Pergamon

Geochimica et Cosmochimica Acta, Vol. 63, No. 23/24, pp. 3959–3966, 1999

0016-7037/99 $20.00 + .00

PII S0016-7037(99)00177-3

Highly isotopically depleted isoprenoids: Molecular markers for ancient methane venting

VOLKER THIEL,[1] JÖRN PECKMANN,[2] RICHARD SEIFERT,[1] PATRICK WEHRUNG,[3] JOACHIM REITNER,[2] and WALTER MICHAELIS[1,*]

[1]Institut für Biogeochemie und Meereschemie, Universität Hamburg, Bundesstr. 55, D-20146 Hamburg, Germany
[2]Institut und Museum für Geologie und Paläontologie, Universität Göttingen, Goldschmidtstr. 3, D-37077 Göttingen, Germany
[3]Institut de Chimie, Laboratoire de Geochimie organique, Université Louis Pasteur, 1 rue Blaise Pascal, F-67008 Strasbourg Cedex, France

(*Received May* 31, 1998; *accepted in revised form May* 10, 1999)

Abstract—We propose that organic compounds found in a Miocene limestone from Marmorito (Northern Italy) are source markers for organic matter present in ancient methane vent systems (cold seeps). The limestone contains high concentrations of the tail-to-tail linked, acyclic C_{20} isoprenoid 2,6,11,15-tetramethylhexadecane (crocetane), a C_{25} homolog 2,6,10,15,19-pentamethylicosane (PME), and a distinctive glycerol ether lipid containing 3,7,11,15-tetramethylhexadecyl (phytanyl-) moieties. The chemical structures of these biomarkers indicate a common origin from archaea. Their extremely ^{13}C-depleted isotope compositions ($\delta^{13}C \approx -108$ to $-115.6‰$ PDB) suggest that the respective archaea have directly or indirectly introduced isotopically depleted, methane-derived carbon into their biomass. We postulate that a second major cluster of biomarkers showing heavier isotope values ($\delta^{13}C \approx -88‰$) is derived from sulfate-reducing bacteria (SRB). The observed biomarkers sustain the idea that methanogenic bacteria, in a syntrophic community with SRB, are responsible for the anaerobic oxidation of methane in marine sediments. Marmorito may thus represent a conceivable ancient scenario for methane consumption performed by a defined, two-membered bacterial consortium: (1) archaea that perform reversed methanogenesis by oxidizing methane and producing CO_2 and H_2; and (2) SRB that consume the resulting H_2. Furthermore, the respective organic molecules are, unlike other compounds, tightly bound to the crystalline carbonate phase. The Marmorito carbonates can thus be regarded as "cold seep microbialites" rather than mere "authigenic" carbonates.

1. INTRODUCTION

Crude oil and gas seepage is a common phenomenon at locations where vertical migration of hydrocarbons occurs from reservoir rocks to surface sediments. Unlike the "hot smokers" known from deep sea spreading centers, the hydrocarbon-dominated "cold seeps" and vents occur at convergent plate boundaries as well as at passive continental margins (Paull et al., 1984; Kulm et al., 1986; Dando et al., 1991). Well studied contemporary examples are known from the Oregon subduction zone, the Florida escarpment, the Gulf of Mexico continental slope offshore Louisiana, and the North Sea (e.g., Kulm et al., 1986; Roberts et al., 1993; Sassen et al., 1993).

The accumulation of hydrocarbons in near-surface sediments results in an unusual geochemical environment that strongly affects the biocommunities observed in such habitats. Hydrocarbon seeps and vents create settings that are dominated by chemosynthetic communities based on the bacterial oxidation of methane or reduced sulphur compounds. Characteristic metazoans associated with cold seeps are tube worms and bivalves (Paull et al., 1984; Kennicutt et al., 1985; Cavanaugh et al., 1987). These animals typically harbor endosymbiotic sulfide oxidizers or methanotrophic bacteria, which supply their hosts with energy and nutrients by oxidizing reduced sulphur species or methane. Cold-seep settings can be regarded as localized, highly productive environments. For example, the bacterioplankton growth rate at the Louisiana hydrocarbon seeps has been reported to be more rapid than in any other marine system (La Rock et al., 1994). It was suggested that methane may serve as a primary nutrient source for these organisms. Benthic communities of bacteria include methanotrophic bacteria as well as different types of sulfate reducers and sulfide oxidizers, such as the mat-forming filamentous bacterium *Beggiatoa* (Sassen et al., 1993; 1994).

Hydrocarbon seeps are often accompanied by massive carbonate deposits that may considerably alter sea floor topography. Sulfate reduction, coupled with anaerobic oxidation of methane, results in an increase in alkalinity and has been suggested as a driving process for the precipitation of carbonate at these sites. Seep-associated carbonates typically show extremely depleted carbon isotope compositions ($\delta^{13}C_{carbonate}$ values as low as $-66.7‰$; Ritger et al., 1987). Low $\delta^{13}C_{carbonate}$ values are due to microbial oxidation of thermogenic ($\delta^{13}C \sim -50‰$) and biogenic (-60 to $-110‰$) methane and the precipitation of the resultant CO_2 (Whiticar et al., 1986; Suess and Whiticar, 1989).

Although cold seep environments and their particular biocommunities have attracted increasing attention during the last decade, little is known about the composition of the resulting biogenic sediments and their contributing organic carbon sources. After their discovery in contemporary settings, different fossil limestones ranging from Carboniferous to Holocene have been interpreted as methane-derived carbonates (Beauchamp et al., 1989; Jørgensen, 1989; von Bitter et al., 1990; Gaillard et al., 1992; Terzi et al., 1994; Kelly et al., 1995). These interpretations are mainly based on $\delta^{13}C_{carbonate}$ values, sometimes supported by the content of preserved fossil assemblages including lucinid and vesicomyid clams, or modiolid mussels, and vestimentiferan worm tubes (Campbell et

* Author to whom correspondence should be addressed (michaelis@geowiss.uni-hamburg.de).

al., 1993, Terzi et al., 1994). The distinct ecological features observed in present-day settings prompted us to study the organic compounds included in their proposed ancient counterparts. We analyzed the lipid composition of a fossil limestone, which is thought to originate from a former cold seep environment. Special emphasis was placed on a search for distinctive molecular markers for signifying the former activity of chemosynthetic communities and thus, ancient hydrocarbon venting.

2. MATERIALS AND METHODS

Blocks of the so-called "calcari a *Lucina*" are widespread throughout the Tertiary sediments of the Apennines (Ricci Lucchi and Vai, 1994). These limestones are interbedded with turbidites and hemipelagites deposited in foredeep basins from the Early to Late Miocene (Terzi et al., 1994). Carbonate precipitation occurred in bathyal depths supposedly deeper than 200 m (Aharon and Sen Gupta 1994). A re-interpretation of the "calcari a *Lucina*" as cold seep deposits was established on limestone lenses near the village of Marmorito in the Monferrato hills, east of Torino (Clari et al., 1988; 1994). These authors reported $\delta^{13}C_{carbonate}$ values ranging from −24.7 to −40.5‰ PDB. This was confirmed by our own measurements, which revealed a $\delta^{13}C_{carbonate}$ of −29.5‰ (mean of five samples) for the material discussed here.

2.1. Sample Preparation

A large limestone block packed with the remains of tube worms was sampled from an outcrop east of Marmorito. Material for analyses was taken from the center of the block, crushed to small pieces, and carefully cleaned by repeated washing with dilute HCl and acetone. Doubly distilled water (200 ml) was added to a 500-g subsample. The carbonate pieces were slowly dissolved using dilute HCl. To avoid transesterification resulting from excess acidification, the addition of HCl was stopped when ca. 80% of the carbonate had been dissolved. The remaining pieces were removed, and the acid–insoluble residue (6.0‰$_{wt}$ of the total rock) was separated by centrifugation. After washing with doubly distilled water, the sample was dried and saponified in 6% KOH in CH_3OH, the supernatant was decanted, and the residue further extracted by ultrasonication in CH_2Cl_2/CH_3OH (3 : 1; v/v) until the solvent became colorless. Subsequently, the combined supernatants were extracted with CH_2Cl_2 vs. water (pH2). The organic components of the CH_2Cl_2 phase were fractionated by column chromatography (i.d.: 15 mm, length: 35 mm; Merck silica gel 60, 70–230 mesh ASTM). A "total hydrocarbon" fraction (6.0 $\mu g/g_{total\ rock}$) was eluted with 2 column volumes (16 ml) of *n*-hexane and an alcohol/ketone fraction (4.0 $\mu g/g_{total\ rock}$) with 3 column volumes (24 ml) of CH_2Cl_2. The alcohol/ketone fraction was treated with acetic acid anhydride and an equal volume of pyridine (14 h, RT) to convert the alcohols to the respective acetates. The hydrocarbons and alcohols/ketones were analyzed by gas chromatography (GC), combined gas chromatography–mass spectrometry (GC-MS), and combined gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS). Component identification was based on comparison of the mass spectra and of GC retention times with those of published data and of reference compounds. 5α(H)-cholestane was used as an internal standard for quantification.

2.2. Free vs. Total Hydrocarbons

A second subsample was ground to a powder and directly extracted with CH_2Cl_2/MeOH (3 : 1; v/v) to yield biomarkers extractable without previous decalcification. Hydrocarbons were obtained from this extract by column chromatography with *n*-hexane as elution agent. The amounts, isotopic compositions, and the relative distribution of these "free" hydrocarbons were compared with those obtained from the total rock using the procedure described above. This comparison was made to differentiate those compounds tightly bound to the crystalline phase, i.e., to distinguish between free (*inter*crystalline) and matrix-bound (*intra*crystalline) organic compounds (Section 3.4.). Note that these terms do not refer to chemical bonding.

2.3. GC, GC–MS, GC–C–IRMS

GC analyses were performed using a 30 m fused silica capillary column (DB5, 0.32 mm i.d., 0.25 μm film thickness and a flame ionisation detector. Carrier gas: H_2. Temperature program: 3 min 80°C; 80°C to 310°C at 4°C/min; 30 min. 310°C

The GC-MS system was a Finnigan MAT CH7A spectrometer interfaced to a gas chromatograph equipped with a 50-m fused silica capillary column (DB5-HT, 0.32 mm i.d., 0.25-μm film thickness). Carrier gas: He. Temperature program: 5 min 80°C; 80°C to 310°C at 4°C/min; 20 min. 310°C.

GC-C-IRMS was performed on a Finnigan MAT 252 instrument controlled by ISODAT software. The isotope ratios reported for individual compounds are the mean of five (hydrocarbons) and three (alcohols) runs. They are given as δ-values ($\delta^{13}C$ [‰], relative to the Pee Dee Belemnite standard, PDB). SD (σ) were less than ±1‰ for all compounds unless stated otherwise.

2.4. GC Elution Behavior of Crocetane

Several stationary phases were tested for their ability to resolve phytane and crocetane under different conditions. Mixtures of synthetic crocetane and phytane were prepared and examined on HP-1, DB5-HT, OV-101, and CpSil 2 CB (squalane) columns. With each column, analyses were run under varying starting temperatures, heating gradients, isothermal conditions, and carrier gas (H_2) pressures.

In general, elution of the phytane/crocetane mixture later than *n*-octadecane was observed for all columns tested. Neither DB5-HT (60 m, 0.32 mm i.d., 0.25 μm film thickness), HP-1 (50 m, 0.32 mm, 0.17 μm,) nor the more fluid OV-101 (50 m, 0.25 mm, 0.23 μm) methylsilicone phases achieved resolution of both compounds. Slightly better results were obtained with a combination of the OV-101 and HP-1 columns (100 m in total). Using this assemblage, a partial separation of $R = 0.7$ was observed when the test mixture was analyzed isothermally at 180°C and 0.8 kg/cm^2 carrier gas pressure. Under these conditions, the retention index of crocetane and phytane, determined by coinjection with *n*-octadecane and *n*-nonadecane, was found to be 1812 and 1814, respectively.

The use of a 25 m CpSil 2CB (squalane) column resulted in a maximum resolution of $R = 0.5$, when the test mixture was examined isothermally at 170°C and 0.7 kg/cm^2 carrier gas pressure. Considering the comparatively short length of this column, we suggest that better separation may occur when a squalane-bonded phase in a column of greater length is used.

These results differ from a report by Robson and Rowland (1993), who observed separation of phytane and crocetane on a OV-1 column. These authors reported a retention index of 1792 for crocetane and thus elution earlier than *n*-octadecane.

3. RESULTS AND DISCUSSION

3.1. Hydrocarbons

Three prominent compounds dominated the hydrocarbons of the Marmorito carbonate. They were identified as the "irregular" tail-to-tail linked acyclic C_{20} isoprenoid 2,6,11,15-tetramethylhexadecane (crocetane), a C_{25}-counterpart 2,6,10,15,19-pentamethylicosane (PME*), and the pentacyclic triterpenoid hop-17(21)-ene. Gas chromatograms of the "free" and "carbonate-bound" hydrocarbons are given in Fig. 1.

*Note that, according to IUPAC nomenclature, a C_{20} alkane is called "icosane" and hence "PMI" would be the correct acronym. However, the compound has commonly been referred to as 2,6,10,15,19-pentamethyleicosane (PME) heretofore. For the reason of compatibility, we further use the abbrevation "PME" (e.g., Schouten et al., 1997).

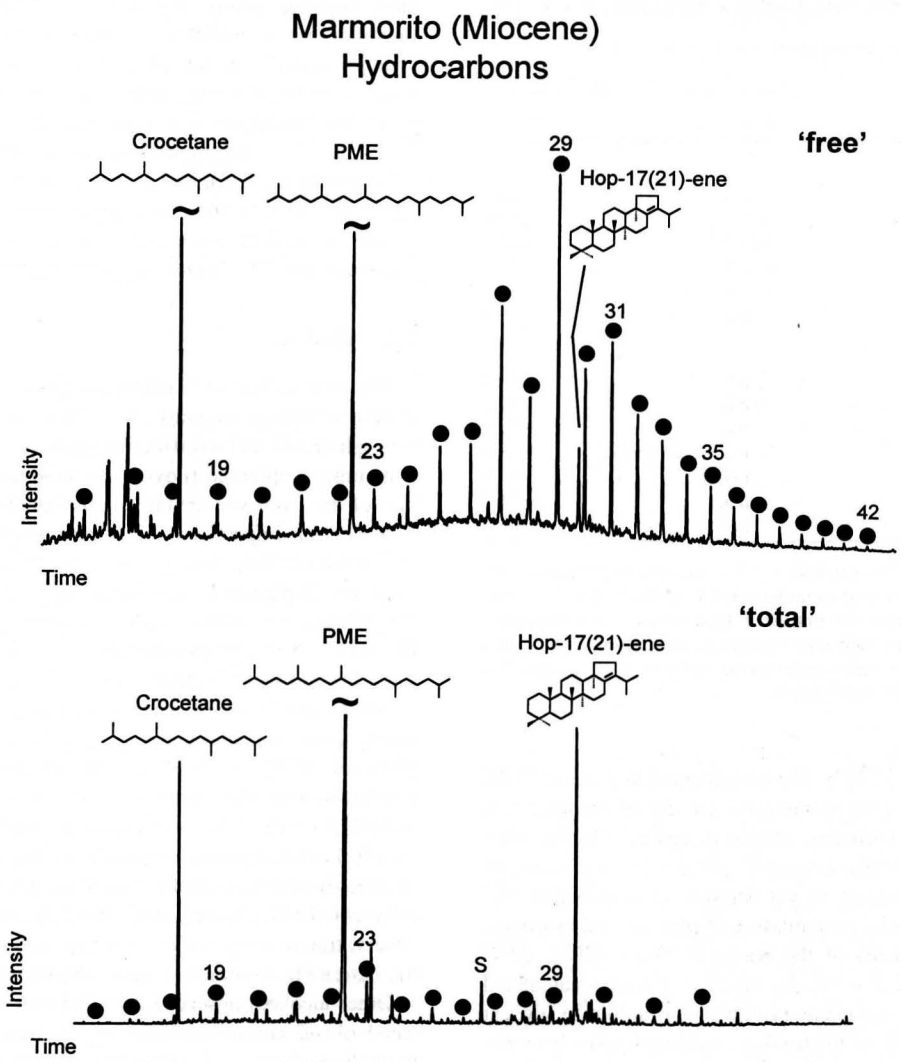

Fig. 1. Gas chromatograms of the hydrocarbon fractions extracted from the total sediment ("free") and from the residual matter after decalcification ("total"). ● = *n*-alkanes (selected carbon numbers denoted); S, 2,6,10,15,19,23-hexamethyltetracosane (squalane). Peak heights of crocetane and PME in the "free" fraction are cut at 75%; the peak of PME in the GC trace of the "total" fraction is cut at 50% peak height.

3.1.1. Crocetane and PME

An intriguing feature of the sample from Marmorito is the occurrence of crocetane as a major component of the hydrocarbon fraction. GC-C-IRMS measurements revealed a $\delta^{13}C$ of $-115.6‰$ (Table 1). This appears to be among the lowest $\delta^{13}C$ values ever measured in a biomarker.

Because the occurrence of crocetane in ancient samples has only been reported once (McCarthy, 1967; cited from Bian, 1994), particular care was taken to ensure the correct assignment of this unusual compound. Synthetic crocetane was used to aid identification by GC-MS. The presence of crocetane was further confirmed by coinjection with the authentic reference compound on DB5-HT and HP-1 GC columns and coelution experiments. Difficulties in the GC and GC-MS based identification arise from its similar chromatographic behavior compared to the widespread head-to-tail linked isoprenoid 2,6,10,14-tetramethylhexadecane (phytane). Coelution may mimic the presence of crocetane or mislead to its identification as phytane (see also Section 2.4.). Nevertheless, the mass spectrum of crocetane (Robson and Rowland, 1993) shows specific characteristics that allow its recognition at least in samples of high abundance relative to phytane.

A second prominent acyclic isoprenoid hydrocarbon was identified as 2,6,10,15,19-pentamethylicosane (PME). Isotopic analysis revealed a $\delta^{13}C$ of $-112.2‰$, a value close to that obtained for crocetane ($-115.6‰$).

In biogeochemistry and oil exploration geochemistry, C_{20}–C_{40} acyclic isoprenoids belong to the most widely used biomarkers for archaeabacteria, in particular methanogens (Brassell et al., 1981; Hahn, 1982; Volkman et al., 1986; Wakeham, 1990; Grice et al., 1998). PME previously has been identified in various recent and ancient sediments as well as in the lipid fractions of methanogenic and thermoacidophilic archaea (Tornabene et al., 1979; Holzer et al., 1979; Risatti et al.,

Table 1. Isotopic composition of selected biomarkers ($\delta^{13}C$ [‰] vs. PDB).

Compound	$\delta^{13}C$ [‰] Free	$\delta^{13}C$ [‰] Total
Hydrocarbons		
n-C_{18}	n.a.	−44.2
Crocetane	−108.3	−115.6
PME	−105.5	−112.2
n-C_{26}	−30.3	−32.0
n-C_{29}	−30.4	−38.4
Hop-17(21)-ene	n.a.	−83.2
Alcohols		
Anteiso-C_{15}	n.a.	−88.3
n-C_{16}	n.a.	−87.6
10-Methyl-C_{16}	n.a.	−87.8
Phytanol	n.a.	−108.5
n-C_{18}	n.a.	−66.8
n-C_{26}	n.a.	−51.3
Ether lipid*	n.a.	−108.2

Standard deviations (σ) are below ±1‰ for all compounds except n-octadecanol (±6.5‰), and n-hexacosanol (±2.8‰); 'free' = compounds extractable from the untreated rock; 'total' = compounds obtained from the total rock after carbonate dissolution; n.a. = not analysed. *: 'Ether lipid' refers to the compound tentatively assigned as 1-O-hexadecyl-2-O-phytanylglycerol.

1984; Schouten et al., 1997). The prominent abundance of PME may therefore point to a pronounced activity of methanogens associated with the particular setting discussed. On the other hand, for the structurally related C_{20}-homolog, crocetane, no discrete source organism is yet known. A remarkable ^{13}C depletion was measured for a mixture of phytane and crocetane from modern sediments of the Kattegat (Bian, 1994). $\Delta^{13}C$ values down to −78.2‰ for the mixture, giving a calculated $\delta^{13}C$ of −104‰ for crocetane, prompted this author to suggest that crocetane arose from the lipids of methanotrophic bacteria. These organisms use thermogenic and/or biogenic methane, which is the only known natural carbon source showing such a prominent ^{13}C depletion. For the Marmorito material, the nearly identical isotopic compositions of crocetane and PME indicate a common origin for both. The exceptional ^{13}C depletion is likewise consistent with a biosynthesis that directly or indirectly involves isotopically light, most likely biogenic, methane as a carbon source for lipid synthesis.

3.1.2. *Hop-17(21)-ene*

The most abundant cyclic hydrocarbon by far present in the Marmorito limestone was hop-17(21)-ene. Other C_{27} to C_{30} hopanoids such as neohop-13(18)-ene and several saturated homologs, were observed in minor or trace amounts. Homologs with carbon numbers $>C_{30}$ accounted for <1% of the hopanoid fraction. This restricted carbon number distribution suggests formation from a common C_{30} precursor. Indeed, hop-17(21)-ene and neohop-13(18)-ene have been shown to originate from the diagenetic rearrangement of hop-22(29)-ene (diploptene), a "primitive" C_{30} triterpenoid that usually co-occurs with the hopanepolyols in prokaryotic lipids (Rohmer et al., 1984; Ageta et al., 1987, Farrimond and Telnaes, 1996; Moldowan et al., 1991). The hop-17(21)-ene from the carbonate is depleted isotopically ($\delta^{13}C$ = −83.2‰) but differs from that of crocetane and PME by approximately 30‰. Apparently, a major portion, if not all, of the hop-17(21)-ene is derived from a bacterial source different to the acyclic isoprenoids. However, the origin of this compound is yet unclear. Because it has not been reported from anaerobic bacteria, including sulfate-reducing bacteria (SRB), a likely source of the hop-17(21)-ene may be methanotrophic bacteria, many of which are known to produce significant amounts of the likely precursor molecule hop-22(29)-ene (diploptene; Rohmer et al., 1984).

3.2. Alcohols

The alcohol/ketone fraction obtained shows a fairly simple pattern with high amounts of 3,7,11,15-tetramethylhexadecan-1-ol (phytanol) and a distinctive glycerol ether lipid compound containing isoprenoid (phytanyl-) moieties (Fig. 2). The structure of the latter was tentatively assigned by GC-MS (Fig. 3). The electron impact mass spectrum, containing a strong m/z 383 fragment ion, clearly indicates a glycerol ether structure with an O-phytanyl substituent at C-2 (Schaeffer, 1993). GC-MS analysis with low ionization energy (33eV) resulted in the detection of a proposed molecular ion at m/z 638, suggesting a C_{16}-substituent ether bound at C-1.

GC–C–IRMS measurements revealed highly depleted, practically identical isotope ratios, for both the ether lipid and phytanol ($\delta^{13}C \sim -108$‰; Table 1). Both compounds have similar isotopically depleted values to crocetane (−115.6‰) and PME (−112.2‰), suggesting a common biological origin for all these compounds. Archaea are the only known sources of ether-bound isoprenoids linked to glycerol (Michaelis and Albrecht, 1979; Chappe et al., 1982; Langworthy, 1985). The observation of the glycerol ether thus also sustains an origin for the extremely isotopically light isoprenoids from archaea.

Other alcohols observed included *iso*- and *anteiso*-pentadecan-1-ol (ω2 and ω3, respectively), n-hexadecan-1-ol, and 10-methylhexadecan-1-ol. The compounds show $\delta^{13}C$ values close to −88‰ (Table 1). They differ from the values found for the acyclic isoprenoids, for hop-17(21)-ene and for other compounds of supposedly allochthonous or mixed origin. Information on the biomarker significance of sedimentary *iso*- and *anteiso*- and mid-chain branched alcohols is scarce. Nevertheless, *iso*- and *anteiso*-branched carbon chains are widespread building blocks within the lipids of bacteria (for a review, see Kaneda, 1991) but seem to be absent from cyanobacteria (e.g., Cohen and Vonshak, 1991). Terminally branched fatty acids are found ubiquitously in modern and fossil sediments and are well accepted as molecular markers for bacterially derived organic matter. They are particularly prevalent in SRB, like the common *Desulfovibrio* (Boon et al., 1977; Edlund et al., 1985). Individual mid-chain branched fatty acids have likewise been observed in several pure cultured prokaryotes. Within common aquatic bacteria, a prominent occurrence of a hexadecyl chain with C-10 methyl branching is 10-methylhexadecanoic acid in *Desulfobacter* sp. (Dowling et al., 1986). Although not exclusively found in SRB, this compound has been used as a marker for organic matter contributions from these bacteria (e.g., Rajendran et al., 1993). We thus suggest an origin of the C_{15}-C_{17} alcohols found from SRB that may have co-existed with archaea at the ancient seep site.

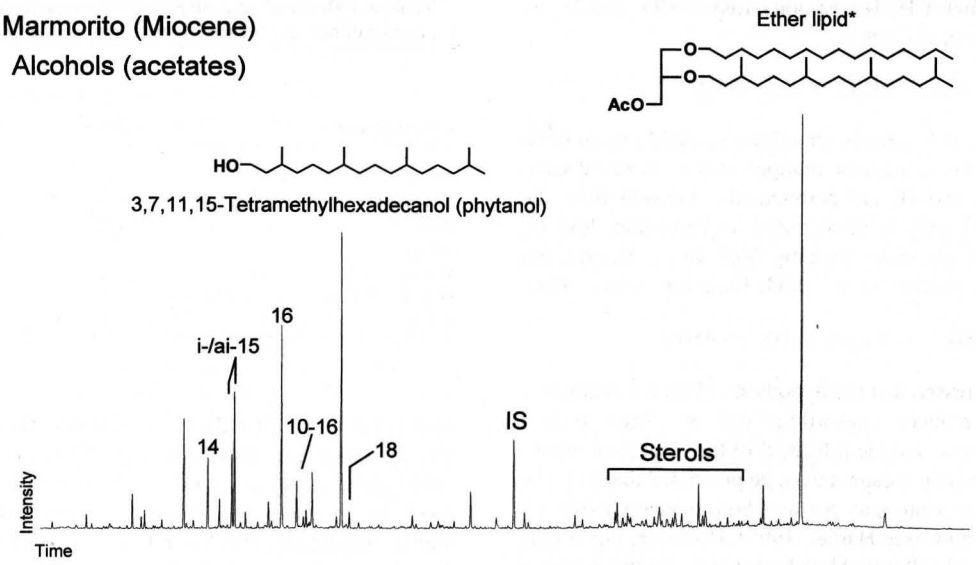

Fig. 2. Gas chromatogram of the alcohol fraction (acetates) extracted from the total rock after decalcification. Numbers denote *n*-alkan-1-ols of the respective carbon chain length; 10–16 = 10-methylhexadecan-1-ol; i/ai-15-*iso*- and *anteiso*-pentadecan-1-ol, respectively; IS-internal standard (5α(H)-cholestane). *"Ether lipid" refers to the compound tentatively assigned as 1-O-hexadecyl-2-O-phytanylglycerol.

3.3. Implications for Biogeochemical Processes

At first glance, the isoprenoids from the Marmorito Miocene limestone exhibit a paradox. They reveal evidence for both archaeal methanogenesis (compound structures) and methanotrophy (environment, isotope ratios). However, it has long been argued that there may be a direct link between methane formation and anaerobic methane oxidation (e.g., Zehnder and Brock, 1980). Recent investigations sustain the idea that certain archaea are able to reverse methanogenesis, although the mechanisms and controls are not yet fully understood. Radiotracer experiments in the laboratory have shown that methanogens re-oxidize a small portion of the dissimilated methane back to CO_2 (Zehnder and Brock, 1979; Harder, 1997). Yet, rates of up to 900 ppm $^{14}CO_2$ formed within 9 days from a $^{14}CH_4$ substrate (Harder, 1997) would not be expected to account for a significant methane consumption in the natural environment.

However, in sediments, efficient *net* anaerobic methane oxidation is typically observed at the base of the sulfate reduction zone. This process is apparently coupled to sulfate reduction (Martens and Klump, 1980; Reeburgh, 1980), although SRB themselves do not appear to use methane as a substrate (Harder, 1997). Based on field and laboratory studies, a model has been

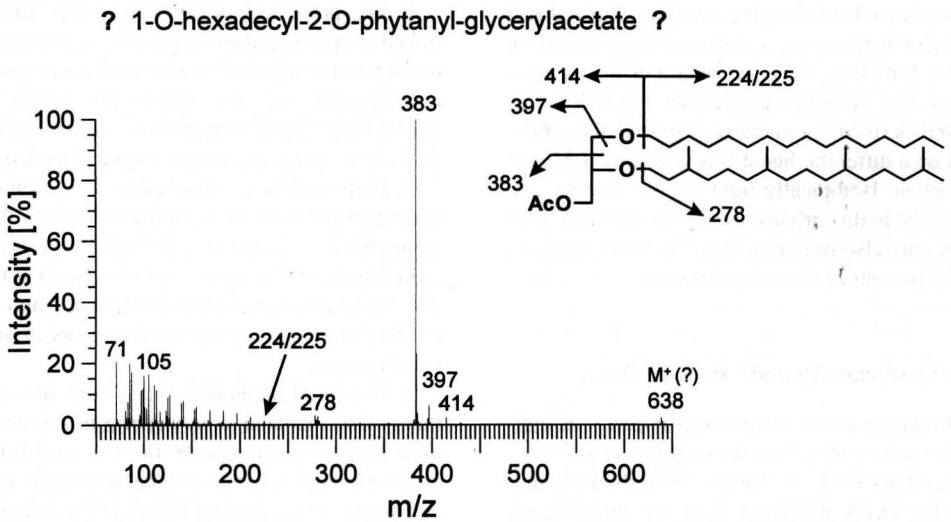

Fig. 3. Mass spectrum (33eV), tentative structure, and fragmentation pattern of the ether lipid present in the Marmorito carbonate. The mass spectrum is consistent with 1-O-hexadecyl-2-O-phytanylglycerol (3-acetate derivative). Scan range m/z 70 to 700.

developed in which CH_4 is first converted to CO_2 and H_2 by archaea (Hoehler et al., 1994):

$$CH_4 + 2H_2O \Rightarrow CO_2 + 4H_2$$

Conditions for this reaction should be favorable in an environment where excess methane is supplied (i.e., at a cold seep) and where CO_2 and H_2 are permanently removed from the system. Whereas CO_2 is precipitated as carbonate, low H_2 partial pressures are maintained by SRB (e.g., Alperin and Reeburgh, 1985; Hoehler et al., 1994; Blair and Aller, 1995):

$$HSO_4^- + 4H_2 \Rightarrow + HS^- + 4H_2O$$

It should be stressed that the hypothesis of such a syntrophic archaea/sulfate reducer consortium has not been proven through the isolation and identification of the respective microorganisms. At present, it cannot even be precluded that a single, yet unknown, bacterium may perform both methane oxidation and sulfate reduction (see Harder, 1997). However, our results from an ancient, ca. 20 my old sediment indicate the presence of a discrete microbial community which controlled the biochemical processes during the formation of the carbonate deposit. The biomarkers found exactly match the idea of a syntrophic methanogen/sulfate reducer consortium being responsible for anaerobic methane consumption. Hence, we consider Marmorito as a conceivable ancient counterpart for the process proposed to occur in recent ecosystems.

It is tempting to take the isotope values from the Marmorito isoprenoids as an indication for a direct transfer of methane carbon into cellular biomass by certain archaea. However, it should be noted that a severe ^{13}C depletion may also result from fractionation effects during uptake of other substrates like acetate or methylamine by archaea. A recent investigation on fractionation effects during the growth of methylotrophic methanogens revealed an isotope effect (ϵ) of $\epsilon \approx 79.9‰$ for the transformation of trimethylamine carbon into phytanyl ether moieties in laboratory cultures of *Methanococcoides burtonii* (Summons et al., 1998). Although these extreme values were observed for nonlimiting substrate conditions that are not likely to occur in the natural environment, the conclusion of a *direct* archaeal utilization of methane as a carbon source should be drawn with caution. Similarly, the exact nature of the material utilized by the associated SRB is as yet unclear. The $\delta^{13}C$ range of supposedly SRB-derived biomarkers suggests that these organisms thrived on a different, but still isotopically depleted, type of organic carbon. Isotopically light organic matter, like low molecular weight hydrocarbons, could be supplied with seepage fluids, but may also originate from the broad range of organisms typically present in such environments (e.g. aerobic methanotrophs).

3.4. "Free" vs. "Carbonate-Bound" Hydrocarbons

Remarkable differences in the relative distributions of individual hydrocarbons can be seen from the comparison of "free" vs. "total" hydrocarbons (Fig. 1; Table 2). High molecular weight *n*-alkanes are easily extracted from the undissolved, powdered rock. In contrast, <15% of the total, free, and total extractable crocetane, PME, and hop-17(21)-ene were obtained from the initial, powdered rock extract. These compounds were preferentially released after carbonate dissolution (>85%). Obviously, crocetane, PME, and hop-17(21)-ene show a particularly tight association with the carbonate rock, most likely as a result of an intracrystalline preservation within the mineral matrix. Apparently, their microbial source organisms have been directly associated with the loci of carbonate precipitation.

Table 2. Calculated percentages of individual hydrocarbons in 'free' (intercrystalline) and 'matrix' bound (intracrystalline) state (see 2.2.).

	[%]	
Compound	Free	Matrix
n-C_{18}	39	61
Crocetane	14	86
PME	13	87
n-C_{26}	69	31
n-C_{29}	67	33
Hop-17(21)-ene	8	92
n-C_{32}	76	24

Two main processes are suggested to cause such a microbially driven formation of carbonate rocks: (1) The precipitation of CO_2 derived from microbial methane oxidation and (2) the presence of organic matrices that attract Ca^{2+} ions from the surrounding sea water and trigger the nucleation of $CaCO_3$ (Mitterer and Cunningham, 1985; Addadi and Weiner 1989). The latter process is proposed to support the growth of microbialitic carbonates in a variety of aquatic environments, where bacterial biofilms provide Ca^{2+}-binding organic macromolecules and increased alkalinity prevails (e.g., due to sulfate reduction; Reitner, 1993). We propose that such conditions characterized the former Marmorito environment and could well account for the observations made for the samples studied. We therefore suggest that a more precise characterization of such carbonates is cold seep "microbialites" rather than mere "authigenic" limestones.

4. CONCLUSIONS

1. The prominent occurrence of the "irregular," tail-to-tail linked acyclic C_{20} isoprenoid 2,6,11,15-tetramethylhexadecane (crocetane) is reported from a geological sample. Crocetane is accompanied by the tail-to-tail linked C_{25} isoprenoid 2,6,10,15,19-pentamethylicosane (PME) and a discrete glycerol ether lipid containing a phytanyl moiety.

2. Their similar, extremely low $\delta^{13}C$ values and their chemical structures indicate a common origin of these biomolecules from archaea. Apparently, these archaea were able to directly or indirectly utilize methane-derived carbon for lipid synthesis. The co-occurrence of these compounds may represent a diagnostic pattern for ancient environments of anaerobic methane consumption.

3. The fossil biomarker and isotope inventories support the theory that methanogenic bacteria in a syntrophic community with SRB are responsible for the oxidation of methane in anaerobic habitats. The biomarker pattern from Marmorito is consistent with a defined bacterial consortium of archaea able to reverse methanogenesis and SRB that oxidize the resulting hydrogen and maintain low H_2 partial pressures favorable for this reaction.

4. A more pronounced association with the carbonate matrix was observed for crocetane, PME, and hop-17(21)-ene than for other members of the hydrocarbon fraction. This implies a constructive role of their source bacteria in carbonate formation and characterizes the Marmorito carbonates as cold seep "microbialites."

Acknowledgments—P. Clari and L. Martire are thanked for support and excellent collaboration during the field work in Italy. P. Albrecht and K. Simon are acknowledged for their assistance with stable isotope measurements. We also thank S. J. Rowland for a sample of synthetic crocetane. C. Roos is acknowledged for performing the GC experiments. We also thank R. Summons, who provided unpublished results as well as valuable comments on the scientific content of the paper. J. M. Hayes and co-workers are acknowledged gratefully for providing unpublished results from their studies on the occurrence of crocetane in recent environments. We appreciate the careful revision of two anonymous reviewers who greatly aided in improving the original manuscript. Our study was supported by the Deutsche Forschungsgemeinschaft (Mi-157/10; Re-665/12; Th-713/1; SFB 468).

REFERENCES

Addadi L. and Weiner S. (1989) Stereochemical and structural relations between macromolecules and crystals in biomineralization. In *Biomineralization* (eds. S. Mann, J. Webb, and R. J. P. Williiams), pp. 133–156. Weinheim (VCH).

Ageta H., Shiojima K., and Arai Y. (1987) Acid-induced rearrangement of triterpenoid hydrocarbons belonging to the hopane and migrated hopane series. *Chem. Pharm. Bull.* **35,** 2705–2716.

Aharon P. and Gupta B. K. S. (1994) Bathymetric reconstructions of the Miocene-age "calcari a *Lucina*" (Northern Apennines, Italy) from oxygen isotopes and benthic Foraminifera. *Geo-Mar. Lett.* **14,** 219–230.

Alperin M. J. and Reeburgh W. S. (1985) Inhibition experiments on anaerobic methane oxidation. *Appl. Environ. Microbiol.* **50,** 940–945.

Beauchamp B., Krouse H. R., Harrison J. C., Nassichuk W. W., and Eliuk L. S. (1989) Cretaceous cold seep communities and methane-derived carbonates in the Canadian Arctic. *Science* **244,** 53–56.

Bian L. (1994) Isotopic biogeochemistry of individual compounds in a modern coastal marine sediment (Kattegat, Denmark and Sweden). M.Sc. thesis. Dept. of Geological Sciences, Univ. Indiana.

Blair N. E. and Aller R. C. (1995) Anaerobic methane oxidation on the Amazon shelf. *Geochim. Cosmochim. Acta* **59,** 3707–3715.

Boon J. J., de Leeuw J. W., Hoeck V. G., and Vosjan J. H. (1977) The significance of taxonomical value of *iso-* and *anteiso*-monoenic fatty acids and branched β-hydroxy acids in *Desulfovibrio desulfuricans*. *J. Bacteriol.* **129,** 1183–1191.

Brassell S. C., Wardroper A. M. K., Thomson I. D., Maxwell J. R., and Eglinton G. (1981) Specific acyclic isoprenoids as biological markers of methanogenic bacteria in marine sediments. *Nature* **290,** 693–696.

Campbell K. A., Carson C., and Bottjer D. J. (1993) Fossil cold seep limestones and associated chemosynthetic macroinvertebrate faunas, Jurassic-Cretaceous Great Valley Group, California. In *Advances in the Sedimentary Geology of the Great Valley Group, Sacramento Valley, California* (eds. S. A. Graham and D. R. Lowe), Vol. 73, pp. 37–50. Society of Economic Paleontologists and Mineralogists, Pacific Section.

Cavanaugh C. M., Levering R. R., Maki J. S., Mitchell R., and Lidstrom M. E. (1987) Symbiosis of methylotrophic bacteria and deep-sea mussels. *Nature* **325,** 346–348.

Chappe B., Albrecht P., and Michaelis W. (1982) Polar lipids of archaebacteria in sediments and petroleum. *Science* **217,** 65–66.

Clari P., Gagiardi C., Governa M. E., Ricci B., and Zuppi G. M. (1988) I calcari de Marmorito: Una testimonianza di processi diagenetici in presenza di metano. *Bollettino del Museo Regionale di Scienze Naturali Torino* **6,** 197–216.

Clari P., Fornara L., Ricci B., and Zuppi G. M. (1994) Methane-derived carbonates and chemosymbiotic communities of Piedmont (Miocene, Northern Italy). An update. *Geo-Mar. Lett.* **14,** 201–209.

Cohen Z. and Vonshak A. (1991) Fatty acid composition of *Spirulina* and *Spirulina*-like cyanobacteria in relation to their chemotaxonomy. *Phytochemistry* **30,** 205–206.

Dando P. R., Austen M. C., Burke R. A., Kendall M. A., Kennicutt M. C. II, Judd A. G., Moore D. C., Schmaljohann R., and Southward A. J. (1991) Ecology of a North Sea pockmark with an active methane seep. *Mar. Ecol. Progr. Ser.* **70,** 49–63.

Dowling N. J. E., Widdel F., and White D. C. (1986) Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide-forming bacteria. *J. Gen. Microbiol.* **132,** 1815–1825.

Edlund A., Nichols P. D., Roffey R., and White D. C. (1985) Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. *J. Lipid Res.* **26,** 982–988.

Farrimond P. and Telnaes N. (1996) Three series of rearranged hopanes in Toarcian sediments (Northern Italy). *Org. Geochem.* **25,** 165–177.

Gaillard C., Rio M., and Rolin Y. (1992) Fossil chemosynthetic communities related to vents or seeps in sedimentary basins: The pseudobioherms of southeastern France compared to other world examples. *Palaios* **7,** 451–465.

Grice K., Schouten S., Nissenbaum A., Charrach J., and Sinninghe Damsté. (1998) Isotopically heavy carbon in the C_{21} to C_{25} regular isoprenoids in halite-rich deposits from the Sdom Formation, Dead Sea Basin, Israel. *Org. Geochem.* **28,** 349–359.

Hahn J. (1982) Geochemical fossils of a possibly archaebacterial origin in ancient sediments. *Zbl. Bakt. Hyg. I. Abt. Orig. C* **3,** 40–52.

Harder J. (1997) Anaerobic methane oxidation by bacteria employing ^{14}C-methane uncontaminated with ^{14}C-carbon monoxide. *Mar. Geol.* **137,** 13–23.

Hoehler T., Alperin M. J., Albert D. B., and Martens C. (1994) Field and laboratory studies of methane oxidation in an anoxic marine sediment: Evidence for a methanogen-sulfate reducer consortium. *Global Biogeochem. Cycles* **8,** 451–463.

Holzer G., Oro J., and Tornabene T. G. (1979) Gas chromatographic-mass spectrometric analysis of neutral lipids from methanogenic and thermoacidophilic bacteria. *J. Chromatogr.* **186,** 795–809.

Jørgensen N. O. (1989) Holocene methane-derived, dolomite cemented sandstone pillars from the Kattegat, Denmark. *Mar. Geol.* **88,** 71–81.

Kaneda T. (1991) *Iso-* and *anteiso*-fatty acids in bacteria: Biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* **55,** 288–302.

Kelly S. R. A., Ditchfield P. W., Doubleday P. A., and Marshall J. D. (1995) An Upper Jurassic methane-seep limestone from the fossil Bluff Group forearc basin of Alexander Island, Antartica. *J. Sed. Res.* **65,** 274–282.

Kennicutt M. C. II, Brooks J. M., Bidigare R. R., Fay R. R., Wade T. L., and MacDonald T. J. (1985) Vent-type taxa in a hydrocarbon seep region on the Louisiana slope. *Nature* **317,** 351–353.

Kulm L. D., Suess E., Moore J. C., Carson B., Lewis B. T., Ritger S. D., Kadko D. C., Thornburg T. M., Embley R. W., Rugh W. D., Massoth G. J., Langseth M. G., Cochrane G. R., and Scamman R. L. (1986) Oregon subduction zone: Venting fauna and carbonates. *Science* **231,** 561–566.

Langworthy T. A. (1985) Lipids of archaebacteria. In *The Bacteria. A Treatise on Structure and Function. VIII: Archaebacteria* (eds. C. R. Woese and R. S. Wolfe), pp. 459–497. Academic Press.

La Rock P. A., Hyun J. H., and Bennison B. W. (1994) Bacterioplankton growth and production at the Louisiana hydrocarbon seeps. *Geo-Mar. Lett.* **14,** 104–109.

Martens C. S. and Klump J. V. (1980) Biogeochemical cycling in an organic-rich coastal marine basin. 1. Methane sediment-water exchange processes. *Geochim. Cosmochim. Acta* **44,** 471–490.

McCarthy E. D. (1967) A treatise in organic geochemistry. Ph.D. thesis. Univ. California.

Michaelis W. and Albrecht P. (1979) Molecular fossils of archaebacteria in kerogen. *Naturwissenschaften* **66,** 420–421.

Mitterer R. M. and Cunningham R. (1985) The interaction of natural organic matter with grain surfaces: Implications for calcium carbonate precipitation. In: *Carbonate Cements* (eds. N. Schneidermann and P. M. Harris), Vol. 36, pp. 17–31. *Soc. Econ. Paleontol. Mineralog.* Spec. Publ.

Moldowan J. M., Fago F. J., Carlson R. M. K., Young D. C., van Duyne

G., Clardy J., Schoell M., Pillinger C., and Watt D. S. (1991) Rearranged hopanes in sediments and petroleum. *Geochim. Cosmochim. Acta* **55,** 3333–3353.

Paull C. K., Hecker B., Commeau R., Freeman–Lynde R. P., Neumann A. C., Corso W. P., Golubic S. Hook J. Sikes E., and Curray J. (1984) Biological communities at Florida Escarpment resemble hydrothermal vent communities. *Science* **226,** 965–967.

Rajendran N., Suwa Y., and Urushigawa Y. (1992) Distribution of phospholipid-linked fatty acid biomarkers for bacteria in the sediment of Ise Bay, Japan. *Mar. Chem.* **42,** 39–56.

Reeburgh W. S (1980) Anaerobic methane oxidation: Rate depth distributions in Skan Bay sediments. *Earth. Planet. Sci. Lett.* **47,** 345–352.

Reitner J. (1993) Modern cryptic microbialite/metazoan facies from Lizard Island (Great Barrier Reef, Australia)—Formation and Concepts. *Facies* **29,** 3–40.

Ricci Lucchi F. and Vai G. B. (1994) A stratigraphic and tectonofacies framework of the "calcari a *Lucina*" in the Apennine Chain, Italy. *Geo-Mar. Lett.* **14,** 210–218.

Risatti J. B., Rowland S. J., Yon D. A., and Maxwell J. R. (1984) Stereochemical studies of acyclic isoprenoids. XII. Lipids of methanogenic bacteria and possible contributions to sediments. *Org. Geochem.* **6,** 93–104.

Ritger S., Carson B., and Suess E. (1987) Methane-derived authigenic carbonates formed by subduction-induced pore-water expulsion along the Oregon/Washington margin. *Geol. Soc. Am. Bull.* **98,** 147–156.

Roberts H. H., Aharon P., and Walsh M. M. (1993) Cold-seep carbonates of the Louisiana continental slope-to-basin floor. In: *Carbonate Microfabrics* (eds. R. Rezak and D. L. Lavoie), pp. 95–104. Springer-Verlag.

Robson J. N. and Rowland S. J. (1993) Synthesis, chromatographic and spectral characterisation of 2,6,11,15-tetramethylhexadecane (crocetane) and 2,6,9,13-tetramethyltetradecane: Reference acyclic isoprenoids for geochemical studies. *Org. Geochem.* **20,** 1093–1098.

Rohmer M., Bouvier-Nave P., and Ourisson G. (1984) Distribution of hopanoid triterpenes in pokaryotes. *J. Gen. Microbiol.* **130,** 1137–1150.

Sassen R., Poberts H. H., Aharon P., Larkin J., Chinn E. W., and Carney R. (1993) Chemosynthetic bacterial mats at cold hydrocarbon seeps, Gulf of Mexico continetal slope. *Org. Geochem.* **20,** 77–89.

Sassen R., MacDonald I. R., Requejo A. G., Guinasso N. L. Jr., Kennicutt M. C. II, Sweet S. T., and Brooks J. M. (1994) Organic geochemistry of sediments from chemosynthetic communities, Gulf of Mexico slope. *Geo-Mar. Lett.* **14,** 110–119.

Schaeffer P. (1993) Marqueurs biologique de milieux evaporitiques. Ph.D. thesis. Université Louis Pasteur, Strasbourg.

Schouten S., van der Maarel J. E. C., Huber R., and Sinninghe Damsté J. S. (1997) 2,6,10,15,19-Pentamethylicosenes in *Methanolobus bombayensis*, a marine methanogenic archaeon, and in *Methanosarcina mazei. Org. Geochem.* **26,** 409–414.

Suess E. and Whiticar M. J. (1989) Methane-derived CO_2 in pore fluids expelled from the Oregon subduction zone. *Palaeogeogr. Palaeoclimato. Palaeoecol.* **71,** 119–136.

Summons R. E., Jahnke L. L., and Roksandic Z. (1994) Carbon isotopic fractionation in lipids from methanotrophic bacteria: Relevance for interpretation of the geochemical record of biomarkers. *Geochim. Cosmochim. Acta* **58,** 2853–2863.

Summons R. E., Franzmann P. D., and Nichols P. D. (1998) Carbon isotopic fractionation associated with methylotrophic methanogenesis. *Org. Geochem.* **28,** 465–475.

Terzi C., Aharon P., Ricci Lucchi F., and Vai B. G. (1994) Petrography and stable isotope aspects of cold-vent activity imprinted on Miocene-age "calcari a *Lucina*" from Tuscan and Romagna Apennines, Italy. *Geo-Mar. Lett.* **14,** 177–184.

Tornabene T. G., Langworthy T. A., Holzer G., and Oro J. (1979) Squalenes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic archaebactera. *J. Mol. Evol.* **13,** 73–83.

Volkman J. K., Allen D. I. Stevenson P. L., and Burton H. R. (1986) Bacterial and algal hydrocarbons from a saline Antarctic lake, Ace Lake. *Org. Geochem.* **10,** 671–681.

von Bitter P. H., Scott S. D., and Schenk P. (1990) Early Carboniferous low-temperature hydrothermal vent communities from Newfoundland. *Nature* **344,** 145–148.

Wakeham S. G. (1990) Algal and bacterial hydrocarbons in particulate matter and interfacial sediment of the Cariaco Trench. *Geochim. Cosmochim. Acta* **54,** 1325–1336.

Whiticar M. J., Faber E., and Schoell M. (1986) Biogenic methane formation in marine and freshwater environments; CO_2 reduction *vs.* acetate fermentation-isotope evidence. *Geochim. Cosmochim. Acta* **50,** 693–709.

Zehnder A. J. B. and Brock T. D. (1979) Methane formation and methane oxidation by methanogenic bacteria. *J. Bacteriol.* **137,** 420–432.

Zehnder A. J. B. and Brock T. D. (1980) Anaerobic methane oxidation: Occurrence and ecology. *Appl. Environm. Microbiol.* **39,** 194–204.