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Archaeal and bacterial lipids in authigenic carbonate crusts from eastern Mediterranean mud volcanoes

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Abstract

We investigated the distributions and $\delta^{13}C$ values of lipid biomarkers in authigenic methane-related carbonate crusts formed on eastern Mediterranean mud volcanoes. A highly diverse suite of microbial membrane lipids was detected. Strongly ^{13}C-depleted lipids diagnostic for archaea include isoprenoid glycerol diethers, isoprenoid glycerol dialkyl glycerol tetraethers and several C_{20}–C_{35} isoprenoid hydrocarbons. Bacterial lipids mainly consist of dialkyl glycerol diethers, branched fatty acids and hopanoids, showing important ^{13}C-depletions, albeit less than archaeal lipids. The data provide further evidence for the involvement of archaea and bacteria in the anaerobic oxidation of methane (AOM) and in the sequestration of methane-derived carbon in authigenic carbonate crusts. Varying distributions and isotopic compositions of ^{13}C-depleted lipids argue for heterogeneous AOM-related microbial consortia, expanding on existing work at cold seeps. Distinctive biomarker features – and inferred microbial assemblages – are observed in these crusts, in agreement with available phylogenetic data for the samples. Our results support the potential of lipids for distinguishing methanotrophic communities. Despite the observed variances, conservative features in the abundances and isotopic offsets of microbial biomarkers occur, likely reflecting the overall relationships between archaea and bacteria and the nature of carbon flow between them.

1. Introduction

There is increasing evidence for episodic massive methane release from dissociated gas hydrates in continental margin sediments during several intervals of global climate warming in the past (Kennett et al., 2000; Maslin et al., 2004). Large releases of methane at the seafloor can not only affect atmospheric methane concentrations, and thus global warming, but also dramatically modify oceanic

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redox conditions (through methanotrophy) and affect the carbon cycle (Hinrichs and Boetius, 2003). Moreover, methane seepage at the seafloor sustains dense benthic communities via oxidation of methane and sulfide (produced through sulfate reduction) coupled to the AOM (Sibuet and Olu, 1998). AOM and the resulting HCO_3^- production favour the formation of carbonate deposits through increases in alkalinity of pore waters which act as an important terminal sink for methane-derived carbon (Bohrmann et al., 1998; Aloisi et al., 2000, 2002 and references therein). Hence, assessment of the fate of methane released at the seafloor, and associated processes, is of major concern and has received substantial focus in recent years.

Early geochemical evidence argued for anaerobic oxidation being the main process of methane consumption in marine sediments (Hoehler et al., 1994). Subsequently, studies utilizing molecular biological and biogeochemical tools have provided direct evidence for the microbial role in AOM (e.g. Hinrichs et al., 1999; Boetius et al., 2000; Pancost et al., 2000, 2001b; Orphan et al., 2001a,b, 2002; Michaelis et al., 2002). Approximately 20–100 Tg of methane, corresponding to 5–20% of the net atmospheric methane flux, are thought to be consumed through AOM (Valentine and Reeburgh, 2000) and other estimates suggest even higher rates (300 Tg methane per year, see Valentine, 2002).

Biogeochemical (molecular and isotopic), phylogenetic and visual evidence have shown that both archaea and bacteria mediate AOM, coupled with sulfate reduction (Boetius et al., 2000; Orphan et al., 2001a; Aloisi et al., 2002; Michaelis et al., 2002; Nauhaus et al., 2002). There is now sound evidence for the involvement of at least two distinct archaeal lineages (ANME-1 and ANME-2) and sulfate reducing bacteria of the *Desulfosarcina/Desulfococcus* branch of the δ-proteobacteria in AOM at cold seep settings. Recently, a third AOM-related archaeal lineage (ANME-3) was reported (Knittel et al., 2005). These microorganisms occur either in physical close association (as syntrophic partners) or as monospecific aggregates (Boetius et al., 2000; Lanoil et al., 2001; Orphan et al., 2001a, 2002; Michaelis et al., 2002). However, questions remain; it is possible that other microbial groups are capable of anaerobic methane consumption (Aloisi et al., 2002; Orphan et al., 2002; Knittel et al., 2003); none of the organisms that mediate AOM have been isolated and the specific mechanisms and biochemical pathways involved in AOM remain poorly constrained.

Field observations in the eastern Mediterranean Sea have identified widespread areas of methane-rich fluid seepage in mud volcano fields (Robertson and ODP Leg 160 Scientific Party, 1996; MEDINAUT/MEDINETH Shipboard Scientific Parties, 2000). Large methane concentrations were measured both at depth in mud-breccia sediments (Emeis et al., 1996; Haese et al., 2003) and in the water column above mud volcanoes (Charlou et al., 2003). Dense benthic communities characteristic of cold seeps were observed (Olu-Le Roy et al., 2004), while carbonate crust pavements up to several decimeters thick were abundant on all the mud volcanoes studied (MEDINAUT/MEDINETH Shipboard Scientific Parties, 2000). These show noticeable ^{13}C depletion, indicating isotopically light methane as the major source for the carbonate carbon (Aloisi et al., 2000, 2002).

We report here detailed molecular and isotopic data obtained from the study of lipid biomarkers in the organic matter encrusted within the mineral lattice of carbonate crusts from eastern Mediterranean mud volcanoes. Lipids diagnostic for archaea and bacteria, along with their carbon isotopic composition, are being increasingly employed in the study of cold seep settings and have provided useful insights into the AOM (e.g. Hinrichs et al., 2000b; Pancost et al., 2000, 2001b, 2005; Pancost and Sinninghe Damsté, 2003; Stadnitskaia et al., 2005). Here, we focus on the abundant and varying distributions of compounds associated specifically with Mediterranean cold seep carbonate crusts. We have previously reported certain aspects of carbonate crust biomarker distributions (Aloisi et al., 2002; Pancost and Sinninghe Damsté, 2003), but this is a complete survey of carbonate crust lipid abundances and isotopic compositions and provides an important basis for comparison with other sites.

2. Material and methods

2.1. Samples

Authigenic carbonate crusts were collected by the *Nautile* submersible during the MEDINAUT cruise (1998) on board the R/V *Nadir* (MEDINAUT/MEDINETH Shipboard Scientific Parties, 2000). Their mineralogy and stable isotope composition have been discussed in detail by Aloisi et al. (2000). Aragonite and high Mg calcite cements account for up to 82% (by weight) of mud breccia carbonate crusts and the carbon isotopic compositions are sig-

nificantly depleted in ^{13}C ($\delta^{13}C < -20‰$), consistent with the suggestion that carbonate carbon is largely derived from methane. This organic geochemical study focusses on a mud breccia crust recovered from the Napoli mud volcano in the Olimpi area south of Crete (MN16BT2, 33°43.548′N, 24°41.0367E, 1945 m water depth), a mud breccia carbonate crust from the Amsterdam mud volcano in the Anaximander Mountains area west of Cyprus (MN13BT4 – 35°19.859′N, 30°16.528E, 2034 m water depth) and a pelagic carbonate crust from the Kazan mud volcano in the Anaximander Mountains area west of Cyprus (MN12BT3 – 35°26.007′N, 24°33.532E, 1706 m water depth). The $\delta^{13}C$ values of carbonate carbon are −28.9‰, −24.8‰, and −34.5‰ for MN16BT2, MN13BT4 and MN12BT3, respectively.

2.2. Extraction and separation of lipid biomarkers

Dried carbonate crusts (60–100 g) were ground and lipids were extracted with a CH_2Cl_2/MeOH mixture (3:1, v/v) under reflux. Total extracts were separated into acetone soluble and insoluble fractions (Jahnke et al., 1995). The acetone soluble fraction was separated using column chromatography on silica gel into four fractions: F1 (aliphatic hydrocarbons with 0–3 double bonds), F2 (aliphatics with $\geqslant 3$ double bonds and (poly)aromatic hydrocarbons), F3 (carbonyl compounds) and F4 (hydroxylated compounds). An aliquot of the total extract was methylated with BF_3/MeOH and separated using column chromatography as described above. Fatty acids as methyl esters were recovered in the third fraction. Hydroxy compounds in F4 were converted to trimethylsilyl ether derivatives by heating in a 1:1 mixture of N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA) and pyridine.

2.3. Hydrogenation and ether bond cleavage

To confirm the identification of unsaturated isoprenoids and to enable structural assignments for the dialkyl glycerol diethers, the corresponding lipid fractions were hydrogenated by stirring for 24 h in ethyl acetate containing 100 mg of Pt charged with H_2 and 100 μl of acetic acid. The fraction containing glycerol diethers and tetraethers was treated with HI to cleave ether bonds. An aliquot was refluxed in 57% HI/$LiAlH_4$ (in H_2O by weight) for 1 h and the generated alkyl iodides were reduced to hydrocarbons with $LiAlH_4$ (Schouten et al., 1998). To elucidate the position at which the alkyl chains were attached to the glycerol moiety, a second HI treatment was performed on an aliquot and the resultant alkyl iodides were quenched with $NaSCH_3$ according to the methods described in Schouten et al. (1998).

2.4. GC and GC–MS

Lipid fractions were analysed using gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). GC–MS was conducted using a Hewlett–Packard 5890 gas chromatograph interfaced to a VG Autospec Ultima Q mass spectrometer operated at 70 eV with a mass range m/z 50–800 and a cycle time of 1.8 s (resolution 1000). A fused silica CP-Sil 5 capillary column (25 m × 0.32 mm, film thickness = 0.12 μm) was used with helium as the carrier gas. Samples were injected at 70 °C and the temperature was programmed to increase at 20 °C/min to 130 °C, at 4 °C/min to 320 °C, and held constant for 15 min. Identification of compounds was based on interpretation of GC–MS spectra and comparison with literature data, where available. Quantitation was based on comparison of GC peak areas with those of internal standards.

2.5. Isotope ratio monitoring GC–MS

Compound specific $\delta^{13}C$ values were determined using irm-GC–MS with a Finnigan Delta C mass spectrometer. A fused silica CP-Sil 5 capillary column (25 m × 0.32 mm i.d., film thickness = 0.4 μm) was used with helium as carrier gas. The temperature programme was the same as that used for GC–MS; $\delta^{13}C$ values are expressed against VPDB, have been corrected for the addition of carbon during derivatization and have an error of less than ±1.0‰.

2.6. High performance liquid chromatography–mass spectrometry (HPLC–MS)

Intact glycerol dialkyl glycerol tetraethers were analysed using HPLC-/atmospheric pressure chemical ionization-MS (HPLC/APCI-MS) using the methodology of Hopmans et al. (2000).

3. Results and discussion

3.1. Archaeal lipids

3.1.1. Ether-bound isoprenoids

Isoprenoidal glycerol diethers are major components of the lipid fraction containing alcohols.

Table 1
Concentrations and isotopic composition of lipids from microbial sources found in carbonate crust samples from eastern Mediterranean mud volcanoes

	MN16BT2		MN13BT4		MN12BT3	
	μg/g	$\delta^{13}C$, ‰	μg/g	$\delta^{13}C$, ‰	μg/g	$\delta^{13}C$, ‰
Archaeol	4.78	−87.9	0.273	−95.7	0.019	−112.2
Hydroxyarchaeol[a]	1.21	−91.4	0.020	−96.0	<0.005	n.d.
Phytane[b]	nq	−88.1	nd	nd	nd	nd
Acyclic biphytane[b]	nq	−90.8	nd	nd	nd	nd
Monocyclic biphytane[b]	nq	−91.1	nd	nd	nd	nd
Bicyclic biphytane[b]	nq	−90.9	nd	nd	nd	nd
Crocetane	0.211	−38.4[d]	0.102	−66.1	<0.005	nd
Crocetene (Cr:1)	0.012	−63.0	<0.005	nd	–	–
PMI:0[f]	0.816	−86.7	0.067	−82.3	<0.005	−69.4
PMI:1	0.484	−88.3	0.019	nd	–	–
PMI:2	0.657	−86.6	–	–	–	–
PMI:3	0.430	82.9	–	–	–	–
PMI:4	0.328	−84.2	–	–	–	–
PMI:5	0.128	nd	–	–	–	–
$C_{30:0}$ isoprenoid	0.066	−63.8	0.053	nd	<0.005	nd
$C_{30:1\text{-}6}$ isoprenoid	0.192	−63.5	0.096[e]	nd	0.010[e]	nd
$C_{35:x}$ isoprenoid	0.084	−71.1	0.066	nd	–	–
Lactone a[c]	0.121	−78.1	0.099	−90.0	–	–
Lactone b[c]	0.045	−72.8	0.030	−84.9	–	–
Phytanoic acid	0.27	−46[g]	nq	−65.7	<0.005	−22.8
Phytanol	0.014	nd	0.023	−87.7	0.015	nd
Series I dialkyl glycerol diethers						
I a	0.155	nd	nd	nd	nd	nd
I b	nd	nd	nd	nd	nd	nd
I c	0.192	−73.9	0.021	nd	0.007	nd
I d	0.111	nd	0.021	nd	0.005	nd
I e	0.154	nd	0.024	nd	0.005	nd
I f	0.605	−71.2	0.061	−78.8	0.022	−99.6
I g	0.442	−67.3	0.026	nd	0.007	nd
I h	0.376	nd	0.074	−83.6	0.024	−94.8
Sum Ia–Ih	2.03		0.227	–	0.070	–
Series II dialkyl glycerol diethers						
II a	1.99	−53.1	0.021	−67.3	<0.005	nd
II b	0.868	−57.7	0.018	nd	<0.005	nd
II c	1.50	−55.6	0.031	nd	<0.005	nd
II d	2.69	−51.9	0.035	−68.8	<0.005	−69.3
Sum IIa–IId	7.05		0.105	–	0.01	–
i-, *ai*-$C_{15:0}$ FAME	0.312	−66.3	0.165	−66.3	0.016	−32.8
i-$C_{16:0}$ FAME	0.045	−59.3	0.022	nd	nd	nd
i-, *ai*-$C_{17:0}$ FAME	0.211	−56.1	0.061	−53.9	0.011	nd
Cyclopropyl-C_{17} FAME	0.121	−50.2	nd	–	–	–
Diploptene	0.038	−41.1	0.057	−36.4	nd	nd
β,β-Homohopane	0.010	nd	0.021	nd	nd	nd
β,β-Bishomohopanol	0.050	nd	0.061	−64.6	<0.005	nd
α,β + β,β-Bishomohopanoic Acid	0.047	−56.3	0.045	−56.2	nd	nd
C_{27}-Trisnorhopanone	0.165	−62.1	0.045	−65.5	nd	nd

nq: not quantified, nd: not determined.
[a] *sn*-3 Hydroxyarchaeaol prevails in MN16BT2, *sn*-2 in MN13BT4, structure not determined in sample MN12BT3.
[b] Released after ether cleavage.
[c] See text.
[d] Coelution with phytane.
[e] $C_{30:6}$ is found.
[f] Numeral refers to the number of double bonds.
[g] Important coelution with unsaturated C_{18}-FAME.

Archaeol (bis-*O*-phytanylglyceroldiether), *sn*-2- and *sn*-3-hydroxyarchaeols (Hinrichs et al., 2000a) were identified in high concentration, reaching 6.0 μg/g in sample MN16BT2 (Table 1). Archaeol is the most abundant GC-amenable lipid in mud breccia carbonate crusts (MN16BT2, MN13BT4). In all crusts hydroxyarchaeols occur in abundances substantially lower than that of archaeol. The *sn*-3- isomer is major in crust MN16BT2, whereas in MN13BT4 *sn*-2-hydroxyarchaeol predominates. The low amounts of any hydroxyarchaeol in sample MN12BT3 prevented the determination of the OH position. Archaeol is a most common core ether lipid in a large variety of archaea and is particularly abundant in methanogens, while hydroxyarchaeol is most commonly found in cultured methanogens, particularly in the orders of *Methanosarcinales* and *Methanococcales* (Koga et al., 1993, 1998; Sprott et al., 1993). Prominent *sn*-2-hydroxyarchaeol is found in archaea of the order of *Methanosarcinales* (Sprott et al., 1993), while predominant *sn*-3-hydroxyarchaeol has been reported in *Methanosaeta concilii* (Ferrante et al., 1988). Both hydroxyarchaeol isomers have been found in *Methanococcus voltae* and *Methanolobus bombayensis* (Sprott et al., 1993).

In carbonate crusts, archaeol and hydroxyarchaeol are profoundly depleted in ^{13}C, with $\delta^{13}C$ values ranging from −88 to −112‰, the lowest encountered among lipids in the respective samples (Table 1). The isotopic compositions of archaeol and hydroxyarchaeol are nearly identical in sample MN13BT4, while in sample MN16BT2 hydroxyarchaeol is slightly more depleted; ^{13}C-depleted archaeol and hydroxyarchaeol have been commonly reported at cold seeps where they are ascribed to archaea that oxidize isotopically light methane under anaerobic conditions (e.g. Hinrichs et al., 1999, 2000b; Elvert et al., 2000; Pancost et al., 2000, 2001b, 2005; Stadnitskaia et al., 2005).

Like archaeol and hydroxyarchaeol, isoprenoid glycerol dibiphytanyl glycerol tetraethers (GDGTs) are major membrane constituents in a variety of archaea (De Rosa and Gambacorta, 1988). They were previously thought to characterise extremophilic (e.g. hyperthermophiles) species, but there is now large evidence for their widespread occurrence in diverse non-extreme marine settings, including cold seeps, where they are ascribed to non-thermophilic, as yet not cultivated, archaea (Hoefs et al., 1997; DeLong et al., 1998; King et al., 1998; Schouten et al., 2000; Pancost et al., 2001b).

In our crusts HPLC/APCI-MS analysis of the polar lipid fractions revealed the presence of intact isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) with $[M + H]^+$ values of *m*/*z* 1302, 1300, 1298, 1296, 1304 (only trace levels), 1294 and 1292. Ether bond cleavage released C_{40} isoprenoid chains from GDGTs, with acyclic, monocyclic and bicyclic biphytanes being the predominant species released, with low levels of a tricyclic biphytane in sample MN12BT3. Phytane, which likely derives from archaeol and hydroxyarchaeol, was also released. Mass spectra, comparison of retention times with those of known GDGTs (Hopmans et al., 2000) and distribution patterns of biphytanes allowed the identification of seven GDGT compounds (see Appendix for structures). GDGTs with 0–4 cyclopentane rings: GDGT-0 (*m*/*z* 1302), GDGT-0′ (*m*/*z* 1304), GDGT-1 (*m*/*z* 1300), GDGT-2 and/or GDGT-2′ (*m*/*z* 1298), GDGT-3 (*m*/*z* 1296), GDGT-4 (*m*/*z* 1294) and crenarchaeol (*m*/*z* 1292). Although intact GDGTs were not quantified, the GC response of released biphytanes is higher than that of phytane released from archaeol and hydroxyarchaeol (Fig. 1) suggesting that GDGTs are more abundant than isoprenoidal glycerol diethers.

Compounds with 0–2 cyclopentanyl moieties (GDGT-0, GDGT-1, and GDGT-2/2′) are most abundant in all crusts, representing >85% of the total GDGTs (Fig. 2). GDGT-3 is an important component in the mud breccia carbonate crusts MN16BT2 and MN13BT4. Crenarchaeol, likely derived from pelagic crenarchaeota (e.g. Schouten et al., 2000), occurs only in sample MN12BT3 at low relative abundance, consistent with the traces of a tricyclic biphytane released. Pelagic crenarchaeota also produce abundant GDGT-0 (DeLong et al., 1998). Distributions in settings where pelagic archaeal sources are inferred are dominated by GDGT-0 and crenarchaeol (Schouten et al., 2000; Pancost et al., 2001b; Wakeham et al., 2003, 2004) and thus differ from the patterns found in our samples. In the study area pelagic GDGT fingerprints were reported in surface mud breccia sediments whereas at depth, where increased amounts of archaeol occur, GDGT-0, GDGT-1, and GDGT-2 prevail (Pancost et al., 2001b).

The carbon isotopic composition of biphytanes released by the HI/$LiAlH_4$ treatment further constrains the archaeal sources of GDGTs. Biphytanes show very low $\delta^{13}C$ values, identical to those measured for archaeol and hydroxyarchaeol and for their HI/$LiAlH_4$ derivative (phytane; Table 1), indi-

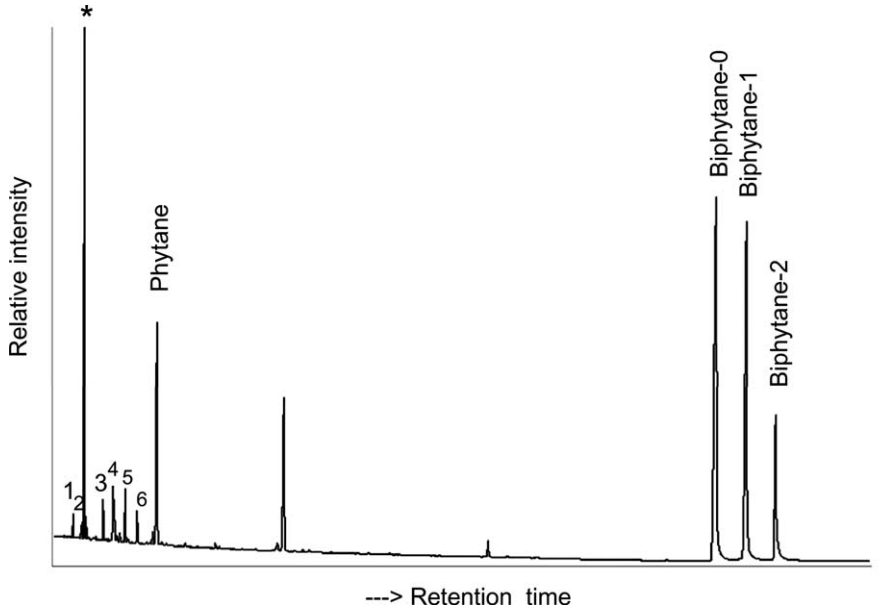

Fig. 1. Mass chromatogram of MN16BT2 (fraction 4) after treatment with HI/$LiAlH_4$. Biphytane-0, -1, -2 refer to acyclic, monocyclic, and bicyclic biphytanes released from glycerol tetraethers. Phytane is released from archaeol and hydroxyarchaeol. Compounds eluting before phytane are alkyl components deriving from Series I and Series II non-isoprenoidal dialkyl glycerol diethers (1: *n*-C_{14}; 2: *ai*- and *i*-C_{15}; 3: *n*-C_{16}; 4: 6-MeC_{17} and 5-MeC_{17}; 5: *n*-C_{17}; 6: cyclohexyl C_{17}; see also Pancost et al., 2001).

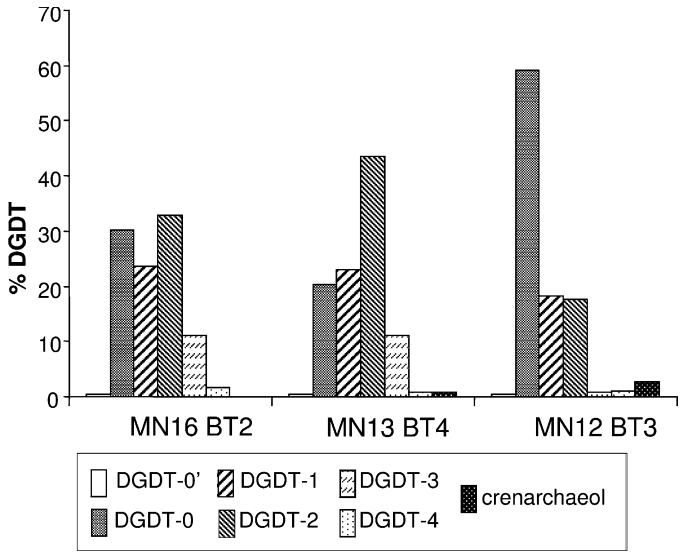

Fig. 2. Distribution of glycerol dialkyl glycerol tetraethers (GDGTs) in carbonate crust samples. Structures of GDGTs are given in Appendix.

cating the DGDTs are generated by archaea that anaerobically consume methane. A rather limited number of investigations in cold seeps have performed intact GDGTs analyses, such as in eastern Mediterranean mud volcanoes (Pancost et al., 2001b; this study), the Gulf of Mexico (Pancost et al., 2005) and the Black Sea (Stadnitskaia et al., 2003, 2005). In several other cases, only ^{13}C-depleted biphytanes released by ether bond cleavage from GDGTs have been reported (e.g. Thiel et al., 2001; Michaelis et al., 2002; Blumenberg et al., 2004). Consistent with our results, these previous investigations have assigned GDGTs to methanotrophic archaea.

3.1.2. Free isoprenoid hydrocarbons

Diverse acyclic irregular, tail-to-tail linked isoprenoid hydrocarbons are present (Fig. 3). In sample MN16BT2 from the Napoli Dome, they are the dominant hydrocarbons and among the most abundant lipids (Table 1). The C_{25} series predominates, reaching concentrations of 2.8 μg/g, six-fold higher than the total (C_{15}–C_{35}) *n*-alkane concentration. They consist of 2,6,10,15,19-pentamethylicosane (PMI) and its mono- to penta-unsaturated counterparts. C_{20}-homologues, namely crocetane (2,6,11,15-tetramethyl-hexadecane) and crocetene, are the second most abundant irregular isoprenoids. PMIs and crocetane also occur in crust MN13BT4 from the Amsterdam Dome, albeit at lower amounts than in MN16BT2 (Table 1). A particular feature of both mud breccia crusts is the presence of significant amounts of irregular C_{30} isoprenoids (2,6,10,15,19,23-hexamethyltetracosane) with 0–6 double bonds and C_{35} isoprenoids (2,6,10,14,19,23,27-heptamethyloctacosane) with 0–7 double bonds (Fig. 1, Table 1). In the pelagic crust MN12BT3 (Kazan Dome) only a few isoprenoids occur as minor hydrocarbon constituents.

PMIs are among the most common biomarkers diagnostic for archaea, and particularly methanogens (Holzer and Oro, 1979; Risatti et al., 1984). Saturated and unsaturated (1–5 double bonds) PMIs have only been observed in cultures of *Methanosarcina mazei* and *Methanolobus bombayensis*, but may occur in other archaea as well (Schouten et al., 1997). Crocetane has not been observed in cultures and specific microbial sources have not been assigned; yet, its common presence and low $\delta^{13}C$ values in cold seep settings has been ascribed to methane-metabolising archaea (Elvert et al., 2000; Hinrichs et al., 2000b; Bian et al., 2001; Pancost et al., 2001b). Isoprenoid hydrocarbons with $C \geqslant 25$ found in our samples may occur in archaea including methanogens (Holzer and Oro, 1979; Risatti et al., 1984). They have not been observed in previous studies at cold seeps, except, recently, in

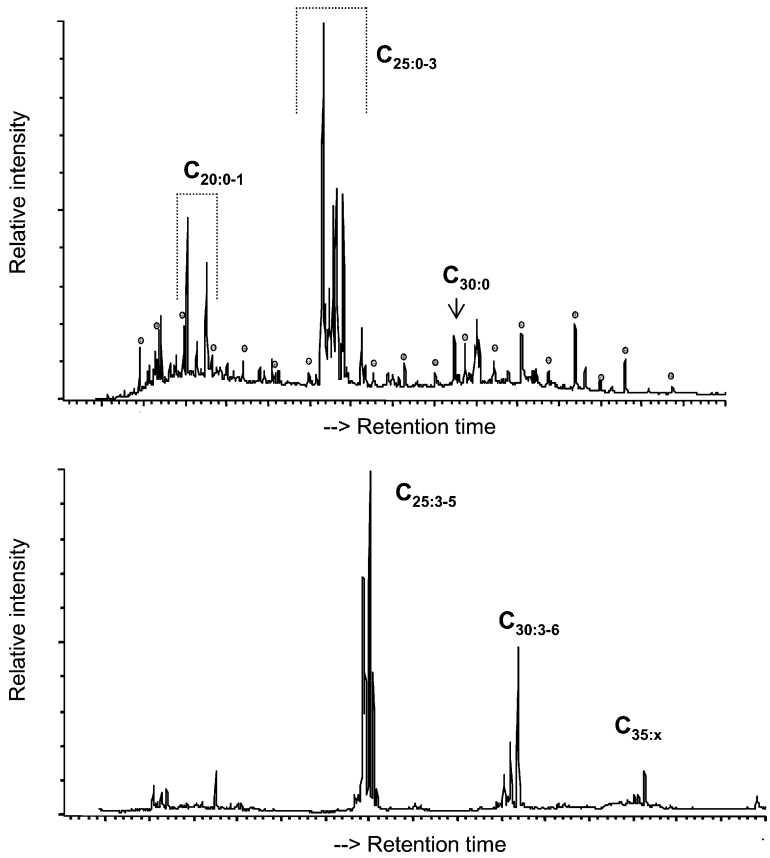

Fig. 3. Gas chromatogramme of MN16BT2 (fractions 1 and 2) showing distribution of saturated and unsaturated isoprenoidal hydrocarbons with 20, 25, 30, and 35 carbon atoms. Dots correspond to *n*-alkanes.

Black sea carbonate crusts (Stadnitskaia et al., 2005).

The isoprenoid hydrocarbons are strongly depleted in ^{13}C (Table 1). In particular, PMIs have $\delta^{13}C$ values as low as −91‰, comparable to those of isoprenoid glycerol diethers. Crocetane/crocetene are ^{13}C-depleted as well, but enriched by ⩾20‰ relative to PMIs (Table 1). Coelution of crocetane with phytane deriving from the "background" organic matter indigenous of the extruded mud breccia most likely contributes to raising the $\delta^{13}C$ value of crocetane (−38.4‰) in crust MN16BT2 and likely affects its isotopic signature in the other crust samples. In MN16BT2, the $\delta^{13}C$ value of the unsaturated homologue (crocetene) is −63‰. The carbon isotopic compositions of intact or as saturated (after hydrogenation) C_{30} and C_{35} isoprenoids range from −63.8 to −71.1‰; ^{13}C-depleted PMIs and crocetanes have been commonly reported at cold seeps (e.g. Hinrichs et al., 1999, 2000b; Elvert et al., 2000; Pancost et al., 2001b, 2005; Thiel et al., 2001; Stadnitskaia et al., 2005) as common biomarkers of archaea (as yet not characterised) responsible for the AOM.

Other free lipids with a phytanyl chain were also detected, i.e. phytanol and phytanoic acid. Phytanol shows very low $\delta^{13}C$ values similar to those of archaeal lipids (Table 1), a feature that excludes an origin from phytoplankton-derived chlorophyll (Volkman and Maxwell, 1986). Phytanoic acid is relatively ^{13}C-enriched, most likely due to coelution with a C_{18} fatty acid with a "normal" C isotope signature. Previously reported ^{13}C-depleted phytanol and phytanoic acid at cold seeps were assigned to archaeal sources, either as a direct input or as decomposition products of phytanyl glycerol ethers (Hinrichs et al., 2000b; Elvert et al., 2000).

Two lactones were found in the fatty acid fractions and show mass spectra and retention characteristics identical to those reported by Barbe et al. (1990) and Grimalt et al. (1991) for the isoprenoid 3,7,11,15-tetramethyl-4-hexadecanolide (lactone a) and 3,7,11,15-tetramethyl-17-hexadecanolide (lactone b). Their occurrence in an alkaline lagoon and evaporitic environments where extremophile microbes thrive and the presence of a phytanyl moiety in their structure argued for an origin from alteration of phytanyl chains of archaeal ether lipids (Grimalt et al., 1991). In our samples, the two isoprenoid lactones show profound ^{13}C-depletion (Table 1), comparable to that of the isoprenoidal ether lipids and consistent with an archaeal origin.

3.2. Bacterial lipids

3.2.1. Dialkyl glycerol diethers (DGDs)

Pancost et al. (2001a) reported novel series of dialkyl (non-isoprenoidal) glycerol diethers (DGDs) in the MN16BT2 carbonate crust (Fig. 1). Similar compounds had also been found in cold seeps at the California Margin (Hinrichs et al., 2000b), but their structures were not determined. In our carbonate crust samples the ether-bound moiety at the *sn*-2 position is either an *anteiso* C_{15} chain (Series I) or a 11,12-methylenehexadecyl chain (Series II). Both series have ether-bound C_{14}–C_{17} alkyl chains at the *sn*-1 position of the glycerol (see Appendix). Several lines of evidence suggested that the DGDs originate from bacteria (mesophilic sulfate reducers) rather than archaea (Pancost et al., 2001a). Subsequently, culture studies demonstrated that mesophilic SRB like *Desulfosarcina variabilis* and *Desulforhabdus amnigenus* biosynthesise (mono) alkyl ether lipids, implying that alkyl ether lipids may be more common membrane lipids of (SRB) bacteria than previously thought (Rütters et al., 2001). However, diethers similar to those found here have yet to be found in cultured mesophiles.

The summed concentrations of the major Series I and II DGDs are high, reaching 9.1 μg/g in crust MN16BT2 (Table 1). In this sample, Series II DGDs (consisting of 4 compounds: IIa–IId; Table 1 and Appendix) largely predominate (7.1 μg/g). Conversely, Series I diethers (8 compounds: Ia–Ih; Table 1 and Appendix) prevail in crusts MN13BT4 and MN12BT3. The carbon isotopic compositions of all dialkyl diethers are low but exhibit a rather large range (from −51.9 to −99.6‰, Table 1). Such values reveal incorporation of methane-derived carbon into the biomass of DGD producers. However, DGDs are not as ^{13}C-depleted as the co-occurring archaeal ether lipids. A significant offset is observed between the two DGD series, with Series I being depleted in ^{13}C by 10–30‰ relative to Series II diethers. These large differences have been interpreted as indicating distinct bacterial (SRB) sources (Pancost et al., 2001a), because $\delta^{13}C$ variations in the lipids of single organisms are typically less that 10‰ (Hayes et al., 1990). Since their first complete structural identification in our sample set (Pancost et al., 2001a), alkyl diethers have been reported for a number of cold seep environments, such as Black Sea (Michaelis et al., 2002; Stadnitskaia et al., 2005) and Gulf of Mexico cold seeps (Zhang et al., 2003; Pancost et al., 2005), albeit not in such a diversity

as in the carbonate crusts discussed here. Alkyl diethers are also found in marine sediments where high rates of sulfate reduction occur (Benguela Upwelling Region; Bouloubassi et al., unpublished data).

3.2.2. Fatty acids

The total fatty acid (FA) composition of the carbonate crusts encompasses ubiquitous non-diagnostic compounds from algal and/or bacterial sources. With regard to components that may be of relevance to methane cycling microbial assemblages, terminally-branched fatty acids (*iso*- and *anteiso*-C_{15} and C_{17}), prevalent in sulfate reducing bacteria (Kaneda, 1991) occur in all the samples. In crust MN16BT2 their concentrations reach 0.5 μg/g, while they are half as abundant in crust MN13BT4 and are only at trace levels in the pelagic crust MN12BT3 (Table 1). Cyclopropyl C_{17} FA, a compound abundant in SRB (Dowling et al., 1986) is also present at concentrations comparable to those of individual *iso*/*anteiso* FAs. Terminally-branched C_{15} and C_{17} and cyclopropyl C_{17} FAs in the mud breccia crusts are all depleted in ^{13}C, albeit significantly less so than most of the archaeal lipids, while they show "normal" $\delta^{13}C$ values in the pelagic crust MN12BT4 (Table 1).The $\delta^{13}C$ values for *iso*/*anteiso* C_{15} are −66‰, while the *iso*/*anteiso* C_{17} series is enriched by ca. 10‰ and the cyclopropyl-bearing C_{17} fatty acidby 16‰. These carbon isotopic offsets are surprisingly large and may argue for varying bacterial sources contributing terminally branched FAs.

3.2.3. Hopanoids

Non-extended and extended hopanoids are found in the mud breccia crusts. They consist of hop-22(29)-ene (diploptene), a C_{27} hopanoid ketone, ββ-homohopane, ββ-bishomohopanol and αβ- and ββ-bishomohopanoic acid, occurring at concentrations reaching 0.17 μg/g (Table 1). Hopanoids are membrane constituents of a large variety of bacteria (Rohmer et al., 1984), including methanotrophs (Summons and Jahnke, 1990). Their reported occurrence at cold seeps characterised by AOM (Elvert et al., 2000; Pancost et al., 2000; Hinrichs, 2001; Thiel et al., 2001; Werne et al., 2002, 2004) has been tentatively ascribed to aerobic bacteria thriving near the oxic/anoxic interface (e.g. H_2S oxidisers, methanotrophs) mainly because hopanoids are considered to be confined to aerobic bacteria (Ourisson and Albrecht, 1992). However, their occurrence in the permanently anoxic deep Black Sea (Thiel et al., 2003) implies (unknown) anaerobic sources, and hopanoids have been found recently in strictly anaerobic bacteria (Sinninghe Damsté et al., 2004; Härtner et al., 2005). In our samples, hopanoids are ^{13}C-depleted, with $\delta^{13}C$ values for the extended hopanoids and the C_{27} hopanoid ketone being between −56 and −66‰, while diploptene is relatively ^{13}C-enriched (Table 1). Consistent with previously reported data, hopanoids derive, therefore, from (unidentified) microbes of the methane-supported community. The C_{27} hopanoid ketone and hopanoid ketones in general, have not been previously reported at cold seep settings other than Mediterranean mud volcanoes and recently in Gulf of Mexico carbonate crusts (Pancost et al., 2005).

3.3. Eukaryote lipids

Tetrahymanol, a C_{30} non-hopanoid pentacyclic triterpenoid, is found at concentrations ranging from 5 to 138 ng/g, the highest amounts being encountered in crust MN16BT2. This compound is commonly observed in diverse marine settings (Venkatesan, 1989) where possible sources may be photosynthetic sulfur bacteria (Kleemann et al., 1990) and marine ciliates thriving on prokaryotic biomass (Harvey et al., 1997). The $\delta^{13}C$ value was determined only for sample MN13BT4 and is very low (−92.2‰), comparable to the values for archaeal lipids, which argues for an origin from ciliates grazing on methane-metabolising archaea; ^{13}C-depleted tetrahymanol was reported in mud breccia sediments and mats of the study area, albeit considerably enriched relative to archaeal lipids, suggesting that, there, ciliates thrive on diverse microbial communities with various $\delta^{13}C$ signatures (Werne et al., 2002; Pancost and Sinninghe Damsté, 2003), whereas in settings associated with carbonate crust precipitation, archaeal biomass likely serves as the primary food source.

Finally, the carbonate crusts contain a number of lipids mostly indigenous to the extruded mud breccia, such as higher plant long chain alkanes and alcohols ($\delta^{13}C$ = ca. −31‰), planktonic sterols and long chain diols ($\delta^{13}C$ = from −25 to −30‰), and non-diagnostic saturated and mono-unsaturated fatty acids that may derive from both planktonic and bacterial sources ($\delta^{13}C$ = from −26 to −31‰).

3.4. Variability in microbial lipids and implications for varying AOM-associated microbial assemblages

The occurrence and distribution of ^{13}C-depleted microbial lipids in authigenic carbonate crusts from

eastern Mediterranean mud volcanoes are consistent with previous investigations of cold seeps, providing evidence for AOM coupled with sulfate reduction mediated by archaea and bacteria. The data presented here, especially for the carbonate crust from the Napoli Dome, represent one of the more extensive lipid data sets reported for a single cold seep setting and further document and expand the body of knowledge of microbial lipids associated with AOM. Microbial ^{13}C-depleted lipids are most abundant in the mud breccia carbonate crusts (Table 1) and highest in crust MN16BT2 where scanning electron microscope investigations revealed authigenic aragonite crystals occurring in close association with organic matter (Aloisi et al., 2002), which, in view of the data presented here, consists mainly of AOM-related archaeal and bacterial biomass. The pelagic carbonate crust (MN12BT3, Kazan Dome) still contains ^{13}C-depleted microbial lipids, albeit at much reduced absolute and relative abundances.

The abundance, distribution and carbon isotopic signature of ^{13}C-depleted lipids varies amongst the investigated carbonate crusts and most likely records the varying structure of the microbial community associated with AOM.

Archaeal lipids: Both *sn*-2- and *sn*-3-hydroxyarchaeol occur, with the latter being only rarely reported in cold seep settings (e.g. Pancost et al., 2001b). Thus, its predominance in the Napoli Dome carbonate crust (MN16BT2) is a peculiar feature, although it is consistent with its presence in unconsolidated mud breccia sediments associated with Napoli Dome cold seeps (Pancost et al., 2001b). In contrast, the more commonly identified *sn*-2-hydroxyarchaeol is predominant in the Amsterdam Dome crust, suggesting differences in the archaeal community structure. The *sn*-2-hydroxyarchaeol is found in cold seeps where the ANME-2 archaeal group (related to *Methanosarcinales* -known to synthesize predominantly this isomer) dominates (Hinrichs et al., 1999; Orphan et al., 2001a). However, its occurrence was recently reported in settings (Black Sea, Guyamas Basin) where the archaeal cluster ANME-1, which is only distantly related to *Methanosarcinales,* dominates (Michaelis et al., 2002; Teske et al., 2002; Stadnitskaia et al., 2005). Circumstantial evidence suggests that both ANME-1 and ANME-2 methanotrophic archaea produce *sn*-2-hydroxyarchaeol. Thus, the major archaea in sample MN16BT2 (where *sn*-3 hydroxyarchaeol predominates) might be distinct from the reported ANME-1 and ANME-2 groups, whereas in sample MN13BT4 at least one of these sequences may occur. In line with the lipid data, 16S rRNA gene surveys conducted on our sample set showed the presence of diverse archaeal communities, distinct in each crust (Aloisi et al., 2002; Heijs et al., 2006). Euryarchaeal sequences related to the ANME-2 group (co-occurring with sequences forming a novel group of *Thermoplasmales*) are found in the Amsterdam Dome crust (MN13BT4) consistent with the presence of *sn*-2-hydroxyarchaeol. In the Napoli crust (MN16BT2) lineages affiliated with both ANME-1 and ANME-1 EEL-TA were found, along with novel *Thermoplasmales,* whereas ANME-2 lineages were absent.

Hydroxyarchaeols show varying abundance relative to archaeol, with the latter being more abundant in sample MN13BT4 [archaeol/hydroxyarchaeol (AH) ratio 13], whereas in the other crusts it is much less predominant (AH ratio = 4). High relative abundance of hydroxyarchaeol (mid values for the AH ratio) has been observed at cold seep sites where ANME-2 archaea dominate, whereas archaeol is much more abundant (high AH ratios) at sites where ANME-1 (and ANME EEL-TA) archaea prevail (Hinrichs et al., 2000b; Orphan et al., 2001a; Teske et al., 2002; Blumenberg et al., 2004; Stadnitskaia et al., 2005). Our data diverge from previous reports since an increased relative abundance of archaeol occurs in the crust where ANME-2 lineages are found (MN13BT4). However, it has to be stressed that varying AH ratios have also been observed at a single site, in mud breccia profiles in the study area (Napoli and Kazan Domes), with the relative abundance of hydroxyarchaeol increasing with depth and being highest at the zone of present day AOM (Pancost et al., 2000; Werne et al., 2002). Difficulties in interpreting the AH ratio as a potential indicator for varying archaeal assemblages may arise from preferential degradation of the more labile hydroxyl-bearing compound (Koga et al., 1993).

Consistent with previous reports for cold seeps, the GDGT profiles in the mud breccia crusts are characterised by the prevalence of GDGT-0, GDGT-1 and GDGT-2/2′, assigned to methanotrophic archaea. In contrast, crust MN12BT3 shows a dominant pelagic signal, in agreement with the very low amounts of ^{13}C-depleted archaeol and hydroxyarchaeol. Interestingly, tetraether GDGT-3 appears to typify the mud breccia crusts (11% of the total GDGTs) while it is virtually absent from crust MN12BT3. This compound is also absent from adjacent sediments, except at sites with high methane venting (Pancost et al., 2001b), and

appears to be particularly diagnostic for active AOM and the archaea involved in it. Subtle differences in GDGT abundances in the two mud breccia crusts also occur. In crust MN16BT2, GDGT-0 and GDGT-2/2′ occur at comparable abundance, whereas in the pelagic crust MN13BT4, GDGT-2/2′ is the most abundant GDGT, twice as abundant as GDGT-0. Differences in GDGT distributions have been reported for other Eastern Mediterranean mud volcano cold seeps and also attributed to variations in archaeal community structure (Pancost et al., 2001b).

PMIs are the second most abundant group of archaeal lipids. Unsaturated PMIs predominate over the saturated PMI in crust MN16BT2 from the Napoli Dome (Table 1). Adjacent sediments show the same pattern only at active sites (Pancost et al., 2000). In contrast, crust MN13BT4 contains predominantly saturated PMI (Table 1), while the pelagic crust MN12BT3 contains very low amounts of only saturated PMI. Prominent (labile) unsaturated PMIs have been interpreted as reflecting active seepage (Elvert et al., 2000), consistent with the data for the Napoli Dome, while other studies suggested that varying PMI patterns may reflect distinct archaeal communities (Stadnitskaia et al., 2005). In our samples the abundance of PMIs relative to other archaeal lipids shows some further intriguing features. In crust MN16BT2, the ratio of PMIs to archaeal diether lipids is 0.5, whereas in the other crusts, their relative abundance is much lower (ratios = 0.3, 0.1). These variations are accompanied by significant isotopic offsets: PMI and ether-bound isoprenoids show similar $\delta^{13}C$ values in crust MN16BT2, while PMI is enriched by ca. 13‰ and 40‰ compared to archaeol in the other samples (Table 1). Disparity in the relative abundances of PMIs and ether-bound lipids has been attributed to differences in community structure, because in some settings PMIs and archaeol/hydroxyarchaeol coexist, while in others either PMI or archaeol/hydroxyarchaeol is reported (e.g. Elvert et al., 2000; Hinrichs et al., 2000b; Pancost et al., 2001b; Thiel et al., 2001). Large isotopic offsets between ether bound isoprenoids and free isoprenoids, similar to those found in our samples, have also been reported (Thiel et al., 1999; Pancost et al., 2001b); they cannot be explained by biosynthetic fractionation within a single organism (usually <10‰), but instead suggest that ether-bound lipids and PMIs derive from different archaea which may not respond similarly to varying methane substrate availability.

C_{20} isoprenoids, crocetane and crocetene, show varying distributions. Sample MN16BT2 contains mainly PMIs, with crocetane/ene being 13 fold less abundant, whereas in sample MN13BT4 PMIs and crocetane/ene abundance is comparable (Table 1). These differences, similar to those reported (e.g. Hinrichs et al., 1999; Orphan et al., 2001a; Thiel et al., 2001), suggest that methanotrophic archaea producing predominantly crocetanes are distinct from those producing predominantly PMIs. Specifically, Blumenberg et al. (2004) argue that high abundances of crocetane/ene are associated with ANME-2 archaea, suggesting a predominance of that group in the Amsterdam Dome crust but not the Napoli crust, which is consistent with the 16S rRNA data obtained for our sample set (Aloisi et al., 2002; Heijs et al., 2006).

Overall, archaeal lipid distributions clearly differentiate between the two mud breccia carbonate crusts and point to the presence of distinct communities of methane-consuming archaea. Archaeal assemblages in the carbonate crust from the Napoli Dome (MN16BT2) consist of species that biosynthesize lipids with the following characteristics: (i) archaeol moderately predominating over hydroxyarchaeol, (ii) dominant *sn*-3 hydroxyarchaeol compared to the *sn*-2 isomer, (iii) very high relative abundance of PMIs compared to crocetane and (iv) abundant GDGTs. Contrary trends are observed for the carbonate crust from the Amsterdam Dome (MN13BT4); 16S rRNA gene surveys in our sample set confirmed the presence of distinct archaeal groups, with ANME-1 archaea occurring in crust MN16BT2 and ANME-2 in crust MN13BT4, along with novel *Thermoplasmales* found in both crusts (Aloisi et al., 2002; Heijs et al., 2006). Archaeal gene sequences were not found in the pelagic carbonate crust MN12BT3 (Heijs et al., 2006), a crust that contained the lowest amounts of archaeal lipids (Table 1). This may reflect the very low DNA yields obtained and/or the preferential degradation of DNA relative to lipids.

Our results are generally consistent with previous investigations associating archaeal lipid distributions to phylogenetic data (Blumenberg et al., 2004). Discrepancies occur, however, stressing that indices like the relative abundances between archaeol and hydroxyarchaeol should be used cautiously. Also, the peculiar occurrence of *sn*-3 hydroxyarchaeol in MN16BT2 is intriguing and its precise archaeal sources remain elusive.

Bacterial lipids: Bacterial lipids from (inferred) sulfate reducers show low $\delta^{13}C$ values but are enriched relative to co-occurring archaeal lipids. This is in accordance with previous investigations on archaeal and bacterial lipids as well as with direct (ion probe) $\delta^{13}C$ determinations of microbial cell aggregates (Hinrichs et al., 2000b; Pancost et al., 2001a; Orphan et al., 2001b; Pancost and Sinninghe Damsté, 2003). While SRB have been shown to oxidise saturated hydrocarbons other than methane (Heider et al., 1999), known sulfate reducers in cultivation have failed to oxidize methane (Harder, 1997) and available evidence suggests that such a process is rather unlikely. In addition, ^{13}C offsets between archaeal and bacterial biomarkers have been discussed by Hinrichs et al. (2000b) and Pancost and Sinninghe Damsté (2003). Several lines of evidence have supported the hypothesis of an autotrophic growth of sulfate reducers on methane-derived carbon pool that may variably be diluted by background (seawater) dissolved inorganic carbon (DIC). Nevertheless, alternative pathways and working hypotheses for the interaction between methane utilizing and sulfate reducing microbes are still under debate.

Like archaeal lipids, compounds from bacterial sources show remarkable variations in the sample set. Among non-isoprenoidal dialkyl glycerol diethers, inferred to derive from sulfate reducers, Series II DGDs predominate by far in crust MN16BT2, while, conversely, Series I DGDs prevail in crust MN13BT4 (Fig. 4B). In the pelagic crust MN12BT3, only Series I DGDs occur as minor components. Series I DGDs are always present in cold seep settings where ^{13}C-depleted DGDs are reported (Hinrichs et al., 2000b; Michaelis et al., 2002; Stadnitskaia et al., 2003). Although Series II DGDs have been found in some settings (e.g. other Mediterranean mud volcano sediments; Werne et al., 2002, Black Sea crusts; Stadnitskaia et al., 2005, Gulf of Mexico crusts; Pancost et al., 2005), only in the Napoli Dome carbonate crust (this study) and in a mud breccia sediment core from the Kazan Dome (in the section of active AOM; Werne et al., 2002) are the Series II DGDs predominant.

Among the *iso*- and *anteiso*-C_{15} and C_{17} fatty acids, inferred to derive from SRB, the C_{15} components are always 10–12‰ depleted in ^{13}C relative to the C_{17} components (Table 1). Intriguingly, terminally branched C_{15} units are the main moieties in Series I DGDs. Thus, it is possible that the source bacteria of Series I DGDs also serve as source(s) of *iso*/*anteiso* C_{15} fatty acids. This would explain the more negative $\delta^{13}C$ values of C_{15} fatty acids, close to the upper range of those encountered for Series I diethers. Such similarities have also been noted by Hinrichs et al. (2000b) at cold seeps in the California margin. However, the branched fatty acids probably do not derive solely from DGD producers; in the Napoli mud volcano DGDs are absent from mud breccia surface sediments, where terminally branched (^{13}C-depleted) FAs occur (Pancost et al., 2000), suggesting that DGDs and *ia*-FAs have at least partially distinct sources. Thus, the

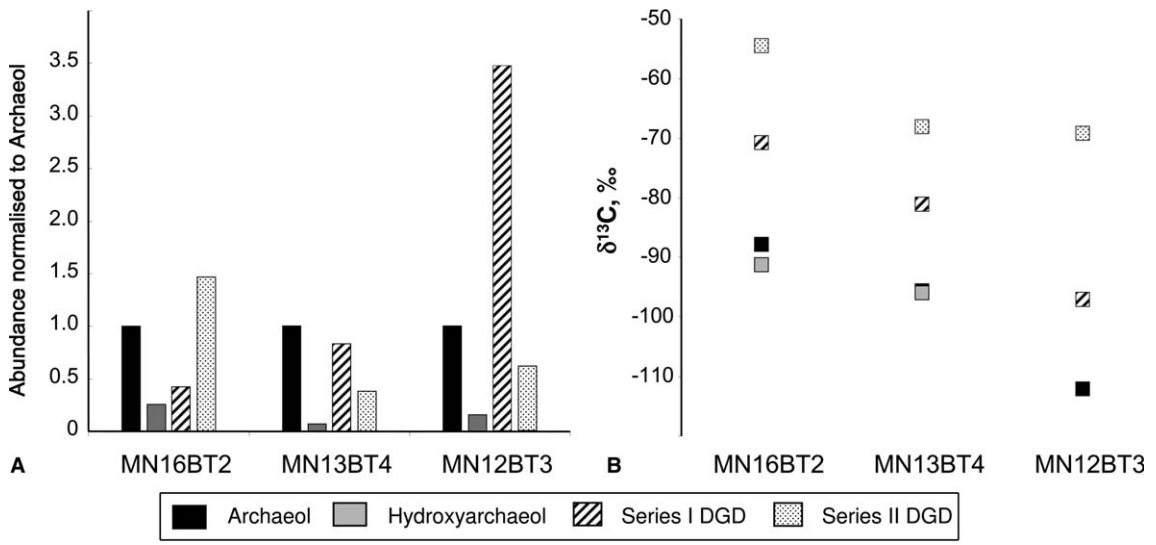

Fig. 4. Relative abundance of glycerol diethers (both isoprenoid and non-isoprenoid) (A) and their carbon isotopic composition (B) in three carbonate crust samples.

measured $\delta^{13}C$ values of branched C_{15} FAs probably result from the admixture of contributions from bacteria that also produce branched C_{17} (relatively ^{13}C-enriched) and from bacteria producing DGDs (relatively ^{13}C-depleted).

DGDs and branched FAs depleted in ^{13}C show variable relative abundances. Interestingly, inferred SRB co-occurring with dominant ANME-1 archaea (crust MN16BT2) show high concentrations of DGDs relative to FAs (DGD/brFAME = 16), while sulfate reducers co-occurring with ANME-2 archaea (crust MN13BT4) show abundances of branched FAMEs comparable to those of DGDs (DGD/brFAME = 1.3). This feature is consistent with the data reported by Blumenberg et al. (2004) for Black Sea microbial reefs. However, in that study the peculiar Series II DGD, that characterise the ANME-1 dominated crust MN16BT2, was not found. The SRB reported by Blumenberg et al. (2004) as likely producers of DGDs and branched FAMEs all cluster in the *Desulfosarcina/Desulfococcus* group, in agreement with several studies of cold seeps (Boetius et al., 2000; Orphan et al., 2001a; Michaelis et al., 2002). However, multiple bacterial groups, such as SRB other than members of the *Desulfosarcina/Desulfococcus* cluster, or bacteria not necessarily limited to classical delta-proteobacterial SRB, have been indicated to co-occur with methanotrophic archaea in various syntrophic partnerships (Orphan et al., 2002; Teske et al., 2002; Knittel et al., 2003).

The presence of ^{13}C-depleted hopanoids, indicates additional bacterial groups that utilize carbon pools affected by methanotrophy. However, the source organisms of hopanoids in our samples and in other cold seeps remain poorly constrained. In contrast with the bacterial lipids discussed above, hopanoid patterns do not distinguish among the crust samples (Table 1). Diploptene shows higher $\delta^{13}C$ values than the other hopanoids, a trend that most likely reflects contributions from various prokaryotes of diverse metabolism, including bacteria that utilize carbon pools unaffected by methanotrophy. The other hopanoids have $\delta^{13}C$ values in the range found for lipids of sulfate reducing bacteria (DGDs, branched fatty acids).

Regardless of the differences in bacterial and archaeal biomarker distributions – and inferred differences in microbial community structure – amongst the crusts, some general patterns do persist, perhaps reflecting more conservative aspects of AOM consortia. First, while absolute concentrations show large variations, the ratio of ether-bound archaeal lipids (archaeol and hydroxyarchaeol) to total hydrocarbon isoprenoids varies minimally, with values of 1.9 and 1.5 in the two mud breccia crusts (MN16BT2, MN13BT4). Isotopic and abundance data for non-isoprenoid DGDs indicate the presence of at least two distinct groups of SRB in the mud breccia crusts (Fig. 4A and B); however, the isotopic offset between Series I DGDs, Series II DGDs and archaeol is similar in all three crusts (Fig. 4B). Finally, the ratio of summed archaeal lipids (isoprenoidal DGDs and hydrocarbon isoprenoids) to the summed SRB lipids (non-isoprenoidal dialkyl glycerol diethers and *ia*-FAs) does not vary (ratios = 0.93 and 0.84). These 'conservative aspects' of the biomarker distributions suggest that, despite important variations in the microbial community mediating AOM, certain aspects of the archaeal-SRB syntrophic community remain unchanged. Different groups, regardless of their relative importance, occupy similar ecological niches (hence, conservation of isotopic relationships) and the relative sizes of archaea and bacterial communities remain the same.

4. Conclusions

Diverse archaeal and bacterial biomarkers with very low $\delta^{13}C$ values dominate the lipid composition of authigenic carbonate crusts from eastern Mediterranean mud volcanoes. Our results are in agreement with previous studies of various cold seep settings showing the involvement of consortia of archaea and SRB in the anaerobic oxidation of methane (AOM). This process releases HCO_3^- into the pore and bottom waters, favouring the precipitation of carbonates and, thus, the sequestration of methane-derived carbon. The diverse biomarker data set in the carbonate crusts displays important variance, providing further evidence for the heterogeneity of archaeal and bacterial communities at cold seeps. The consortia mediating AOM at two sites from eastern Mediterranean mud volcanoes (Napoli Dome, Amsterdam Dome) present distinctive biomarker features, pointing to the occurrence of different archaeal assemblages. This is further confirmed by available phylogenetic data. Biomarker data also suggest varying bacterial communities co-occurring with methanotrophic archaea. The reasons for the development of heterogeneous microbial communities at cold seeps are not clear, but environmental factors and primarily

patterns of methane supply should play an important role in favouring preferential growth of distinct assemblages. Our data suggest that microbial assemblages involved (directly or indirectly) in AOM may be not restricted to those currently known for archaea/SRB consortia. Consistent variations in the distributions of archaeal and bacterial lipids among various (active or past) fluid seepage sites could reflect major molecular characteristics of the microbes involved in AOM and thus help constrain the presence of distinct assemblages. However, further studies are needed to improve the use of lipids as chemotaxonomic tools for AOM-related microbial consortia. Despite the variability in microbial lipid abundances, and thus the archaeal and bacterial community structure, in the carbonate crusts, there remain some conservative relationships (archaeal vs. bacterial lipids, isotopic offsets) that reflect the overall relationships between archaea and bacteria and the nature of carbon flow between them.

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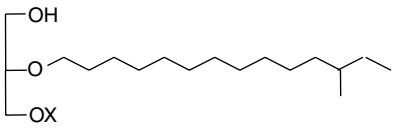
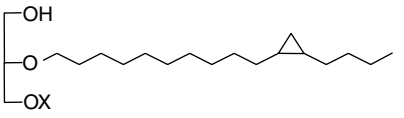
Appendix

Series I DGD



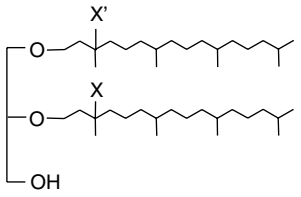
Ia: X=*i*-C_{14}, **Ib**: X=*n*-C_{14}, **Ic**: X=*n*-C_{14}, **Id**: X=*i*-C_{15}
Ie: X=*i*-C_{15}, **If**: X=*a*-C_{15}, **Ig**: X=cyclopropyl C_{16},
Ih: X=cyclopropyl C_{17}

Series II DGD



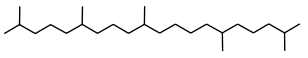
IIa: X=*n*-C_{14}, **IIb**: X=*n*-C_{15},
IIc: X=*n*-C_{16}, **IId**: X=ω-cyclohexyl C_{17}

Isoprenoidal Glycerol Diethers

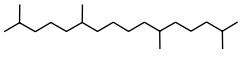


Archaeol: X = H, X' = H
***sn*-3-hydroxyarchaeol**: X = H, X' = OH
***sn*-2-hydroxyarchaeol**: X = OH, X' = H

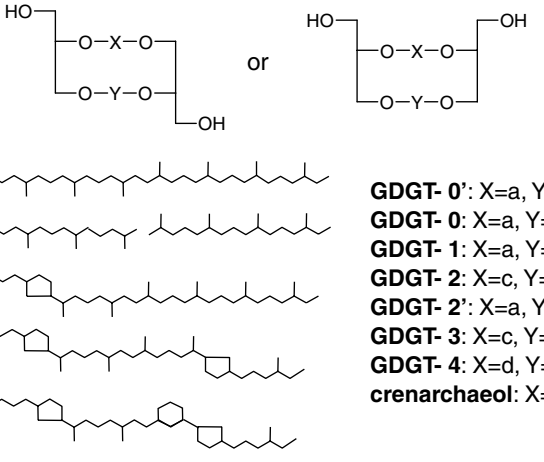
PMI



Crocetane



GDGT



GDGT- 0': X=a, Y=b
GDGT- 0: X=a, Y=a
GDGT- 1: X=a, Y=c
GDGT- 2: X=c, Y=c
GDGT- 2': X=a, Y=d
GDGT- 3: X=c, Y=d
GDGT- 4: X=d, Y=d
crenarchaeol: X=d, Y=e

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