrogen into the coulomatic cell, which was set to a known pH (pH 9.2), resulting in a decrease in pH. The magnitude of the current needed to restore the original cell pH was then determined and used to calculate the amount of CO_2 released from the sample. TOC concentrations were determined by subtracting the IC content from the total carbon content. Reported values of elemental analyses represent means of duplicate measurements.

2.3. Sample Preparation

[9] After freeze-drying, about 20–25 g of each of the 15 sediments were solvent extracted with a Soxhlet apparatus using a mixture of dichloromethane (DCM):methanol (MeOH) 2:1 (v/v) for 24 h. The obtained total lipid extracts were rotary evaporated to near dryness and elemental sulfur was removed using activated Cu. An aliquot of the TLE was separated into three fractions on a pre-washed solid phase extraction (SPE) column (glass column; 500 mg aminopropyl-functionalized silica; 50 μm particle size; 60 Å porosity; ISOLUTE UK). The fractions were obtained by eluting with DCM:iso-propanol 2:1 (v/v) (neutrals), 2% acetic acid in diethyl ether (free fatty acids) and MeOH (polar lipids), respectively. The neutral lipids were further separated into an apolar and alcohol fraction over activated Al_2O_3 using n-hexane:DCM 9:1 (v/v) and DCM:MeOH 1:2 (v/v) as eluents.

[10] Five samples from above, within and below the SMTZ were selected for further analysis of the non-extractable lipid fraction. To this end the dried residues were extracted a second time to make sure all extractable lipids were removed, although recent work suggests that extraction of intact polar GDGT lipids might not be exhaustive [*Huguet et al.*, 2010b]. To obtain ester-bound organic compounds, the residue was base hydrolyzed using 0.3M KOH in MeOH (5% H_2O) at 70°C for 2h; the product was subsequently extracted using an ultrasonic bath with MeOH, MeOH:DCM 1:1 (v/v)

and DCM (3 × 5 min each) and, after neutralizing the pH using HCl, the organic fraction was extracted by washing with di-ethyl ether (3×) and dried over Na_2SO_4. The base hydrolysis procedure was repeated once more to ensure all mildly bound organic matter was removed. Finally, the remaining residue was acid hydrolyzed to obtain more strongly bound ester-bound organic components: the sediment was heated at 70°C for 3h in a 5M HCl:MeOH 1:1 (v/v) solution and subsequently neutralized using KOH in MeOH. After centrifugation the supernatant was collected and the sediment extracted further with MeOH, MeOH:DCM 1:1 (v/v) and DCM (3 × 5 min each) in an ultrasonic bath. The combined extracts were dried over Na_2SO_4.

[11] For GDGT analysis, known amounts of a C_{46} GDGT standard [*Huguet et al.*, 2006b] were added to the alcohol fraction that was separated from the neutral fraction, the base hydrolysis extracts and the acid hydrolysis extracts. These were subsequently dried, re-dissolved in n-hexane:iso-propanol 99:1 (v/v) to a concentration of ca. 2 mg ml^{-1} and filtered over a 0.45 μm mesh PTFE filter (Altech) prior to analysis.

2.4. GDGT Analysis

[12] The samples were analyzed for their GDGT content using a high performance liquid chromatograph interfaced to a mass spectrometer (HPLC/MS; Agilent 1100 series), equipped with ChemStation software (Agilent) and operated according to the procedures described by *Hopmans et al.* [2000] and *Schouten et al.* [2007]. Sample injection was 10 μl and separation of compounds was achieved on an analytical Alltech Prevail Cyano column (150 mm × 2.1 mm; 3 μm) kept at 30°C by eluting with n-hexane:iso-propanol 99:1 (v/v), isocratically for the first 5 min, thereafter with a linear gradient to 1.8% iso-propanol in 45 min with a flow rate of 0.2 ml min^{-1}. The column was subsequently rinsed with 10% iso-propanol in backflush mode for 10 min and re-equilibrated with n-hexane:iso-propanol 99:1 (v/v) for 10 min. Detection by the mass spectrometer was performed in selected ion monitoring (SIM) mode, scanning for the masses of interest using settings as described by *Schouten et al.* [2007]. GDGTs were quantified by comparing the area underneath the $[M + H]^+$ (protonated GDGTs) peaks in the selected ion chromatograms with that of the C_{46} standard and correcting for differences in ionization efficiency. Based on duplicate HPLC/
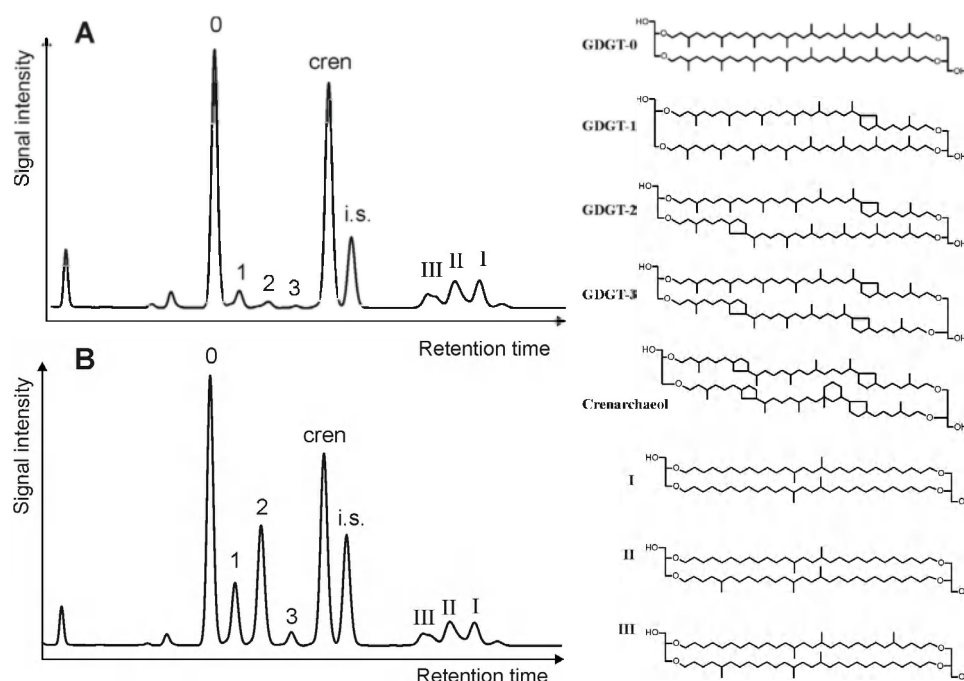
Figure 3. LC/MS base peak chromatograms of Aarhus Bay sediment from (a) 224 cm depth, i.e., outside the SMTZ, and (b) from 185 cm depth, i.e., within the SMTZ. GDGT structures are shown on the right hand side with isoprenoid GDGTs in Arabic numerals and branched GDGTs in Roman numerals; cren = crenarchaeol; i.s. = internal standard (C$_{46}$ GDGT).

MS runs, the analytical uncertainty in the quantifications is ±2%.

[13] The BIT index was used as defined by *Hopmans et al.* [2004]:

$$BIT = \frac{[GDGT\,I] + [GDGT\,II] + [GDGT\,III]}{[GDGT\,I] + [GDGT\,II] + [GDGT\,III] + [Cren]} \quad (1)$$

TEX$_{86}$ was used as defined by *Schouten et al.* [2002]:

$$TEX_{86} = \frac{[GDGT\,2] + [GDGT\,3] + [Cren']}{[GDGT\,1] + [GDGT\,2] + [GDGT\,3] + [Cren']} \quad (2)$$

where the numerals refer to GDGTs shown in Figure 3, [Cren] is the crenarchaeol concentration and [Cren'] the concentration of the region-isomer of crenarchaeol (the latter not indicated in Figure 3). The TEX$_{86}$ value was translated into SST using the calibration formula provided by *Kim et al.* [2008]:

$$SST = -10.78 + 46.9 \times TEX_{86} \quad (3)$$

The analytical reproducibility of the TEX$_{86}$ analyses is generally better than 0.01, corresponding to <0.5°C [*Schouten et al.*, 2007], and the standard

error of derived temperature estimates is 1.7°C for the *Kim et al.* [2008] calibration. A recent modification of the TEX$_{86}$ proxy has been proposed by *Kim et al.* [2010] that separates the calibration into a low (<15°C; TEX$_{86}^{L}$) and high (>15°C, TEX$_{86}^{H}$) temperature calibration which improves the correlation for sub-polar regions and the extrapolation to greenhouse worlds. Since SSTs reconstructed in this study are around this boundary between TEX$_{86}^{L}$ and TEX$_{86}^{H}$, and in order to enable direct comparison with previously published TEX$_{86}$ values for AOM sites, we used the 2008 calibration.

3. Results

[14] TOC values are ca. 3.3% for the top half meter of the core and decrease to 0.8% on top of the SMTZ (ca. 1.5 m; Figure 2c). Further down the core the values are rather stable, varying between 0.9 and 1.3%.

[15] In the solvent extractable fraction, the distribution of isoprenoid GDGTs is dominated by both GDGT-0 and crenarchaeol with concentrations between 12 and 37 μg g^{-1} TOC (Figures 3 and 4). Concentrations of GDGT-1 and -2 are lower and vary between 0.5 and 11 μg g^{-1} TOC. Concentrations of the regio-isomer of crenarchaeol (not
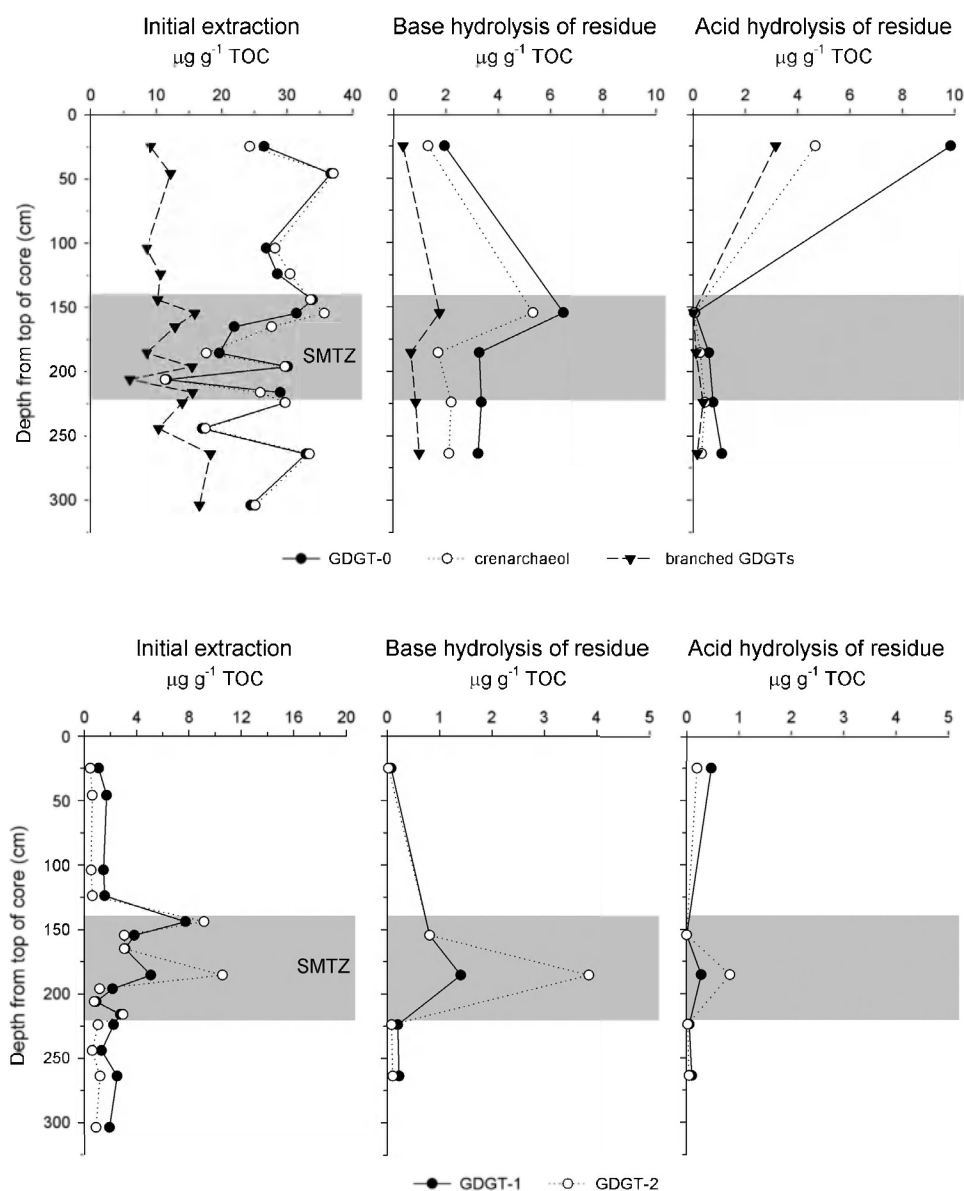
Figure 4. GDGT concentrations in core 174GC from Aarhus Bay for the free extractable lipid pool and the fractions obtained upon base and acid hydrolysis; GDGT-0, crenarchaeol and branched GDGTs in the top plots and GDGT-1 and GDGT-2 in the bottom plots. Note: GDGT-3 shows a similar pattern as GDGT-2 but concentrations are generally very low (<1 μg g^{-1} TOC) and therefore not plotted here.

shown) are even lower with values on average of 0.2 μg g^{-1} TOC, but with a depth distribution pattern similar to that of crenarchaeol. Branched GDGTs show intermediate concentrations ranging between 6 and 18 μg g^{-1} TOC.

[16] Concentrations of GDGTs in the base hydrolyzed fractions are substantially lower than those in the solvent-extractable fractions by about a factor of 10, and abundances in the acid hydrolyzed fractions are even lower (except for the shallowest sample; Figures 4 and 5). Due to the low con-

centrations of GDGTs in the acid hydrolyzed fraction, some of them were present at or below detection limit, hampering calculation of TEX$_{86}$ for these samples. Although GDGT distributions are generally similar between the different extracts, the GDGT-0/crenarchaeol ratio is somewhat elevated in the extracts obtained upon base and acid hydrolysis relative to the free extractable fraction (Figure 6). The clearly elevated GDGT-0/crenarchaeol ratio at 265 cm depth might be a result of the very low yields upon acid hydrolysis.
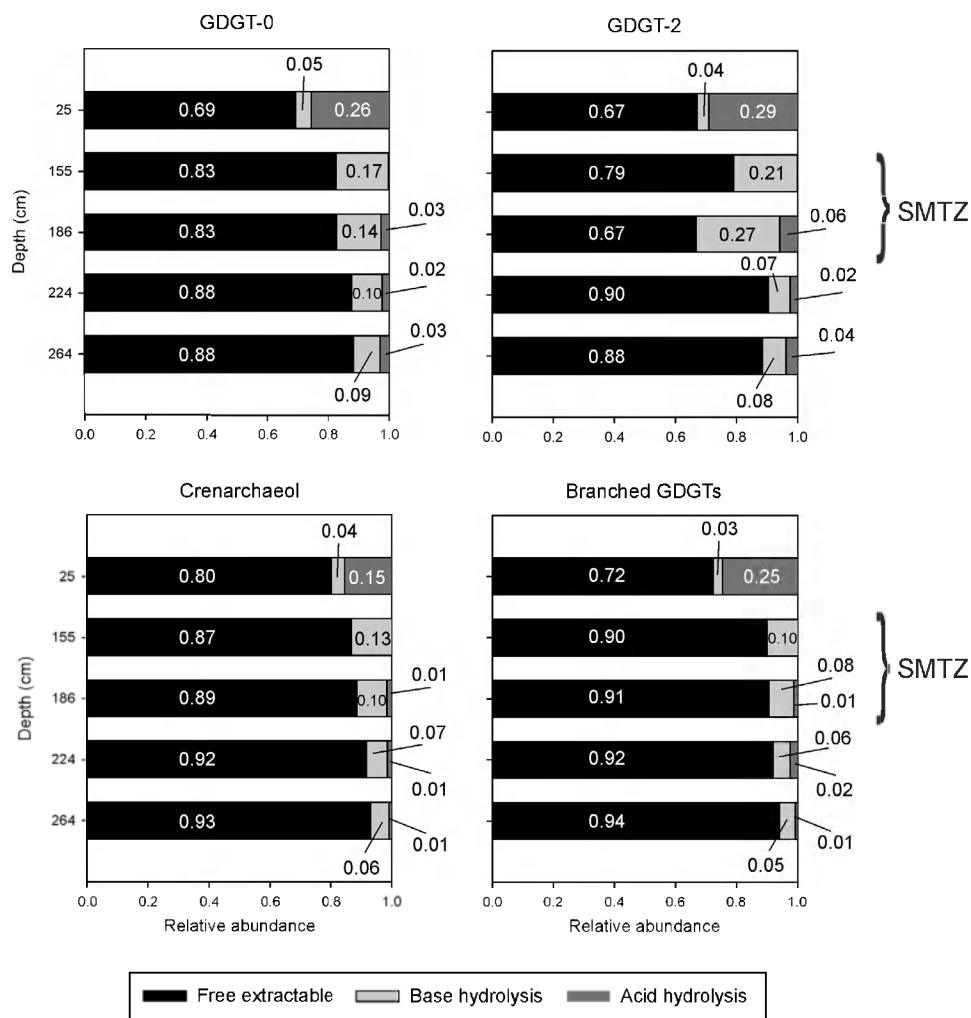
Figure 5. Relative abundance of GDGT-0, GDGT-2, crenarchaeol and branched GDGT lipids released upon regular solvent extraction, base hydrolysis and acid hydrolysis at different depths.

[17] At the SMTZ, GDGT-0, crenarchaeol (and its regio-isomer) and the branched GDGT concentrations are similar to those in shallower and deeper horizons, whereas GDGT-1 and especially GDGT-2 concentrations are generally higher within the SMTZ than above and below (Figures 3 and 4). The relatively strong increase in GDGT-2 concentrations in the SMTZ is also reflected in the GDGT-2/crenarchaeol ratio, which is generally higher in the SMTZ in comparison to both deeper and shallower sediments where it is ca. 0.03 (Figure 6). In the extracts obtained by base and acid hydrolysis of the residue, the GDGT-2/crenarchaeol ratio is even higher at the SMTZ, especially for the sample at 185 cm, compared to the free extractable fraction, with increases by about a factor of 4 and 5, respectively (Figure 6).

[18] The TEX$_{86}$ values for sediments outside the SMTZ all fall between 0.39 and 0.43. The sediments within the SMTZ give substantially higher TEX$_{86}$ values ranging from 0.45 to 0.69 (Figure 7). For the extracts retrieved upon base hydrolysis, the TEX$_{86}$ values of the uppermost sediment and of the sediments below the SMTZ are only slightly lower than those of the free-extractable fraction, i.e., 0.39 to 0.41. The sediments within the SMTZ, in contrast, exhibit higher TEX$_{86}$ values of 0.54 and 0.75, respectively (Figure 7). For the extract obtained after acid hydrolysis, a TEX$_{86}$ value could only be calculated for the uppermost sediment and it is 0.40, similar to the other two extracts from this sediment horizon.

[19] The BIT index, which is a ratio of soil derived branched GDGTs to crenarchaeol, predominantly derived from pelagic Thaumarchaeota [*Hopmans*
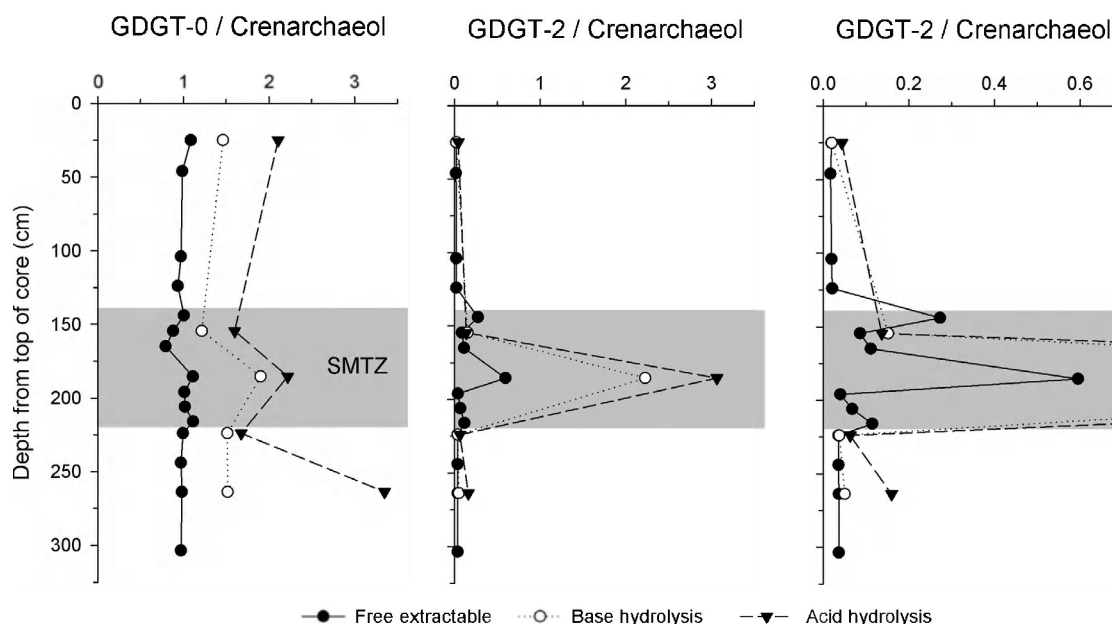
Figure 6. GDGT-0/crenarchaeol and GDGT-2/crenarchaeol ratios for the free extractable lipid pool and the fractions obtained upon base and acid hydrolysis in core 174GC at Aarhus Bay. Note that the panel on the right-hand side is the same as the middle panel, but with a blown up *x* axis to better visualize the pattern of the GDGT-2/crenarchaeol record for the free extractable lipid pool through the SMTZ.

et al., 2004], shows rather stable values above the SMTZ of ca. 0.25, but from the top of the SMTZ to the lowermost horizon a gradual increase to 0.40 occurs with no noticeable deviation in the SMTZ

(Figure 7). This pattern is similar for the extract obtained upon base hydrolysis, although the values are slightly lower, i.e., ranging from 0.23 to 0.32. After acid hydrolysis the uppermost horizon gives a
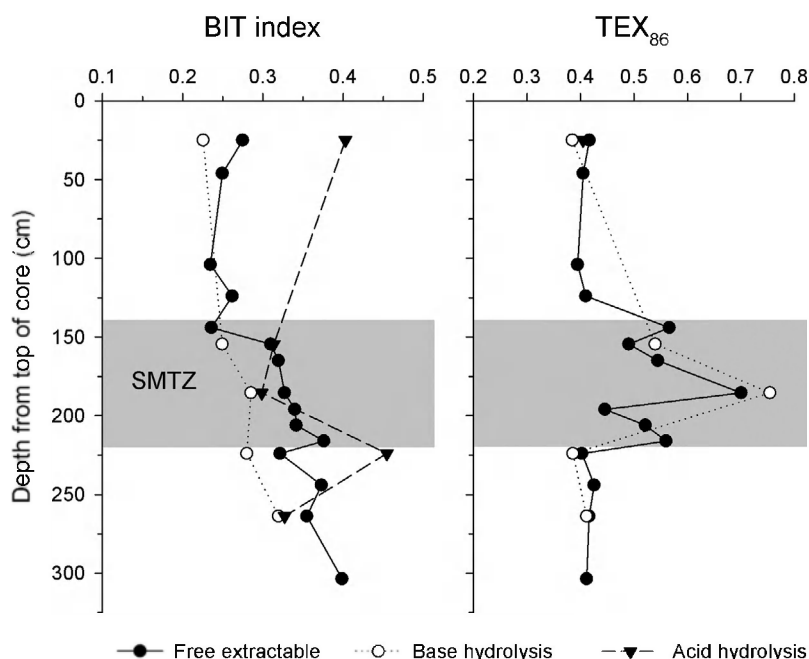

Figure 7. BIT indices and TEX$_{86}$ values for the free extractable lipid pool and the fractions obtained upon base and acid hydrolysis in core 174GC at Aarhus Bay.

BIT index of 0.4, which is high relative to the other treatments. Further down core, BIT values in the acid hydrolyzed fractions are more variable relative to those obtained for the solvent-extractable and base hydrolyzed fractions, probably due to analytical limitations associated with the low yields.

4. Discussion

4.1. GDGTs in the SMTZ of Aarhus Bay

[20] In an earlier study, *Aquilina et al.* [2010] reported a sharp increase of hydroxyarchaeol and PMI (2, 6, 10, 15, 19-pentamethylicosane) concentrations at the SMTZ at Aarhus Bay. Both biomarkers are present in methanogenic archaea albeit not exclusively. However, in settings like this they are often substantially depleted in $\delta^{13}C$, indicating that they are derived from anaerobic methanotrophs [e.g., *Pancost et al.*, 2001, and references therein]. Thus, in conjunction with AOM rate measurements and the ^{13}C-depleted isotopic composition of these lipids, an AOM source for these lipids could be assigned [*Aquilina et al.*, 2010]. The distribution of isoprenoid GDGTs at the SMTZ at Aarhus Bay is clearly different from that in sediments outside the SMTZ (Figures 3 and 6). This change in distribution is caused by an increase in the concentrations of GDGTs-1 to −3 whereas concentrations of GDGT-0, crenarchaeol and also branched GDGTs remain rather constant relative to the adjacent sediments (Figure 4). This strongly suggests that against a background signal of isoprenoid GDGTs derived from pelagic Thaumarchaeota (or derived from soil organic matter in case of the branched GDGTs), there is additional input of isoprenoid GDGTs within the SMTZ, most likely from the archaea involved in AOM.

[21] Crenarchaeol is considered a biomarker for Thaumarchaeota involved in ammonia oxidation [e.g., *Pitcher et al.*, 2011] and not known to be synthesized by archaea involved in AOM. Crenarchaeol should, therefore, behave conservatively through the SMTZ. This seems to be supported by the BIT index, which does not show a decline at the SMTZ (Figure 7). This would be the case if additional crenarchaeol is produced. Comparison of the ratios of isoprenoid GDGT-0 to −3 to crenarchaeol for the SMTZ sediments with that in adjacent sediments shows the relative increase in abundance within the SMTZ of that specific GDGT (Table 1). For this site specifically an estimate of pelagic derived and AOM derived GDGT concentrations

could be made using these ratios and the measured GDGT concentrations. This is a rough estimate based on the assumption that crenarchaeol-normalized inputs of pelagic GDGTs were stable across the SMTZ. Indeed, based on this approach GDGT-0 appears not to be produced by AOM related archaea which seems unrealistic, confirming the limitations of this calculation. Nevertheless, it does indicate that the AOM source dominantly produces GDGT-2, then followed by GDGTs-1 and some GDGT-3 (Table 1), similar to what has been observed in some cold seep settings [*Pancost et al.*, 2001].

[22] AOM is typically mediated by a syntrophic consortium of Euryarchaeota and sulfate-reducing bacteria, with the Euryarchaeota generally being methanogens operating in reverse and belonging to the so-called clusters of ANaerobic MEthanotrophs ANME-1, ANME-2 and ANME-3 [e.g., *Boetius et al.*, 2000; *Hoehler et al.*, 1994; *Niemann et al.*, 2006]. *Pancost et al.* [2001] showed that considerable variability exists in the distribution and carbon isotopic composition of lipids at different AOM sites and even within sites, pointing to different archaeal species or communities involved in AOM. This work was elaborated on by *Blumenberg et al.* [2004], who showed, using additional molecular biological techniques, that the presence of isoprenoid GDGT lipids in AOM consortia is mainly, if not only, confined to AOM consortia that comprise ANME-1 archaea and not ANME-2 archaea (although the AMNE-2c sub cluster might be an exception [*Niemann and Elvert*, 2008]). This was subsequently confirmed in other studies, including those by *Stadnitskaia et al.* [2005], *Schubert et al.* [2006], and *Rossel et al.* [2008], who all reported the co-occurrence of ANME-1 archaea and elevated abundances of GDGTs 1 to 3 relative to total isoprenoid GDGTs in the absence of ANME-2 archaea. At site M1 in Aarhus Bay, phylogenetic 16S rRNA analysis indeed showed that archaeal strains belonging to ANME-1a are present, while members of ANME-2 were not detected [*Aquilina et al.*, 2010], strongly suggesting that the ANME-1 archaea here at Aarhus Bay are the source of the additional input of isoprenoid GDGTs.

4.2. TEX$_{86}$ Across the SMTZ at Aarhus Bay

[23] The TEX$_{86}$ values for the sediments unaffected by AOM yield SST estimates of ca. 12.3°C (using equation (3)), slightly higher than the present-day

Table 1. Overview of Isoprenoidal GDGT Abundances Relative to Crenarchaeol, Their Relative Increase in the SMTZ and an Estimate of the Relative Distribution of GDGTs Produced in the SMTZ by Euryarchaeota Involved in AOM

	GDGT-0	GDGT-1	GDGT-2	GDGT-3	Cren
Ratio relative to crenarchaeol outside SMTZ (average)	0.99	0.06	0.03	0.01	n.a.[a]
Ratio relative to crenarchaeol inside SMTZ	0.99	0.14	0.18	0.02	n.a.
Relative increase in SMTZ by factor	1.0	2.3	6.7	2.4	n.a.
Hypothetical pelagic contribution SMTZ[b] ($\mu g\ g^{-1}$ TOC)	25.6	1.6	0.7	0.2	26.0
Hypothetical AOM contribution SMTZ[c] ($\mu g\ g^{-1}$ TOC)	0.0	2.0	3.7	0.3	0.0
Hypothetical AOM contribution normalized to 100%	0	34	61	5	0.0

[a]Not applicable.

[b]Obtained by multiplying the ratio of the individual GDGTs to crenarchaeol outside the SMTZ with the crenarchaeol concentration inside the SMTZ.

[c]Obtained by subtracting the hypothetical pelagic contribution from the measured concentration.

annual mean SST of 9.5°C (data from World Ocean Atlas database). For the extracts obtained after base hydrolysis of the residues, the SST estimates above and below the SMTZ are only slightly lower than those of the free extractable fraction. For the acid hydrolyzed fraction, only a value for the uppermost sample could be obtained; it is 12°C and similar to the other fractions obtained from the uppermost horizon. Due to the substantial input of AOM-related GDGTs with a different distribution dominated by GDGT-2 (Table 1), it is inappropriate to derive SST estimates from TEX$_{86}$ values within the SMTZ interval. At this site in Aarhus Bay, the excess GDGT-2 would apparently bias TEX$_{86}$ based SST estimates for all fractions toward much higher temperatures.

[24] Intriguingly, the elevated concentrations of AOM-related GDGTs and consequently the large change in the TEX$_{86}$ signal disappear below the SMTZ, both in the free and non-extractable lipid fractions, and does not seem to be preserved in the sedimentary record (Figure 7). Such preservation might be expected if it is assumed that AOM is a continuous process and that following the rate of sedimentation, the SMTZ has moved upward through the sediment column. We cannot exclude, however, that deeper sediments experienced relatively less or no AOM in the past. Alternatively, this absence of an AOM signal in GDGT lipids below the SMTZ could indicate that lipid biomass produced in situ within the sediments is relatively rapidly remineralized, perhaps by other benthic microorganisms, or recycled by the AOM community. *Liu et al.* [2011] analyzed both intact polar (IP) GDGT lipids (presumably derived from extant biomass) and core (C) GDGT lipids in shallow sediments of an active methane seep area in the Black Sea. They concluded that IP GDGTs produced in situ by an AOM community were, upon cleavage of the polar head groups, transferred to the C GDGT lipid pool, suggesting that IP GDGT

production will have an impact on the sedimentary record of fossil C GDGTs [*Liu et al.*, 2011]. Their record, however, did not extend further than 30 cm depth, which in their setting is likely situated within the SMTZ [*Liu et al.*, 2011, Figure 1], and it is possible that the AOM signal in the GDGT lipid distribution (both IP and C GDGTs) of Black Sea sediments also disappears below the SMTZ. Indeed, *Stadnitskaia et al.* [2005] observed that elevated concentrations of GDGT-2 not only disappeared away laterally from a cold seep in the Black Sea, but also vertically. Thus, as yet the extent to which AOM in diffusive (as well as active) methane flux settings can permanently affect TEX$_{86}$ derived SSTs remains unclear.

4.3. Free Versus Non-Extractable GDGTs

[25] Isoprenoid GDGTs released upon base and acid hydrolysis together represent between 10 and 30% of the total isoprenoid GDGTs analyzed. We caution that not all material released upon hydrolysis is by definition part of macromolecular structures; part of it could also represent difficult-to-extract material such as IP lipids. Although it is likely that many IP lipids were extracted during the initial Soxhlet extraction (and not subsequently analyzed), any non-extractable IP lipids would have been released by base and acid hydrolysis treatments. Indeed, recent work [*Huguet et al.*, 2010b] suggests that differences in IP GDGT extraction efficiency exist depending on the type of extraction used. If present as IP lipid, we might expect these GDGTs to preferentially be released as core lipids during acid hydrolysis, given the seemingly prevalence of archaeal GDGTs bearing glycosidic moieties [*Koga and Morii*, 2005; *Pitcher et al.*, 2010, 2011; *Schouten et al.*, 2008]. That is, however, not the case for the bulk of our sediments. The sole exception is the uppermost horizon (25 cm), from which only small amounts of GDGTs are released upon base hydrolysis of the

residue, whereas larger amounts are released upon acid hydrolysis (Figure 5). This seems unlikely to be an analytical artifact, because the base hydrolysis step was repeated prior to acid hydrolysis of the residue. Instead, we suggest that indeed in these shallow sediments, this higher yield arises from the presence of non-extracted glycosidic IP lipids that would be released by acid hydrolysis but not by base hydrolysis. The fact that this acid-hydrolysable component does not persist to greater depths suggests that in deeper sediments IP lipids have been degraded to a larger extent and that we are observing covalently bound GDGTs rather than non-extractable IP lipids. Regardless of its exact nature, the total pool of non-extractable isoprenoid GDGTs is substantial but certainly smaller than the free extractable pool.

[26] For branched GDGTs, the yield upon hydrolysis of the sediment residues is somewhat lower, between 6 and 10%, except for the uppermost sample (30%). A similar treatment of a French and Amazonian podzol soil by *Huguet et al.* [2010a] resulted in ca. 25(±15)% of branched GDGT lipids being retrieved upon hydrolysis and for an English grassland soil ca. 18% of branched GDGTs was obtained upon hydrolysis of the soil residue after extraction (J. W. H. Weijers et al., unpublished results, 2009). These studies could document the incorporation of branched GDGTs into macromolecules [*Huguet et al.*, 2010a] or into organo-siliciclastic associations in soils and preservation of those associations during transport, or alternatively they could reflect the presence of non-extractable IP lipids. The difference in the proportion of non-extractable isoprenoid versus branched GDGTs in Aarhus Bay sediments causes BIT indices to be slightly lower in the extract obtained by base hydrolysis than in the free extractable fraction (Figure 7). This could either mean that crenarchaeol, i.e., isoprenoid GDGTs, are bound into macromolecular structures to a larger extent than branched GDGTs, or branched GDGTs, since derived from soils, might be much more intimately associated with particles than pelagic derived isoprenoid GDGTs and are not being released using a base hydrolysis.

[27] The ratio of GDGT-0/crenarchaeol is generally higher in the extract obtained by base hydrolysis than in the free extractable fraction and seems to further increase in the extract obtained by acid hydrolysis (Figure 6). GDGT-0 is a common lipid produced by virtually all Archaea and, thus, in addition to being produced by pelagic Thau-

marchaeota, GDGT-0 might also be produced by methanogenic Archaea and by ANME Archaea in the sediments. GDGT-0 produced in situ could be more prone to incorporation into macromolecular structures (or occur as non-extractable IP lipids) since there has been less time for degradation of functional head groups after cell lyses. A similar increase in the GDGT-0/crenarchaeol ratio has been observed by *Lipp and Hinrichs* [2009] and *Liu et al.* [2011] in the hydrolyzed IP lipid fraction compared to the core lipid fraction in SMTZ sediments.

[28] Outside the SMTZ, the GDGT-2/crenarchaeol ratio and the TEX$_{86}$ values (Figures 6 and 7) are similar for both the free and non-extractable lipid fractions. This most likely underscores the similar biological source (i.e., pelagic Thaumarchaeota) and thus similar diagenetic behavior of the molecules, which contrasts with the GDGT-0/crenarchaeol ratio and BIT index. This is consistent with a study by *Schouten et al.* [2004] who performed an experiment in which marine sediment was artificially matured using hydrous pyrolysis. At increasing temperatures, when the isoprenoidal GDGTs were released, the TEX$_{86}$ did not change substantially. The equal ratios in both free and non-extractable fractions also attest to the absence of preferential incorporation into macromolecular matrices of specific GDGT lipids.

[29] At the SMTZ, GDGTs-1, −2 and −3 are, relative to the total amount of isoprenoid GDGTs, more abundant in the extract obtained by hydrolysis than in the free extractable fraction. As a consequence, for the sample in the center of the SMTZ (185 cm, see also Figure 2), the ratio of GDGT-2/crenarchaeol increases from 0.6 in the free extractable fraction to 2.2 and 3.1 in the extracts obtained after base and acid hydrolysis, respectively, an increase not observed outside the SMTZ (Figure 6). Also the TEX$_{86}$ value for that sample is clearly higher in the extract after base hydrolysis (0.75) than in the initial extract (0.69; Figure 7). The explanation is likely similar as for GDGT-0 in the GDGT-0/crenarchaeol ratio, i.e., production of an additional suite of GDGTs 1–3 by AOM Archaea residing within the sediment. These lipids, due to the presence of reactive functional head groups, are either more prone to become incorporated in a macromolecular matrix or more difficult to extract.

[30] Overall, the hydrolysis of Aarhus Bay sediments after regular extraction shows that there are

lipids that are closely associated with sediments (either non-extractable IP lipids or covalently bound into a matrix) and that this association with sediments happens early, probably when functionalized lipids are available. This need for functionalized lipids results in a gradient of increasing potential for incorporation: from distal soil derived lipids to pelagic derived lipids and finally benthic/ sedimentary derived lipids. Future work is needed to better understand the implications of this for quantifying these lipids in different environments.

5. Implications and Conclusions

[31] In their original paper presenting the TEX_{86} proxy, *Schouten et al.* [2002] clearly indicated the limitation of this SST proxy at sites where anaerobic oxidation of methane occurs. They highlighted AOM hot spots, such as cold seeps. This study now suggests that isoprenoid GDGTs synthesized by Archaea involved in AOM can also affect the GDGT distributions in sediments characterized by diffusive methane flow and shallow sulfate-methane transition zones, despite the lower rates of AOM. The extent of alteration of the TEX_{86} signal depends on the abundance of ANME Archaea and thus on the rate of AOM, which in turn is determined by the production and diffusion of methane in marine sediments. AOM occurs, though to different extents, at many continental shelves [*Hinrichs and Boetius*, 2002, and references therein; *Valentine*, 2002]. Aarhus Bay, with particularly high TOC contents (1–3%; Figure 2) and AOM rates, might represent a particularly strong continental shelf end-member site. However, similar locations are described in the literature, many around Denmark and the Baltic Sea but also in the Black Sea and at Saanich Inlet and Skan Bay in North America [e.g., *Hinrichs and Boetius*, 2002, and references therein]. Such settings might also be characterized by a considerable change in TEX_{86} values as observed here, but it is unclear whether sites with lower AOM rates – and presumably archaeal biomass – will exhibit similar but more subtle biases. Clearly, further investigations of such settings are necessary. It is also interesting to note that this bias does not appear to be preserved in deeper sediments; the reasons for this are unclear but also highlight the need for further investigation of such settings. Until then, this study emphasizes the original arguments by *Schouten et al.* [2002] that GDGT distributions that have been affected by a contribution of AOM-related GDGTs should not be used for reconstructing SSTs with TEX_{86}.

Acknowledgments

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