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Archaeal lipids in Mediterranean Cold Seeps: Molecular proxies for anaerobic methane oxidation

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Abstract—We investigated the distributions and $\delta^{13}C$ values of biomarkers for Archaea associated with anaerobic methane oxidation in disparate settings throughout two Eastern Mediterranean mud dome fields. All major classes of archaeal lipids are present in the studied sediments, including isoprenoid glycerol diethers, isoprenoid glycerol dialkyl glycerol tetraethers, and irregular isoprenoid hydrocarbons. Of the compounds present, many, including a novel glycerol tetraether and *sn*-3-hydroxyarchaeol, have not been previously reported for settings in which methane oxidation is presumed to occur. Archaeal lipids are depleted in ^{13}C, indicating that the Archaea from which they derive are either directly or indirectly involved with methane consumption. The most widespread archaeal lipids are archaeol, PMI, and glycerol tetraethers, and these compounds are present at all active sites. However, archaeal lipid abundances and distributions are highly variable; ratios of crocetane, PMI, and hydroxyarchaeol relative to archaeol vary from 0 to 6.5, from 0 to 2, and from 0 to 1, respectively. These results suggest that archaeal communities differ amongst the sites examined. In addition, carbon isotopic variability amongst archaeal biomarkers in a given mud breccia can be as large as 24 ‰, suggesting that even at single sites multiple archaeal species perform or are supported by anaerobic methane oxidation. *Copyright © 2001 Elsevier Science Ltd*

INTRODUCTION

Recent work has highlighted the potential climatic impact of methane (e.g., Dickens et al., 1995; Henriet and Meinert, 1998; Petit et al., 1999), prompting considerable interest in the controls on methane production and consumption. In marine sediments, anaerobic methane oxidation is the dominant pathway for methane consumption (Blair and Aller, 1995; Borowski et al., 1996; Burns, 1998; Iverson and Jørgensen, 1985; Reeburgh, 1980), and consequently, the flux of methane to the atmosphere from marine sediments is small compared to other sources (Reeburgh, 1996). However, it is unclear whether this has always been true or if this process can be disrupted by future climate change, and thus, it is important to understand the mechanism of anaerobic methane oxidation. However, the inability to culture anaerobic methane-oxidizing organisms combined with the apparently low energy yield of this reaction (Hoehler et al., 1994) has prevented previous workers from successfully identifying the responsible organisms.

Multiple lines of evidence show that in such diverse settings as Cape Lookout Bight (Hoehler et al., 1994), Hydrate Ridge (Elvert et al., 1999), the California Margin (Hinrichs et al., 1999), and Mediterranean mud domes (Pancost et al., 2000), methane is consumed anaerobically in a microbial process mediated by Archaea. This represents a profound step forward in addressing a problem that has perplexed geochemists and microbiologists for several decades. However, the specific Archaea involved in anaerobic methane oxidation remain uncultured and unknown. Hoehler et al. (1994) argued on the basis of inhibition studies that the Archaea are methanogens that operate in reverse under certain conditions. In general, Elvert et al. (1999) agreed with this idea, and Pancost et al. (2000) and Thiel et al. (1999) provided further support for the Hoehler et al. (1994) proposal by showing that methane-consuming Archaea co-existed with sulfate reducers and other bacteria in cold seep sediments. In contrast, phylogenetic analyses revealed that Archaea present in California Margin sediments consist of an unidentified *Methanosarcina* species and apparently two new orders of Archaea (Hinrichs et al., 1999).

One manner in which to gain further insight into the organisms responsible for anaerobic methane oxidation is by identifying biomarkers that are diagnostic for certain species. This is particularly useful when coupled to compound-specific carbon isotope analyses. Because methane tends to be highly depleted in ^{13}C, the carbon isotopic composition of archaeal lipids can be used to examine carbon flow in such communities and further evaluate the ecology of the Archaea from which such lipids are derived (Hinrichs et al., 1999; Elvert et al., 1999; Pancost et al., 2000). Archaea are distinguished from the Bacteria and Eukarya partly because they contain ether-bound membrane lipids with isoprenoidal carbon skeletons rather than ester-linked alkyl lipids (Woese et al., 1990). Such isoprenoid ether lipids or their degradation products are present in diverse environments (Teixidor et al., 1993; Pauly and van Fleet, 1986; Hoefs et al., 1997; DeLong et al., 1998; Schouten et al., 1998) and been shown to be useful in studying archaeal communities in methane seeps (Hinrichs et al., 1999; Pancost et al., 2000). Additional diagnostic compounds for the Archaea include pentamethylicosane (PMI; Brassell et al., 1981; Schouten et al., 1997) and probably crocetane (Elvert et al., 1999) and the corresponding unsaturated components (Sinninghe Damste et

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Table 1. Locations and methods used for collection samples.

Dome	Site	Sampling device[a]	Latitude	Longitude	Depth[b]
Olimpi Field					
Napoli	Seep	Bordeaux Core	33°43.545E	24°41.041N	1945
	Breccia	Bordeaux Core	33°43.543E	24°41.041N	1945
	Brine	Biological Box	33°43.565E	24°41.164N	1946
	Mat	Biological Box	33°43.565E	24°41.164N	1946
	Crust	Sediment Grab	33°43.564E	24°41.166N	1946
Milano	Seep	Bordeaux Core	33°44.059E	24°46.695N	1958
	Breccia	Bordeaux Core	33°44.062E	24°46.660N	1956
Anaximander Field					
Amsterdam	Seep/Breccia?	Bordeaux Core	35°19.897E	24°16.745N	2021

[a] A detailed description of sampling is given in the text.
[b] In meters below sea surface.

al., 1998). Recent advances in the use of high performance liquid chromatography–mass spectrometry (Hopmans et al., 2000) now allow non-GC amenable tetraethers to also be examined.

Initial work suggests that ether lipid distributions and possible species compositions of cold seep Archaea can be highly variable. For example, Hinrichs et al. (1999) and Pancost et al. (2000) observed abundant hydroxyarchaeol in some samples, but Elvert et al. (1999) and Thiel et al. (1999) did not. Based on comparison of their data with that of Elvert et al. (1999), Hinrichs et al. (1999) suggested that at least two archaeal assemblages could be responsible for anaerobic methane oxidation in different settings. Understanding the underlying basis of this variability is fundamental to the development of molecular proxies for identifying these organisms in ancient and modern sediments.

Mud domes of the Eastern Mediterranean Sea are ideal settings in which to study the diversity and heterogeneity of lipids generated by methane-oxidizing Archaea. Multiple lines of evidence suggest that methane is oxidized anaerobically (de Lange and Brumsack, 1998) in a process mediated by a consortium of prokaryotes, of which Archaea are a significant component (Pancost et al., 2000). Based on molecular and isotopic data (Pancost et al., 2000), we previously concluded that the Archaea in these sediments are methanogens operating in reverse and co-existing with sulfate-reducing bacteria in a consortium similar to that described by Hoehler et al. (1994). Mud dome sediments are highly heterogeneous, containing variable quantities of methane, brines, and biologic communities. Because samples were collected via submersible, several unique settings could be examined for potential variations in the content of archaeal lipids. Here we determine the diversity and compound-specific $\delta^{13}C$ values for a wide variety of archaeal lipids in these settings to ascertain whether multiple Archaea species are present at some sites and whether archaeal assemblages are the same at all sites. To explore the breadth of archaeal lipids present in our samples, we do not discuss hopanoids, fatty acids and other lipids derived from bacteria even though it is likely that such organisms also play important roles in the microbial methane oxidizing community (Pancost et al., 2000).

2. GEOLOGICAL SETTING

Mud domes in the Mediterranean Sea are created by tectonic compression resulting in extrusion of methane-rich mudflows (mud breccias) along apparently regional-scale fault planes (Limonov et al., 1996; Woodside et al., 1998). These mud breccias are rich in methane (Emeis et al., 1996), which is apparently oxidized in the upper 1m of the mud breccia profile (Haese et al., in preparation). Samples (Table 1) were collected from surface sediments (upper 30 cm) of several mud flows in the Olimpi and Anaximander mud dome fields using the submersible *Nautile* during the 1998 cruising of the R/V *Nadir*. Sampling via submersible provided an opportunity to obtain sediments from a variety of highly specific settings (Table 1).

2.1. Mud Breccias

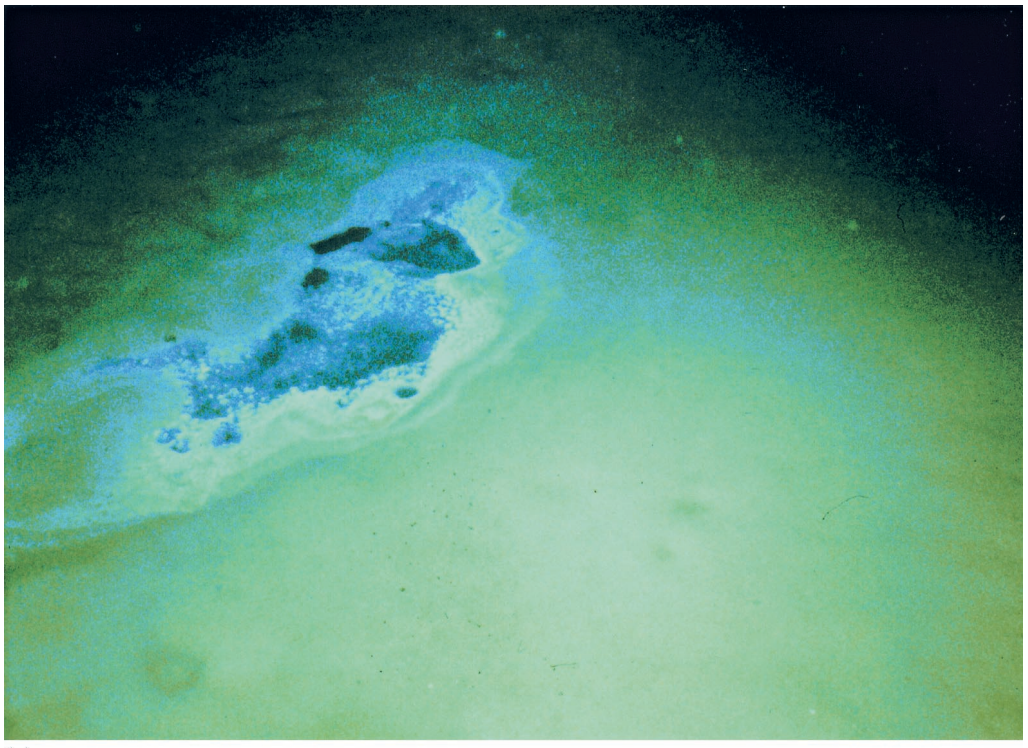
The dominant lithology of all Mediterranean mud domes is mud breccia—a matrix of sediment containing diverse lithic fragments that can be as large as 1 m in diameter. We collected mud breccia profiles from Napoli and Milano mud domes using a bordeaux coring device employed by the arm of the *Nautile*.

2.2. Cold Seeps

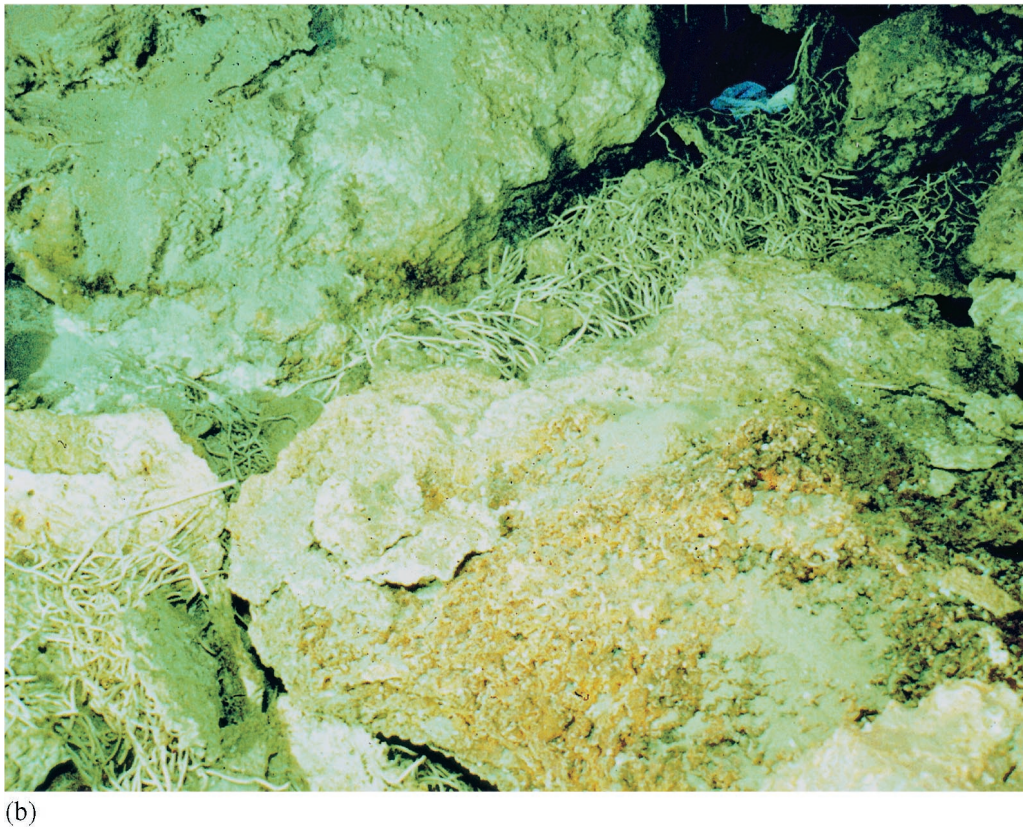
Apparent methane seeps (Fig. 1a,b) are scattered across all of the mud domes explored with the *Nautile*. Using a bordeaux core, seeps were sampled on Milano, Napoli, and Amsterdam mud domes. On Napoli, the cold seep and mud breccia samples were only 1 m apart. The sediment associated with the cold seeps is much more fluid than the surrounding mud breccia, inhibiting the collection of sediment profiles, and the data presented here is for bulk mixed surface (upper 2–8 cm) sediment. Visual evidence for fluid expulsion and bottom-water methane concentrations (Charlou et al., unpublished data) indicate that seeps are the most active sites of methane release.

2.3. Brine Pool

Brine expulsion is clearly associated with mud volcanism in the Olimpi area and brine pools and streams, apparently originating from seeps, are present across the summit of Napoli mud dome. We used the arm of the Nadir to grab a bulk



(a)



(b)

Fig. 1. Photographs taken by the onboard camera of the *Nautile* during the 1998 Medinaut expedition and sailing of the R/V *Nadir*. (A) Cold seep on Napoli mud dome (Olimpi mud dome field); white material could derive from salts precipitated from brines excreted from the seep and the black material is a microbial mat similar to the one described in this work. (B) Seep area on Amsterdam mud dome. Note that the seep is surrounded by extensive and thick (up to 20 cm) carbonate crusts and abundant tube worms.

sediment sample from beneath one such brine pool. This sediment was transported to the surface in a sealed biologic box.

2.4. Microbial Mats

Black, gelatinous, inferred microbial mats were observed on top of many methane seeps and floating in the aforementioned biologic box containing brine sediments. Here we discuss analyses performed on mat material recovered from the biologic box.

2.5. Carbonate Crusts

Carbonate crusts (Fig. 1b) are ubiquitous on the Mediterranean mud domes and likely formed by oxidation of methane to carbon dioxide and the consequential enhancement of bottom water super-saturation with respect to calcium or, less commonly, magnesium carbonate. Here we report the analyses of lipids isolated from a carbonate crust collected from the summit of Napoli mud dome (near the area from which the cold seep and breccia samples were collected).

3. METHODS

3.1. Extraction and Separation

After sample collection, samples were stored at −20° until analyzed. Samples (8–25 g sediment) were freeze-dried and extracted via sonication in a sequence of solvent mixtures with increasing dichloromethane (DCM): methanol (MeOH) ratios (0:1 three times, 1:1 three times, 1:0 three times; solvent volume was ca. three times the sediment volume). For two samples (Amsterdam seep and Milano seep), 100 g of sediment were extracted for 24 h using a Soxhlet apparatus and a DCM:MeOH mixture (7.5:1 v:v). Elemental sulfur was removed from the total extracts by adding ca. 100 mg of activated copper and stirring the sample for 4 h. Aliquots (65%) of total extracts were separated into acetone soluble and insoluble fractions (Jahnke et al., 1995). The soluble component was further separated on an alumina column (activated for 2 h at 150°C; 3–30 g alumina depending on sample size) into apolar and polar fractions using, three times the column volume of hexane:DCM (9:1; v:v) and methanol as the eluents, respectively. In the polar fraction, fatty acids were methylated by refluxing at 60°C for 5 min in BF_3 (in MeOH), and alcohols were converted to trimethylsilyl-derivatives using 25 μL each of bis(trimethylsilyl)trifluoracetamide (BSTFA) and pyridine heated at 60°C for 30 min.

To confirm identification and assist in compound-specific carbon isotope analyses, apolar fractions containing unsaturated PMIs were hydrogenated by stirring the samples for 24 h in ethyl acetate containing 100 mg of platinum charged with H_2 and 100 μL of acetic acid.

To facilitate determination of tetraether structures and carbon isotopic compositions, samples were treated with HI to cleave ether bonds. Polar fractions were refluxed in 57% HI/$LiAlH_4$ (in H_20 by weight) for 1 h and generated alkyliodides were reduced to hydrocarbons with $LiAlH_4$.

3.2. Analysis of Biomarkers

Apolar and polar fractions and apolar compounds released upon HI treatment were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) for identification. 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 to 800 and a cycle time of 1.8 s (resolution 1000). A fused silica CP-Sil 5 capillary column (25 m × 0.32 mm, d_f = 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. Compound identifications were based on mass spectra and retention times. The structures of *sn*-2- and *sn*-3-hydroxyarchaeol were confirmed by comparison to standards isolated from methanogens (Hinrichs et al., 2000; Hinrichs, personal communication). Abundances of compounds were determined by GC using a flame ionization detector and the same run conditions as described above. Quantification was based on comparison of peak areas to the internal standard (2,3-dimethyl-5-(1,1-d_2-hexadecyl)thiophene) added to the sample after column chromatography.

3.3. Isotope-Ratio-Monitoring Gas Chromatography-Mass Spectrometry (IRM-GC-MS)

Irm-GC-MS was performed on a Finnigan Delta C and used to determine compound-specific $\delta^{13}C$ values. The GC conditions are the same as those used during GC-MS analyses. $\delta^{13}C$ values are expressed against VPDB, have been corrected for the addition of carbon during derivatization, and have an error of less than ±1.0‰ unless otherwise noted (based on analytical accuracy and precision of measurements of co-injected standards—C_{20} and C_{24} perdeuterated *n*-alkanes—and considering the probable influence of co-elution in some cases).

3.4. High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

To determine the distribution of intact diglycerol tetraethers, samples were examined by HPLC-MS using a recently developed method specific for such compounds. Run conditions and a full description of methods has been previously published by Hopmans et al. (2000).

4. RESULTS AND DISCUSSION

4.1. Indigenous Biomarkers

To evaluate biomarkers generated by Archaea thriving in mud dome sediments, we must be able to distinguish them from lipids indigenous to the extruded mud breccias. To do this we evaluated surface sediment samples from a Milano mud breccia where anaerobic methane oxidation appears to be minimal. Biomarkers in these samples include steroids, hopanoids, and fatty acids typical of pelagic inputs. However, with the exception of known pelagic crenarchaeal tetraethers discussed below, archaeal biomarkers are absent. Thus, even though methanogens could have present in the subsurface and account for the high methane content of mud breccias, their lipid signatures are no longer present and we interpret the majority of archaeal lipids as deriving from organisms that grew after deposition of the mud flows.

4.2. Occurrence of Glycerol Diethers

The most abundant diether lipid in all samples is archaeol (bis-*O*-phytanylglyceroldiether, I; see Appendix for structures) (Table 2). However, a variety of other glycerol diethers, including hydroxyarchaeol (II), are also present in many samples (Fig. 2). We also observed two distinct series of non-isoprenoidal glycerol diethers, but these are likely derived from bacteria rather than Archaea and will be discussed in a subsequent publication.

4.2.1. Archaeol

Archaeol (I) has been identified in halophiles, thermophiles, and methanogens and is the most common and ubiquitous of the archaeal diethers (see Koga et al., 1998). Similarly, archaeol is present in all but four of our samples and at many sites is the most abundant GC-amenable lipid (Table 2). Highest concentrations of archaeol occur in a fragment of a microbial

Table 2. Abundances (μg/g of dry sediment) of archaeal biomarkers in Mediterranean mud dome sediments.

			Hydroxyarchaeol		Unsat.	PMI		Crocetane		Biphytanes (Released with HI)			
Site	Depth (cm)	Archaeol I[a]	*sn*-3- IIa	*sn*-2- IIb	Archaeol III	Saturated XXIV	Unsat. XXV[b]	Saturated XXVI	Croc: 1 XXVII	VIII	IX	X	XI
Napoli Mud Breccia	0–2	0.4[c]	—[d]	—	—	0.7	—	—	—	n.d.[e]	n.d.	n.d.	n.d.
	2–5	0.6[c]	—	0.1	—	0.6	—	—	—	n.d.	n.d.	n.d.	n.d.
	10–12	0.8[c]	—	0.1	—	1.1	—	—	—	n.d.	n.d.	n.d.	n.d.
	17–20	0.9[c]	—	0.5	—	1.3	—	—	—	n.d.	n.d.	n.d.	n.d.
Napoli Seep	s[f]	17.9[c]	12.9	3.8	2.1	3.8	4.0	12.4	1.4	n.d.	n.d.	n.d.	n.d.
Napoli Crust	s	2.5[c]	—	0.1	—	0.5	—	1.7	—	n.d.	n.d.	n.d.	n.d.
Napoli Brine Pool	s	14.0[c]	—	10.2	3.9	2.4	2.7	14.3	2.9	n.d.	n.d.	n.d.	n.d.
Napoli Mat	s	52.9[c]	—	0.0	17.9	21.0	—	55.4	—	n.d.	n.d.	n.d.	n.d.
Milano Breccia	0–4	—	—	—	—	—	—	—	—	n.d.	n.d.	n.d.	n.d.
	5–9	Tr	—	—	—	—	—	—	—	n.d.	n.d.	n.d.	n.d.
	24–27	0.4[c]	—	—	—	—	—	—	—	n.d.	n.d.	n.d.	n.d.
Milano Seep	s	8.2[c]	—	0.5	0.1	1.7	16.0	2.0	—	1.1	0.3	0.2	0.1
Amsterdam Seep	s	6.9[c]	2.7	4.3	1.1	2.8	6.3	2.4	—	18.3	15.9	16.2	9.3

[a] Roman numerals refer to compounds identified in text and Appendix.
[b] The abundance of the sum of all unsaturated PMI compounds.
[c] Abundances of these compounds were previously reported in Pancost et al. (2000).
[d] Not detected in our samples.
[e] Not determined.
[f] s = Surface sediments.

mat recovered from a brine pool (over 50 μg/g dry sediment), but high concentrations are also present at cold seeps on Napoli (18 μg/g), Amsterdam (6.9 μg/g) and Milano (8.2 μg/g) mud domes. The carbon isotopic composition of archaeol is highly variable in these settings, ranging from −20‰ in shallow sediments on Napoli dome to −96‰ at a seep on Amsterdam mud dome (Table 3).

Archaeol concentrations were also determined for two mud breccia profiles (Pancost et al., 2000). In a profile from Napoli mud dome, archaeol concentrations increase from 0.4 μg/g of dry sediment in surface sediments to 0.9 μg/g in sediments at 17 to 20 cm depth (Fig. 3) with a concomitant decrease in $\delta^{13}C$ value from −20‰ to −40‰. In a profile developed for Milano mud dome, archaeol is absent in samples from 0 to 4 and 5 to 9 cm, but is present (0.4 μg/g) in the deepest sample from 24 to 27 cm.

4.2.2. Hydroxyarchaeol

Hydroxyarchaeol (II) has the same core structure as archaeol but also contains an additional hydroxy group on the third carbon of the phytanyl moiety ether-linked to either the third (IIa; *sn*-3-hydroxyarchaeol) or second (IIb; *sn*-2-hydroxyarchaeol) glycerol carbon. The two hydroxyarchaeol isomers in our samples have similar mass spectra, characterized by predominant peaks at *m/z* 143, 341, and 517 (Hinrichs et al., 2000). These likely represent the *sn*-2 and *sn*-3 isomers of hydroxyarchaeol. Indeed, analyses of authentic standards reveals that such fragmentation is expected for both the *sn*-2 (Hinrichs et al., 2000) and *sn*-3 (Hinrichs, pers. commun.) isomer. Based on subtle differences in the fragmentation of the authentic standards and a comparison of retention times to compounds in *M. mazeii*, a methanogen known to contain predominantly the *sn*-2 isomer, it appears that the later-eluting isomer is *sn*-3-hydroxyarchaeol.

Hydroxyarchaeol has been observed almost exclusively in cultures of methanogenic Archaea of the orders *Methanococcales* and *Methanosarcinales*. Exceptions include the methanogens *Methanobacteriaceae methanosphaera* (Koga et al., 1998) and *Methanobrevibacter smithii* (Sprott et al., 1999), the halophile genus *Natronobacterium* (Upasani et al., 1994), and some thermoacidophiles, albeit in very low quantities (Sprott et al., 1997).

Hydroxyarchaeol is present in most samples but is always less abundant than archaeol and there is no relationship between their concentrations (Fig. 4a). For example, hydroxyarchaeol is only slightly less abundant than archaeol in the Napoli seep sample, but in other samples where archaeol is abundant, hydroxyarchaeol is present in low abundances or absent (Napoli microbial mat and carbonate crust, Milano seep). This variation in hydroxyarchaeol abundances relative to archaeol is particularly clear in the mud breccia profile from Napoli mud dome (Fig. 3), in which the ratio of hydroxyarchaeol to archaeol increases from 0 to 0.5 between the 0 to 2 cm and 17 to 20 cm samples.

Curiously, the relative abundances of the *sn*-2- and *sn*-3-hydroxyarcheols are also highly variable with the inferred *sn*-2-hydroxyarchaeol predominating in all samples except for the Napoli seep. *Sn*-2-hydroxyarchaeol predominates in methanogens of the order *Methanosarcinales* and, consistent with this, Hinrichs et al. (1999) found *sn*-2-hydroxyarchaeol in the same samples for which phylogenetic analyses revealed the presence of novel *Methanosarcina* species. In contrast, *sn*-3-hydroxyarchaeol has not been observed in any other natural settings and appears to be much less common. Organisms known to contain *sn*-3-hydroxyarchaeol include *Methanococcus voltae* (Sprott et al., 1993) and *Methanolobus bombayensis* (Schouten, unpublished results), both of which also produce

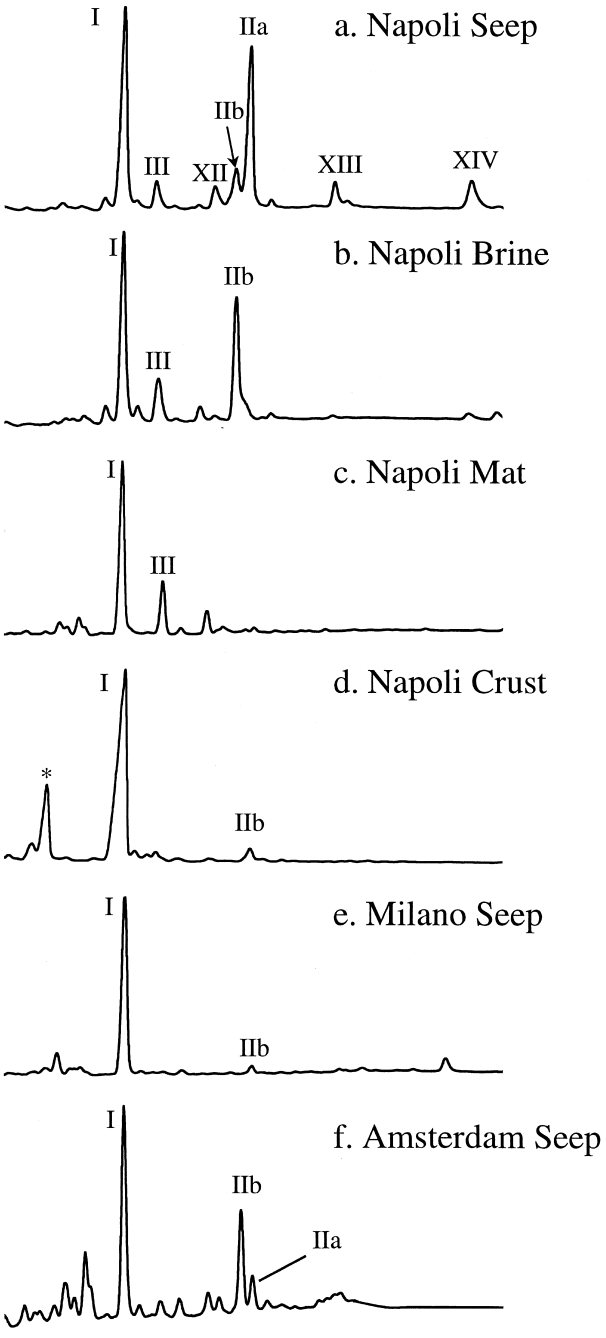


Fig. 2. Partial gas chromatograms of the derivatized (trimethylsilylated alcohols and methylated acids) polar fractions isolated from six sites on the Napoli, Milano, and Amsterdam mud domes. Roman numerals denote structures shown in the Appendix.

sn-2-hydroxyarchaeol, and *Methanosaeta concilii* (Ferrante et al., 1988) which produces predominantly the *sn*-3-hydroxyarchaeol. Thus, the presence of *sn*-3-hydroxyarchaeol in several of our samples suggests that methanogenic Archaea distinct from *Methanosarcina* species are present.

The carbon isotopic compositions of hydroxyarchaeol (two isomers integrated as a single peak) range from −57‰ to −105‰ and covary with those of archaeol (Fig. 5a). However, there are significant deviations from the simple 1:1 relationship. In some samples, such as the Napoli seep and crust, hydroxyarchaeol and archaeol $\delta^{13}C$ values are the same, suggesting a single source for both compounds. In contrast, in the deepest Napoli mud breccia sample and the brine pool, hydroxyarcheaol is depleted by 15‰ and 23‰, respectively, relative to archaeol.

Previous workers (Nichols et al., 1993; Koga et al., 1993) have commented on the relatively labile nature of hydroxyarchaeol. Specifically, Nichols et al. (1993) reported that loss of the hydroxy group during mild acidic treatments could occur and result in the formation of a monounsaturated archaeol (III), characterized by an enhanced *m/z* 445 fragment. Indeed, this compound is present in several samples. In most cases, it is less abundant than hydroxyarchaeol, but in some samples (e.g., microbial mat) it is significantly more abundant than hydroxyarcheaol. Because there appears to be no consistent relationship between the abundance of these two compounds, we suggest that the monounsaturated archaeol in our samples is not an analytical artifact but is present as such in the sediments. Whether or not it was initially present as hydroxyarchaeol is unclear. However, there is no evidence from abundant culture experiments that the monounsaturated archaeol will be directly biosynthesized. The only previously reported unsaturated diether is bis-*O*-geranylglyceroldiether (IV; *Sulfolobus solfataricus*, De Rosa et al., 1983; *Methanopyrus kandleri*, Sprott et al., 1997), which is significantly different than the compound observed here. Moreover, in the brine sample, $\delta^{13}C$ values of hydroxyarchaeol and monounsaturated archaeol are similar (−87 and −82‰, respectively) and depleted relative to archaeol (−63‰), suggesting a common origin. Whether sedimentary degradation of hydroxyarcheaeol can explain the presence of monounsaturated archaeol in all samples—especially the microbial mat—is unclear.

In addition, both 1-*O*-phytanyl glycerolether (V) and 2-*O*-phytanyl glycerolether (VI) were tentatively identified in some samples. 1 to 0-phytanyl glycerolether has been previously reported in the Archaea *Methanthermus fervidus* and *Methanosarcina barkeri* (see review by De Rosa and Gambacorta, 1988). However, Koga et al. (1993) showed that mild acid treatment could result in loss of the hydroxy-bearing phytanyl chain, and argued that this could be the source of the monoether in *M. barkeri*. If such degradation can occur under natural conditions that would provide an explanation for the presence of both compounds in some of our samples. 1-*O*-phytanyl glycerolether (characterized by a pronounced *m/z* 205 fragment; Liefkens et al., 1979) results from loss of the *sn*-2 chain and thus could be derived from *sn*-2-hydroxyarchaeol. Similarly, 2-*O*-phytanyl glycerolether (characterized by a pronounced *m/z* 218 fragment; Liefkens et al., 1979) could be derived from *sn*-3-hydroxyarcheol. The fact that these monoethers are both depleted in ^{13}C (microbial mat sample; −87‰) is consistent with an origin from hydroxyarchaeol. In the Napoli seep sample, the 2-*O*-phytanyl glycerolether is about four times more abundant than the 1-*O*-phytanyl glycerolether, consistent with the observation that the *sn*-3-hydroxyarchaeol is the more abundant isomer at that site.

Table 3. $\delta^{13}C$ values of archaeal biomarkers in Mediterranean mud dome sediments

Site	Depth (cm)	Archaeol I[a]	Hydroxy-archaeol IIa + IIb	PMI XXIV	ΣPMI XXV[b]	Croc XXVI	Croc:1 XXVII	Biphytane (Released with HI) VIII	IX	X	XI
Napoli Mud Breccia	0–2	—	—	−43.6[c]	—	—	—	—	—	—	—
	2–5	−20.2[c]	—	−34.2[c]	—	—	—	—	—	—	—
	10–12	−32.7[c]	—	−43.3[c]	—	—	—	—	—	—	—
	17–20	−41.3[c]	−57.1[c]	−52.0[c]	—	—	—	—	—	—	—
Napoli Seep	s[d]	−76.6[c]	−77.1	−64.8[c]	−67.1	−53.9[e]	−63.5	—	−77.2[f]	−75.2[f]	—
Napoli Crust	s	−87.9[c]	−87.7	−84.3[c]	—	−80.8	—	—	—	—	—
Napoli Brine Pool	s	−63.1[c]	−86.4	−62.2[c]	—	−56.7[e]	−72.5	—	—	—	—
Napoli Mat	s	−84.1[c]	−87.2	−76.2[c]	—	−88.7	—	—	—	—	—
									—	—	—
Milano Breccia	24–27	−57.2[c]	—	—	—	—	—	—	—	—	—
Milano Seep	s	−81.1[c]	—	−66.9[c]	−92.6	−44.5[e]	—	−75	−78.2	−55.0	—
Amsterdam Seep	s	−95.8[c]	−105.1	−91.4[c]	−107	−51.1[e]	—	−61.7	−77.1	−53.6	−19.3

[a] Roman numerals refer to compounds identified in text and appendix.
[b] The $\delta^{13}C$ values are for the sum of all saturated and unsaturated PMI compounds.
[c] $\delta^{13}C$ values of these compounds were previously reported in Pancost et al. (2000).
[d] s = Surface sediments
[e] Crocetane values include co-eluting phytane.
[f] Biphytane diols.

4.3. Occurrence of Gycerol Tetraethers and Biphytanediols

Like diethers, glycerol tetraethers (glycerol dialkyl glycerol tetraether; GDGT) are diagnostic for and ubiquitous amongst the Archaea. Nonetheless, the distributions of GDGTs appear to vary significantly amongst different Archaea (De Rosa et al., 1986) and could be useful for elucidating species variability in mud flows. The high molecular weight of these compounds precludes analyses by conventional lipid extraction and subsequent GC-MS. Instead we analyzed total lipid extracts using HPLC-MS to determine distributions of GDGTs (Hopmans et al., 2000). For selected samples, we used HI/$LiAlH_4$ treatment of polar lipid fractions to cleave ether bonds (cf. Schouten et al., 1998), releasing GC-amenable C_{40} isoprenoid chains so that carbon isotopes and GDGT abundances could be determined.

HPLC-MS analyses reveal that GDGTs are present in all of the samples analyzed. All seep, crust, and brine samples contain at least three GDGTs, characterized by $[M+H]^+$ values of *m/z* 1302, 1300, and 1298 (Fig. 6). In addition, some samples contain GDGTs characterized by $[M+H]^+$ values of *m/z* 1296, 1294, and 1292. A limitation of the HPLC-MS technique is that the primary products of the chemical ionization are the protonated molecule and ions directly related to fragmentation of the glycerol moiety such as loss of a hydroxyl group (cf. Hopmans et al., 2000). Thus, information regarding the structure of the tetraether carbon chains is limited. Compound identifications, then, are based on the following information: (i) the molecular ion which reveals the number but not the location of cyclic groups in the tetraether; (ii) comparison of retention times to those of GDGTs in *Sulfolobus solfataricus* for which the dominant tetraethers are known (De Rosa et al., 1986); and (iii) HI/$LiAlH_4$ treatment, which reveals the total distribution of the tetraether C_{40} isoprenoid moieties. HI/$LiALH_4$ treatment on four samples yielded various abundances of phytane (VII; largely from archaeol and hydroxyarchaeol), acyclic (VIII), monocyclic (IX), and bicyclic (X) biphytanes. The only tricyclic biphytane (XI) generated is directly related to a GDGT (XII) derived from non-extremeophilic crenarchaeota, which can be directly assigned to the $[M+H]^+$ *m/z* 1292 peak and has been isolated and rigorously characterized (Schouten et al., 2000). Thus, we conclude that the peaks in the mass chromatograms of $[M+H]^+$ *m/z* 1302, 1300, 1296, and 1294 are almost certainly compounds XIII, XIV, XVII, and XVIII, respectively. We are confident of these interpretations because they represent the only possible combination of carbon chains with zero, one, or two cyclic groups that could generate the respective *m/z* signals. The $[M+H]^+$ *m/z* 1298 signal at 23 min is less clear because it could be generated by both XV and XVI; based on the relatively high abundances of the bicylic component in our HI treated samples, it seems likely that this peak is comprised of a mixture of both compounds.

In most of our samples (Fig. 6), the predominant GDGTs contain 0 to 2 cyclopentane rings (i.e., XIII, XIV, and XV/XVI). Nonetheless, there are some differences in tetraether distributions. For example, XVII (monocyclic and bicyclic chains) is relatively abundant in the Napoli seep and crust and the Amsterdam seep but is present in only low abundances in the other samples (Fig. 3). The Napoli seep sample also contains minor components of unique glycerol tetraethers, XIX and XX. The former compound contains a biphytane chain ether-bound to both glycerol groups and two phytane chains each ether-bound to a single glycerol. This compound has previously been observed only in cultures of *Sulfolobus solfataricus* (De Rosa et al., 1986), a thermophilic chemoautotrophic archaeon that is likely not present in these settings. The second, previously unreported compound has a similar structure but also contains a single cyclic group in the biphytane chain (Hopmans et al., 2000).

Most samples also have some background component of pelagic Archaea ether lipids represented by GDGTs XII and, to a lesser degree, XIII. XIII is ubiquitous, having been reported in a wide variety of organisms including methanogens and

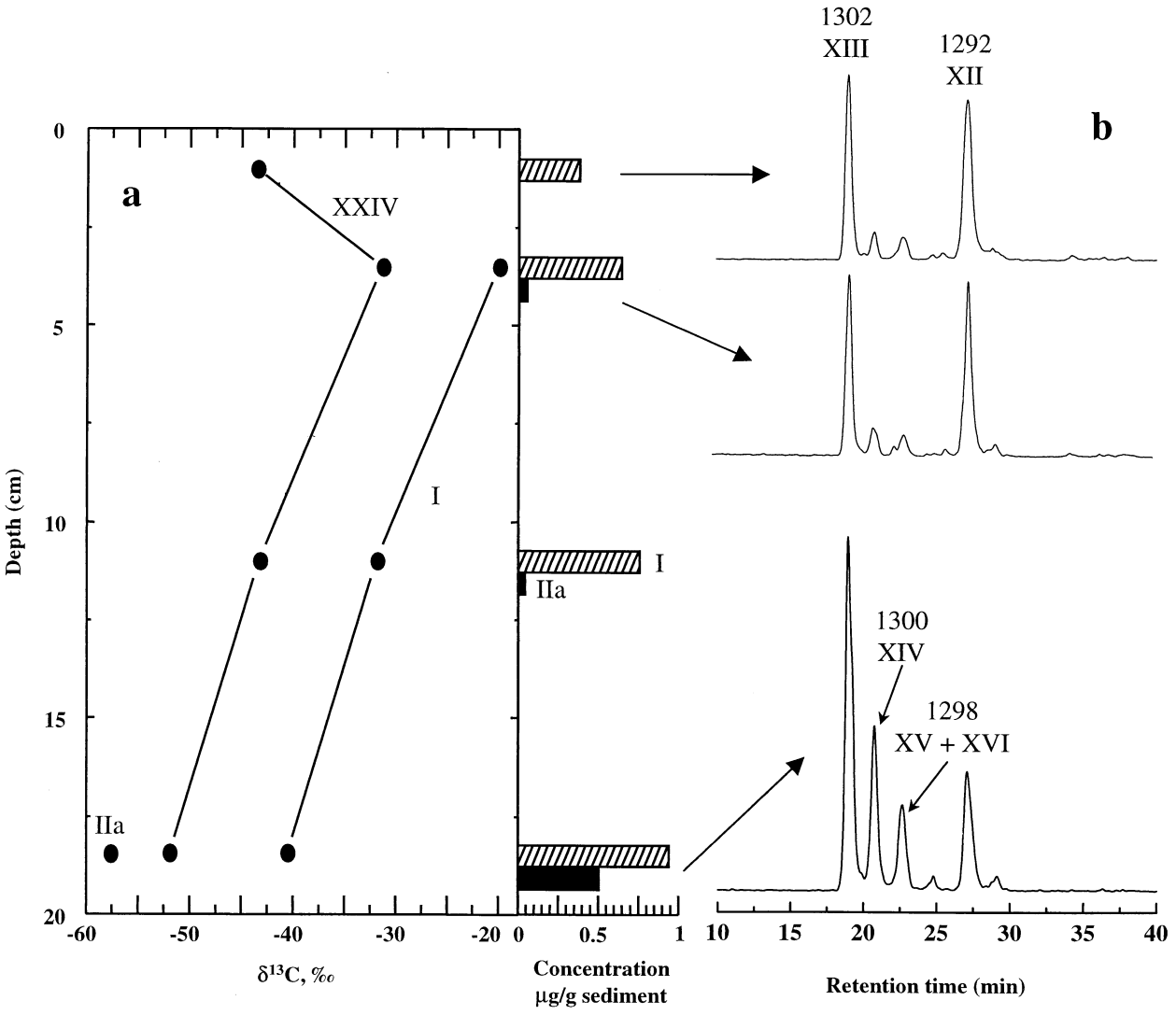


Fig. 3. Distribution and of archaeal lipid in a Napoli mud breccia depth profile (partly after Pancost et al., 2000). (a) Profiles show decreases in PMI (XXIV) and archaeol (I) 104 $\delta^{13}C$ values with depth. Bar plots show abundancies of archaeol (I) and *sn*-3-hydroxyarchaeol (IIa). (b) Base peak chromatograms of the acetone-soluble fractions of the extracts of samples representing a vertical profile through a Napoli mud breccia show the distribution of GDGTs. The numbers above each peak denote the dominant $[M22H]^{22}$ ion and the roman numerals refer to structures in the Appendix.

thermophiles (Kates, 1993). Thus, while XII is derived from strictly pelagic crenarchaeota, XIII likely derives from both pelagic crenarchaeota and Archaea present in cold seeps—a conclusion supported by carbon isotopic data discussed below. The contribution of GDGTs from multiple sources is illustrated by the mud breccia profile (Fig. 3). At shallow depths, the GDGT distribution is dominated by XII and XIII and is similar to that observed in marine water and sediment samples from diverse locations (Schouten et al., 2000). Thus, it is likely that similar pelagic sources contributed these compounds to Mediterranean sediments, which were subsequently extruded as part of the mud breccia. Deeper in the mud breccia profile, corresponding to highest glycerol diether concentrations, compounds XIII, XIV, and XV/XVI ($[M+H]^+ = 1298$) are enhanced indicating additional archaeal inputs.

Further insights into GDGT sources are provided by the $\delta^{13}C$ values of biphytanes released by $HI/LiAlH_4$ (Table 2). The carbon isotopic compositions of XI derived from GDGT XII is highly enriched in ^{13}C relative to other biphytanes and are consistent with those observed in a wide variety of other settings, in which a pelagic archaeal origin has been inferred (Hoefs et al., 1997; DeLong et al., 1998; Schouten et al., 1998). The $\delta^{13}C$ values of X are intermediate between those of XI and other biphytanes, consistent with the interpretation that X occurs as both a constituent of GDGTs generated by methane-consuming seep organisms (XVI and XVII) and the aforementioned pelagic GDGT (XII). $\delta^{13}C$ values for the acyclic biphytane VIII are intermediate between those of IX, the most depleted biphytane in these samples, and XI, also consistent with a contribution from both methane-consuming and pelagic sources. The compound for which no pelagic contributions are inferred is the monocyclic biphytane, IX, and we propose that

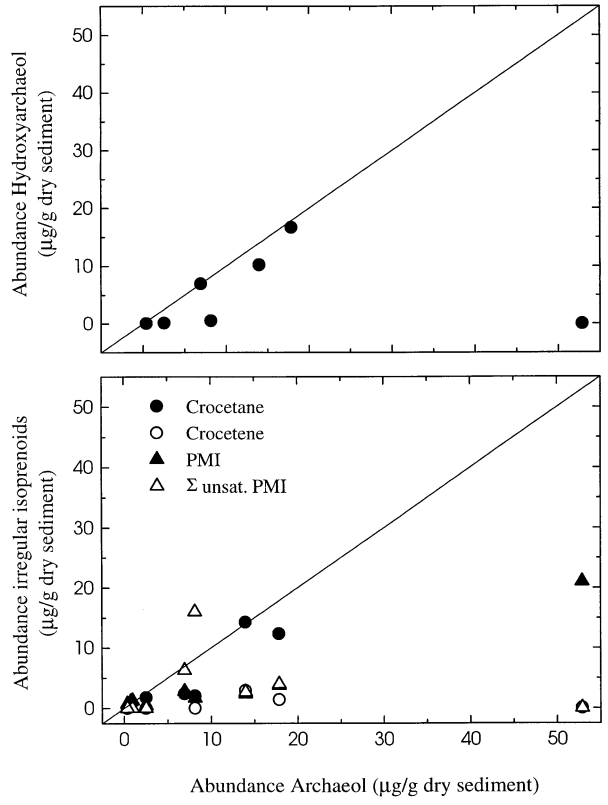


Fig. 4. Cross-plots showing the relationships among the abundances of different archaeal lipids in the sites studied. A 1:1 line is shown.

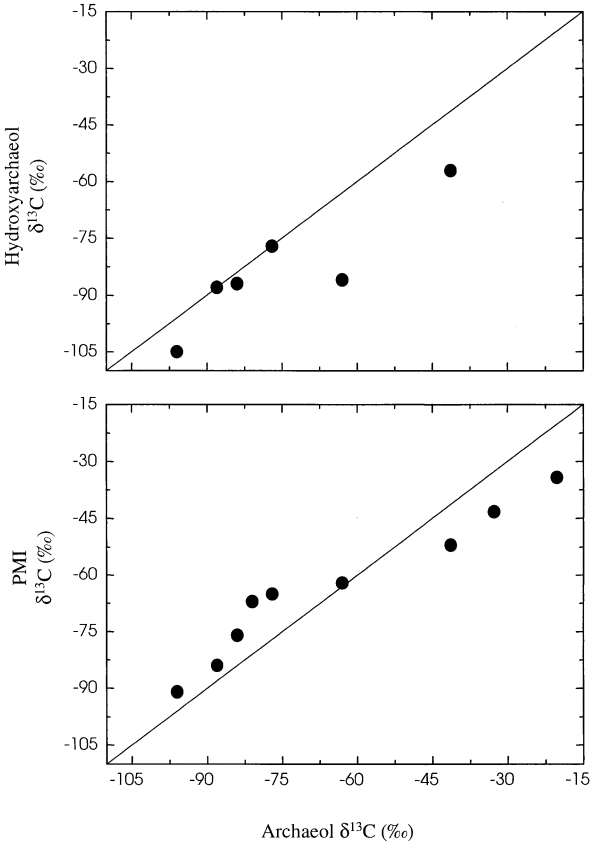


Fig. 5. Cross-plots showing the relationships among the $\delta^{13}C$ values of different archaeal lipids in the sites studied. A 1:1 line is shown.

this compound best reflects the carbon isotopic composition of GDGTs generated by methane-consuming Archaea. Although the $\delta^{13}C$ value of IX in the Milano seep is comparable to those observed for diether archaeal lipids, in the Amsterdam seep, IX is enriched in ^{13}C relative to glycerol diethers.

Concentrations of biphytanes released by ether bond cleavage (Table 2) are comparable to those of other archaeal lipids. In the Milano seep the concentrations of diethers are greater than those of tetraethers (8.2 and 1.1 μg/g for archaeol and VIII, respectively), while in the Amsterdam seep the opposite relationship is observed (6.9 and 18.2 μg/g for archaeol and VIII, respectively). Because of sample size limitations, concentrations could not be calculated for samples from Napoli mud dome; however, comparison of the abundances of XIII, XIV, and XV/XVI to those of the pelagic GDGT (XII), indicates that significant quantities of GDGTs are generated by the Archaea growing in these settings.

In addition to the intact GDGTs, biphytanediols are present in the Napoli seep sample. Because biphytanediols have terminal oxygen functionalities and the same carbon skeleton as the carbon chains of GDGTs—including the presence of pentacyclic groups—they are likely related to the GDGTs (Schouten et al., 1998). These compounds are absent in all other samples, but in the seep, biphytanediol concentrations are comparable to that of other archaeal biomarkers (ca. 2 μg/g dry sediment). The biphytanediol distribution is distinct from that observed for pelagic sediments (Schouten et al., 1998). That and the fact that Napoli biphytanediol $\delta^{13}C$ values are identical to those of archaeol and hydroxyarchaeol suggest that all compounds derive from the same or closely related sources.

4.4. Occurrence of Other Archaeal Lipids

4.4.1. Saturated and unsaturated PMI

The irregular isoprenoid 2,6,10,15,19-pentamethylicosane (PMI; XXIV) is biosynthesized by *Methanosarcina barkeri* (Holzer et al., 1979; Risatti et al., 1984) and is commonly considered to be a methanogen biomarker (Brassell et al., 1981). Recently, Schouten et al. (1997) and Sinninghe Damsté et al., (1998) unambiguously identified unsaturated PMIs (defined here as a group as XXV) in cultures of *Methanolobus bombayensis* and *Methanosarcina mazei*, further illustrating the utility of these compounds as biomarkers for methanogenic Archaea. Both saturated and unsaturated PMIs have been found in cold seep sediments and attributed to anaerobic methane-oxidizing Archaea (Elvert et al., 1999; Pancost et al., 2000). In our samples (Fig. 7 and Table 2), PMI is present in abundances slightly less than but comparable to those of archaeol (Fig. 2b). Highest concentrations of PMI occur in the microbial mat and it is absent in the Milano mud breccias.

Although not as common as PMI, unsaturated PMIs are present in the seep samples from Napoli, Milano, and Amsterdam mud domes and in the Napoli brine pool sediments (Fig. 8; retention indices shown in Table 4). On Napoli, the predom-

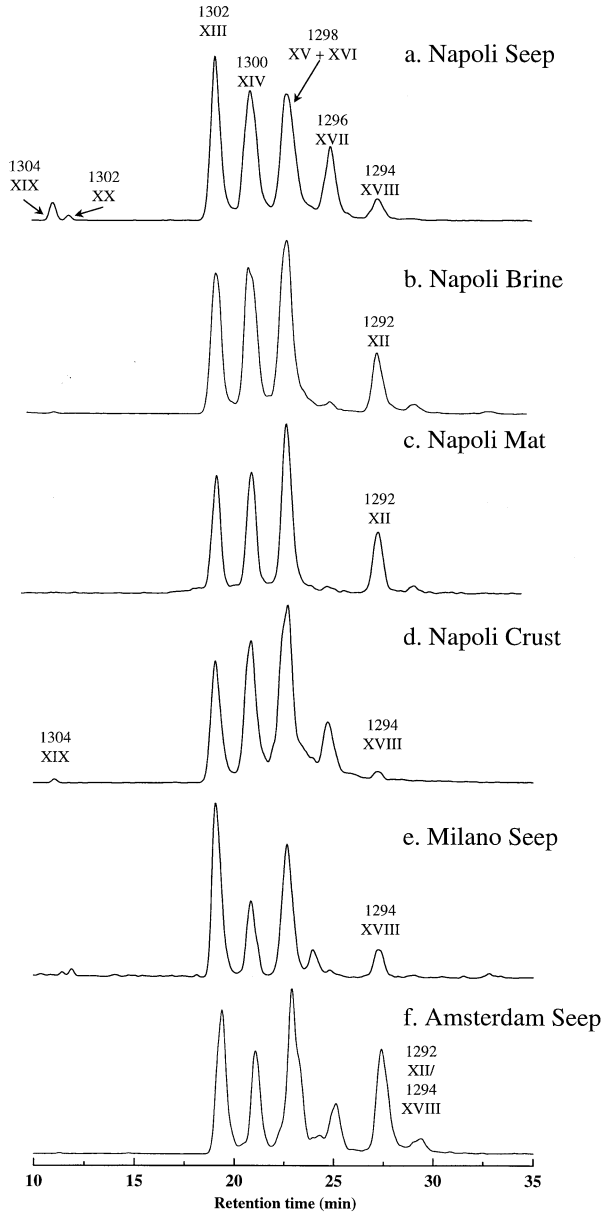


Fig. 6. Base peak chromatograms of the acetone-soluble fractions of the extracts of selected Mediterranean mud dome samples. The numbers above each peak denote the dominant $[M+H]^+$ ion and the roman numerals refer to structures in the Appendix (see the text for discussion of the assignment of peak identities).

inant unsaturated PMIs contain either one or two unsaturations. However, in the Amsterdam seep numerous PMIs with one to five double bonds are present. In the Milano seep, a similar range of unsaturated PMIs is present but those with either four or five double bonds predominate. In the Milano seep, unsaturated PMIs are particularly abundant (16 μg/g) such that, in sum, they are the most abundant archaeal biomarkers and twice as abundant as archaeol. The abundances of unsaturated PMI do not correlate with those of PMI; this is particularly striking in the microbial mat sample, in which PMI is abundant (21 μg/g), but unsaturated PMIs are absent.

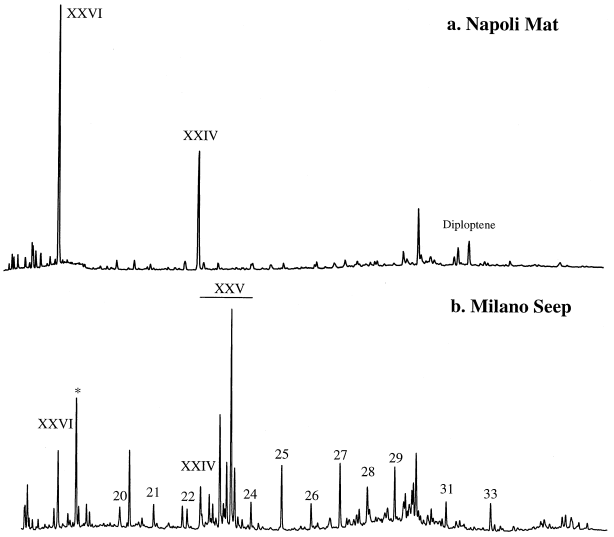


Fig. 7. Gas chromatograms of the apolar fractions extracted and isolated from two sites on the Napoli and Milano mud dome fields. Roman numerals denote structures shown in the Appendix. * denotes contamination, and arabic numerals denote the chain length of *n*-alkanes.

Although diverse unsaturated PMIs have also been observed in the marine archaeon *Methanolobus bombayensis*, many of the unsaturated PMIs in our samples are not present in this methanogen and vice versa (Table 4). Other investigated methanogens either have low concentrations of unsaturated PMIs (tentatively identified in *Methanobacterium ruminantium* and *Methanococcus vannielii*, Holzer et al., 1979; *Methanosarcina barkeri*, Summons et al., 1998) or biosynthesize predominantly only one unsaturated PMI (*Methanosarcina mazei*, Schouten et al., 1997). Thus, these compounds could have possible chemotaxonomic utility as our understanding of PMI distributions in cultured methanogens improves.

δ^{13}C values for PMI range from -35‰ in shallow Napoli mud breccia samples to -91‰ in the Amsterdam seep. Although the correlation between archaeol and PMI δ^{13}C values is strong ($r = 0.97$), PMI δ^{13}C values exhibit a narrower range (Fig. 5b). Consequently, in some samples (e.g., Napoli mud breccia) PMI is 15‰ depleted in ^{13}C relative to archaeol, while in others (Milano seep) it is 14‰ enriched in ^{13}C. The PMI δ^{13}C values determined after hydrogenation are lower than those of saturated PMI, indicating that unsaturated PMIs are depleted relative to saturated PMI.

4.4.2. Crocetane

2,6,11,15-tetramethylhexadecane (crocetane; XXVI; Robson and Rowland, 1986) is also found in several of the studied samples (Fig. 7). Crocetane is analogous to PMI in that it is a C_{20} as opposed to C_{25} irregular isoprenoid and has also been invoked as a biomarker for methanogens operating as methane consumers (Bian, 1994; Elvert et al., 1999). As with PMI, some cold seep sediments containing crocetane also contain unsaturated crocetene components (defined here as a group as XXVII) (Elvert et al., 1999). Although neither crocetane nor any crocetenes have been observed in cultured Archaea and their

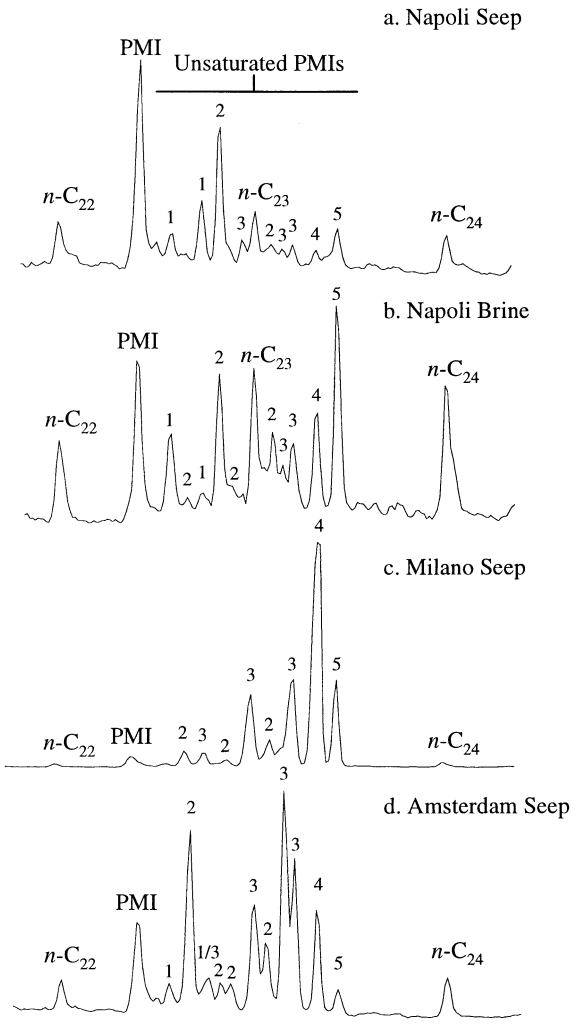


Fig. 8. Summed mass chromatograms of m/z 69 + 83 derived from GC-MS runs showing the distribution of unsaturated PMIs in Amsterdam, Napoli, and Milano seeps and the Napoli brine pool. Numbers above the peaks denote the number of double bonds.

precise source remains unknown, their structural similarity to PMI and occurrence in cold seep settings strongly suggest that these are archaeal biomarkers. In our samples, crocetane abundances are variable. In the bacterial mat, crocetane is the most abundant apolar compound (55 μg/g) and is more abundant than any other archaeal biomarker, but it is absent (or undetectable due to co-elution with abundant phytane) in all mud breccia samples. Monounsaturated crocetene was observed only in the Napoli seep and brine pool samples.

The carbon isotopic compositions of crocetane must be interpreted with caution; crocetane co-elutes with phytane, which in these settings is probably indigenous to the extruded mud breccia. This, in large part, explains why the crocetane $\delta^{13}C$ values in Table 3 are higher than those of other archeal biomarkers. In samples in which the concentration of crocetane is sufficiently high that phytane contamination is minimal, $\delta^{13}C$ values are quite low (−88.7‰ in the microbial mat) and comparable to those determined for PMI.

4.5. Archaeal Lipid Variability Amongst Sites

4.5.1. Milano and Amsterdam mud domes

The uppermost mud breccia samples on Milano contained very low abundances of archaeal lipids, similar to that reported for a Napoli mud breccia (Pancost et al., 2000). Only in the deepest sample (24–27 cm) was archaeol—but no other archaeal lipids—detected. Similarly, in the Milano seep archaeol is abundant but the hydroxyarchaeol concentration is low. In contrast to the breccia, in the seep sample PMI and especially unsaturated PMIs are among the most abundant compounds present.

The Milano and Amsterdam seep archaeal lipid distributions have several similarities: the dominant hydroxyarchaeol is the *sn*-2 isomer; archaeol, crocetane, and PMI are all present in approximately equal concentrations; and multiple unsaturated PMI isomers are abundant at both sites. Moreover, the tetraether distributions are similar with the predominant compounds being XIII and XV/XVI with lesser quantities of XIV. Differences between the two sites include the predominance of PMIs with four of five unsaturations in the Milano seep and relatively greater abundances of *sn*-2- and especially *sn*-3-hydroxyarchaeol in the Amsterdam seep.

4.5.2. Napoli mud dome

The Napoli seep exhibits several profound dissimilarities with the seeps on Amsterdam and Milano mud domes. Primarily, the abundances of almost all archaeal lipids are higher at the Napoli seep. The exceptions are the unsaturated PMIs; predominantly mono- and diunsaturated PMIs are present in the Napoli seep and their abundances are low compared to those found at the Amsterdam and Milano seeps. In contrast, monounsaturated crocetene is present in the Napoli seep but is absent on other examined mud domes. Also, *sn*-3-hydroxyarchaeol and acyclic, monocyclic, and bicyclic biphytanediols are present but are absent in all other samples. Similarly, the concentrations of XIV and XVII relative to other GDGTs are elevated compared to other mud domes. Variations in archaeal lipid distributions amongst sample locations on Napoli mud dome are also profound. Relative to the seep and brine samples, the crust, mud breccia, and microbial mat samples all contain markedly less hydroxyarchaeol relative to archaeol. Moreover, the *sn*-3 isomer is predominant only in the seep, distinguishing it from all other sites.

4.6. Implications for Anaerobic Methane Oxidation

It is well established that the mud breccias contain abundant methane and commonly accepted that this methane is part of the original extruded mud flow (Emeis et al., 1996). Such interpretations are supported by observations that methane is lost from the mud flow with time (determined by the thickness of overlying pelagic sediments) and that the fault planes believed to source mud domes contain methane seeps (submerged and subaerial) even where mud flows are absent. Although we have no geochemical pore-water profiles associated with our specific samples, multiple profiles (Emeis et al., 1996; de Lange and Brumsack, 1998; Haese et al., in preparation) indicate that this indigenous methane is oxidized anaerobically and in con-

Table 4. Occurrence of unsaturated PMI compounds in cold seeps and brine pool.

Compound	Retention index[a]	Sample sites and cultures: Amsterdam Seep	Milano Seep	Napoli Seep	Brine pool	*Methanosarcina mazei*	*Methanolobus bombayensis*
PMI:1	2257	√	√	√	√	—	—
	2274	√	√	√	√	—	—
PMI:2	2266	√	√	—	√	—	—
	2283	√	√	√	√	—	—
	2288	√	√	—	√	—	—
	2306	√	√	√	√	—	—
PMI:3	2276	√	√	—	—	—	—
	2290	—	—	√	—	—	√
	2301	√	√	?[b]	?	—	—
	2314	√	√	√	√	—	√
	2321	√	√	√	√	—	—
PMI:4	2326	—	—	—	—	—	√
	2333	√	√	√	√	—	√
	2336	—	—	—	—	√[c]	—
PMI:5	2347	√	√	√	√	√	√
	2366	—	—	—	—	—	√

[a] Retention indices are calculated from *m/z* 69 + 83 summed mass chromatograms and calculated based on the retention times of *n*-C_{22} (2200) and *n*-C_{24} (2400).
[b] Because of co-elution with *n*-C_{23}, the presence or absence of this unsaturated PMI could not be determined for the Napoli mud dome samples.
[c] Structure rigorously determined by NMR (Sinninghe Damsté et al., 1998).

junction with sulfate reduction. Consequently, ^{13}C depleted archaeal lipids in these same settings were interpreted as being generated by anaerobic methane oxidizing Archaea (Pancost et al., 2000). We suggested (Pancost et al., 2000) that even archaeal lipids that are not significantly depleted in ^{13}C (surface mud breccia samples on Napoli) could derive from oxidation of methane that has become enriched via a Rayleigh distillation.

This does not preclude the possibility that Archaea could be involved in other reactions in these sediments. Sulfate reduction and sulfide oxidation also occur, and it is possible that where sulfate has been reduced methanogenesis occurs as either an autotrophic (H_2/CO_2) or heterotrophic (acetate) process. In the subsequent sections, we discuss, along with other explanations, the possibility that Archaea performing these processes could partially explain the pronounced heterogeneity in archaeal lipid distributions and $\delta^{13}C$ values. However, even where such processes occur, it should be remembered that the vastly predominant reduced constituent of extruded mud flows is methane and oxidation of methane likely serves as the underlying basis for mud dome chemistry and microbial life.

4.6.1. Variation in archaeal lipid $\delta^{13}C$ values

The heterogeneity of archaeal lipid $\delta^{13}C$ values suggests that at most sites the archaeal community consists of multiple organisms. Archaea apparently use diverse metabolic pathways during biosynthesis and only a single report on methylotrophic methanogens provides any insight into partitioning of carbon isotopes amongst lipid components of Archaea (Summons et al., 1998). This lack of data is compounded by the fact that almost nothing is known about the organisms—let alone their metabolic pathways—involved with anaerobic methane oxidation. Summons et al. (1998) reported that the difference in $\delta^{13}C$ values between the phytanyl side chain of archaeol and PMI varied from +3.4 to −3.6‰ in *Methanosarcina barkeri* cultured using tetramethylamine as a carbon source. This is but a single archaeon that has been studied under very specific conditions that certainly do not apply here. Nonetheless the values are consistent with the general observation that isoprenoidal lipids—ostensibly generated by the same or related metabolic pathways—should have similar $\delta^{13}C$ values in a single organism (Hayes, 1993). Thus, the fact that in a single sample we observe differences as great as 24‰ amongst isoprenoidal archaeal lipids is striking.

When considering all sites, the $\delta^{13}C$ values for different compounds correlate well (Fig. 5), but this is probably at least partially due to the carbon isotopic variability of methane amongst locations due to (i) different sources of methane at different sites and (ii) enrichment of methane via Rayleigh distillation as methane is progressively oxidized (Pancost et al., 2000). These processes should affect all organisms participating in the consortium responsible for anaerobic methane oxidation, and the latter explanation is particularly consistent with the differences in archaeol and PMI $\delta^{13}C$ values amongst the Napoli seep and the four samples in the Napoli mud breccia (Fig. 3). Specifically, even though the four mud breccia samples span a depth range of only 20 cm and the mud breccia core was collected from only 1 m away from the seep, we observe a 60‰ variation in archaeol $\delta^{13}C$ values. Although we lack the necessary data to validate this hypothesis and cannot exclude other explanations such as multiple sources for archaeol and PMI, the data is certainly consistent with a Rayleigh distillation. Because of these uncertainties we make no attempt to interpret differences in archaeal lipid $\delta^{13}C$ values amongst different sites.

However, changes in the isotopic composition of substrate methane cannot explain why PMI is enriched in ^{13}C relative to

archaeol in the seep but depleted relative to archaeol in the mud breccia. Similarly, hydroxyarchaeol is profoundly depleted in ^{13}C relative to archaeol in the lowermost mud breccia sample but has the same $\delta^{13}C$ value in the seep. This variability is considerably greater than that previously reported, which is always less than 7‰ (Elvert et al., 1999; Hinrichs et al., 1999; Thiel et al., 1999). However, this likely reflects either the lower diversity of biomarkers or fewer sites examined in those previous studies, and we expect that this phenomenon will be widely observed in future investigations.

Isotopic variations could indicate that different compounds are generated by the same organisms but under different environmental conditions (methane availability or carbon isotopic composition, availability of organic substrates that could be consumed heterotrophically) that also affected the lipid $\delta^{13}C$ values. Although this explanation cannot be excluded, the fact that there is no consistent difference in $\delta^{13}C$ values of different lipids (PMI is sometimes depleted and sometimes enriched relative to archaeol), suggests that other factors probably also play a role. Alternatively, despite the fact that all are isoprenoids, there could be differences in biosynthetic pathways among archaeal lipids. This could be particularly important when comparing the carbon isotopic compositions of glycerol ethers to isoprenoid hydrocarbons, because glycerols are synthesized by different pathways than isoprenoids. However, because glycerols contribute only 3 carbon atoms for every 40 isoprenoid carbon atoms, we suspect this effect is minimal. A third explanation is that differences in archaeal lipid $\delta^{13}C$ values originate after the death of the source organisms. However, this explanation requires a dramatic (greater than 20‰) isotope effect during degradation that has not previously been reported for any lipid. In the study of oxidized and unoxidized organic matter-rich turbidites no significant (i.e., $<$1‰) carbon isotopic fractionation effect was noted for alkenones even though $>$99% was oxidized (Sinninghe Damsté, unpublished results). Thus, although some of the above processes probably account for some of the reported variation in archaeal lipid $\delta^{13}C$ values, we suspect that they cannot explain the whole range.

Instead, we propose that the significant variations in archaeal lipid $\delta^{13}C$ values arise because these compounds are generated by multiple archaeal species or strains. In this case, the differences in $\delta^{13}C$ values amongst some organisms could arise from different carbon isotopic responses to varying methane supply. Thus, when inferred methane flux is high (seep samples) hydroxyarchaeol and archaeol $\delta^{13}C$ values are similar, but at lower methane concentrations (mud breccia), archaeol becomes progressively enriched in ^{13}C relative to hydroxyarchaeol. Such an explanation is speculative and no background data is available to validate it. Nonetheless, it is consistent with the commonly observed phenomenon that the response of carbon isotope fractionation to changing concentrations of substrate carbon (either CO_2 or CH_4) can differ amongst organisms, and we propose this as a guide to future research.

Alternatively or additionally, the variations in biomarker $\delta^{13}C$ values could reflect different ecological roles for different archaeal species. The commonly depleted $\delta^{13}C$ values indicate that methane serves as a carbon source for most of these organisms. However, although anaerobic methane oxidation certainly appears to be the dominant process in mud flows, some Archaea could assimilate this depleted carbon indirectly. For example, Archaea could live as heterotrophs (e.g., methanogens) consuming ^{13}C-depleted organic compounds derived from methanotroph biomass. Or some Archaea could be autotrophs, such as methanogens or sulfide oxidizers (utilizing HS^- generated during sulfate reduction), and assimilate CO_2 generated in part from methane oxidation. It is impossible at this time to distinguish amongst these and other explanations, but we suggest that anaerobic methane oxidation is performed in sediments containing a diverse assemblage of Archaea. If so, then the integration of phylogenetic and biomarker analyses (Hinrichs et al., 1999)—despite the obvious benefits of such research—should be done cautiously. Indeed, phylogenetic analyses by Hinrichs et al. (1999) revealed at least two groups of Archaea in California seep samples, and it is difficult to confidently state from which group of organisms the co-occurring archaeal biomarkers derive.

4.6.2. Archaeal variability

The reasons for the profound variability in archaeal lipid distributions amongst sites are difficult to ascertain. It is possible that the differences reflect differential degradation of the lipids generated by now-deceased organisms, although this has yet to be demonstrated to occur under anoxic conditions. Elvert et al. (2000) argued that the number of double bonds in PMI and crocetane compounds is greatest at active locations with high methane fluxes. That explanation is consistent with our data in that our most active sites all contain unsaturated PMIs and the less active sites do not. However, amongst the four highly active sites (three seeps and brine), the number of unsaturations still varies significantly. Moreover, in the Napoli seep and brine samples unsaturated crocetane is more abundant but unsaturated PMIs are less abundant than in the Amsterdam and Milano seeps. Differences in methane fluxes also seem inadequate to explain the profound differences in diether and tetraether distributions between the Napoli seep and seeps on other mud domes.

It is known that archaeal membrane lipid distributions (diethers and tetraethers) can vary with environmental conditions to maintain membrane fluidity and an appropriate proton permeability (van de Vossenberg et al., 1998). For example, increasing temperature results in an increasing number of cyclic groups in the tetraether membrane lipids of *S. solfataricus* (De Rosa et al., 1991) and influences the proportional abundances of tetraethers and diethers in *Methanococcus jannaschii* (Sprott et al., 1991). At all sample sites bottom-water temperatures are similar (ca. 14°C) and thus, temperature is an unlikely cause for the observed variation. Similarly, changes in pressure, also invoked as causes for variations in archaeal membrane lipid distributions (van de Vossenberg et al., 1998), cannot apply here because all samples come from approximately the same depth. Other environmental parameters such as salinity or methane fluxes could influence lipid distribution (van de Vossenberg et al., 1998). For example, only on Napoli mud dome are brines present, and thus, a more halotolerant archaeon could be present there. Indeed, the Napoli brine pool archaeal lipid distribution shares some similarities with that present in the seep—such as the presence of crocetene. However, causes for variations in PMI and crocetane abudances are

particularly difficult to determine because their function in Archaea remains unknown.

Alternatively or additionally, the variations in membrane lipid distributions could simply reflect variations in archaeal assemblages amongst different settings. Such a potential has already been proposed by previous workers (Hinrichs et al., 1999; Elvert et al., 2000). For example, it is possible that in some settings methanogens operating in reverse predominate (Hoehler et al., 1994), while in others, obligate methanotrophs are present. Indeed such variations could explain the variability in methanogen abundance as determined by most-probable-number (MPN) counts of mud dome sediments (Pancost et al., 2000). MPN counts revealed that the potential for methanogenesis is present in several samples and thus, archaeal biomarkers in those samples were interpreted as being derived from methanogens assimilating methane (i.e., reverse methanogenesis). However, in the Milano and Amsterdam seeps, methanogen abundances determined by MPN counts were low. Although limited MPN data makes interpretations speculative, it is possible that the methane-oxidizing Archaea present in Milano and Amsterdam samples are not methanogens operating in reverse but are instead obligate methanotrophs.

Regardless of the reasons, our results clearly show that archaeal lipid distributions in sites of anaerobic methane oxidation are diverse and could reflect archaeal community structures. We propose that sites can be divided on the basis of the relative abundances of the following major compound classes: (i) archaeol, (ii) hydroxyarchaeol, (iii) PMI, (iv) crocetane, and (v) GDGTs. Moreover, within each of these compound classes significant variability and further chemotaxonomic information is available: (i) the relative abundances of *sn*-2- and *sn*-3-hydroxyarchaeol; (ii) the location and number of unsaturations in PMI and crocetane; and (iii) the relative abundances of the XVII and XIX GDGTs and the presence of heretofore undescribed tetraethers (XX).

Ultimately, however, the cause of the archaeal lipid variability amongst our samples and compared to those previously reported remains unclear. Likely, salinity and methane flux are important controls on Archaea populations and thus archaeal lipid distributions, but clearly, the full causes of such heterogeneity will only be elucidated after more extensive culture work. Nonetheless, these results show that in sediments where anaerobic methane oxidation occurs, archaeal communities are adaptable, and possibly highly variable, which could explain the apparent ubiquity of this process in diverse settings.

4.6.3. GDGTs as biomarkers for methane-oxidizing Archaea

Despite the variations in archaeal lipid distributions, our results indicate that GDGTs are important lipid components of Archaea and could be used to distinguish methane-oxidizing species from others. Unlike other archaeal biomarkers these compounds were found at all sites examined (excepting the surface mud breccia samples) and had similar apparently diagnostic distributions. Specifically, in all samples except for the mud breccias, GDGTs XIII, XIV, and XV/XVI are major components. In contrast, peat deposits in which terrestrial methanogenic Archaea are present contain few cyclic GDGTs, GDGTs of thermophilic organisms typically contain more cyclic groups, and the aformentioned pelagic Archaea are characterized exclusively by the XII and XIII GDGTs (Schouten et al., 2000). Thus, these compounds could be useful in further investigations of anaerobic methane oxidation in modern and ancient settings.

In addition, we observed "open" tetraethers (XIX and XX) in the Napoli seep. These compounds appear to be quite uncommon; the acylic component has only been found in *S. solfataricus* (De Rosa et al., 1986) and the monocyclic compound has not been previously reported. Thus, these two compounds could be particularly useful, if identified in future culture work, as chemotaxonomic indicators of the Archaea thriving in this setting.

5. CONCLUSIONS

Results presented here expand on previous work in four fundamental ways. First, we show the potential of some GDGTs in the identification of methane-oxidizing Archaea. In addition, acyclic and monocyclic "open" tetraethers are present in our samples and the latter compound, although present in relatively low concentrations, has not been previously identified. These uncommon compounds could have particular chemotaxonomic utility if their source organisms are identified. Second, in addition to GDGTs, we have identified a variety of uncommon and potentially diagnostic archaeal lipids or lipid distributions, including abundant and diverse unsaturated PMIs and *sn*-3-hydroxyarchaeol. Third, we present evidence for dramatic carbon isotopic variability amongst archaeal lipids within a single sample. The observed variability of up to 24‰ suggests that at some sites, a diverse community of Archaea either mediates or prospers from anaerobic methane oxidation. Fourth, we report that archaeal lipid distributions vary profoundly between sites even though the overall processes of methane release from mud dome sediments and subsequent anaerobic consumption are similar. This, combined with previous results from Hydrate Ridge and the California Margin, clearly indicates that the Archaea populations responsible for anaerobic methane oxidation are more variable than previously suggested.

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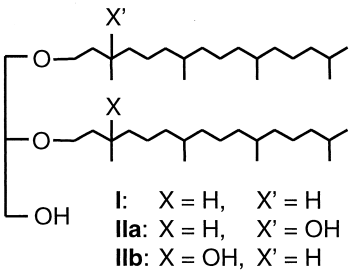
APPENDIX

Diethers



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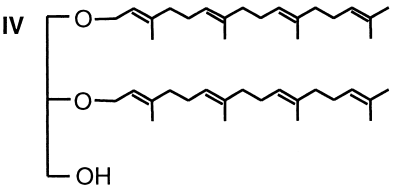
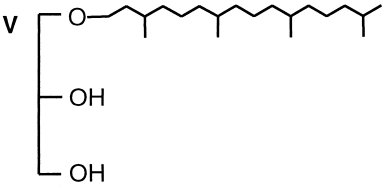
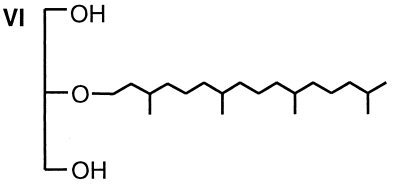
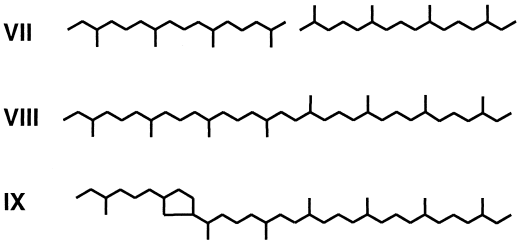
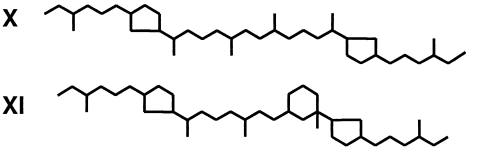


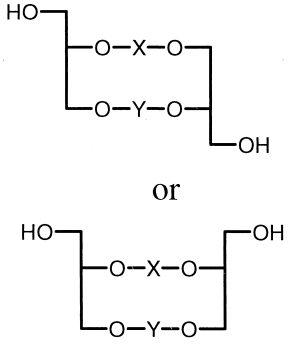




Biphytanes and GDGTs

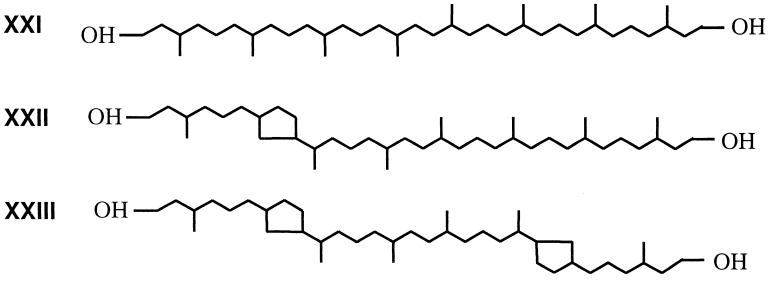






GDGT no.	X	Y	GDGT no.	X	Y
XII	X	XI	**XVII**	IX	X
XIII	VIII	VIII	**XVIII**	X	X
XIV	VIII	IX	**XIX**	VII	VIII
XV	VIII	X	**XX**	VII	IX
XVI	IX	IX			

Biphytanediols



Other Irregular Isoprenoids