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Chemical Geology 195 (2003) 29–58

CHEMICAL GEOLOGY
INCLUDING
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Carbon isotopic compositions of prokaryotic lipids as tracers of carbon cycling in diverse settings

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Received 15 October 2000; received in revised form 15 July 2001

Abstract

Recent work has highlighted the ubiquity of bacteria and archaea and continues to affirm their significance in diverse biogeochemical processes. The $\delta^{13}C$ values and distributions of diagnostic lipids derived from such microorganisms can provide insights into prokaryotic processes in modern settings and are one of the few means by which past prokaryotic processes in depositional settings can be elucidated. Here we present a brief review of prokaryotic lipids commonly observed in sediments and the controls on their carbon isotopic compositions. We then present two case studies—one of Mediterranean cold seeps at which anaerobic oxidation of methane occurs and one of a Holocene peat deposit in which a variety of aerobic and anaerobic microorganisms govern the degradation of organic matter. These two studies illustrate the potential utility of compound-specific carbon isotope analyses to deconvolute pathways of carbon flow in microbial communities.

Keywords: Bacteria; Archaea; Carbon isotopes; Peat; Cold seeps; Biomarkers; Hopanoids; Fatty acids; Ether lipids

1. Introduction

Carbon dioxide and methane are two of the most significant greenhouse gases, and controls on their production and consumption are fundamental to our understanding of ancient climate, the biogeochemical cycling of carbon, and the potential for future changes in greenhouse gas concentrations as a consequence of anthropogenic activity. In both marine and terrestrial settings, bacteria and archaea are involved with a range of processes that directly impact the global carbon cycle and, thus, atmospheric levels of these greenhouse gases. Such processes include photosynthesis by cyanobacteria, methane oxidation, and multiple aerobic and anaerobic respiration reactions including methanogenesis. Consequently, biomarker and isotope proxies for prokaryotic processes in ancient and modern settings can be used to evaluate environmental conditions and elucidate pathways of carbon flow.

The lipids of prokaryotes and their carbon isotopic compositions have been shown to be highly useful in such endeavors. Prokaryotic lipids are among the most abundant compounds present in marine and terrestrial, modern and ancient sediments. In fact, it has been suggested that the hopanoids, pentacyclic triterpenoid membrane lipids of bacteria (Ourisson et al., 1987), are the most abundant organic compounds on earth (Our-

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doi:10.1016/S0009-2541(02)00387-X

isson and Albrecht, 1992). Moreover, recent work has highlighted the ubiquity of archaeal lipids in marine settings (Hoefs et al., 1997; Schouten et al., 1998, 2000). Because these lipids have diverse functionalities and carbon skeletons, biomarkers diagnostic for specific organisms and potentially specific processes have been and will continue to be developed. Thus, the structure of prokaryotic lipids can be significant repositories of information on ancient environmental conditions and provide windows into prokaryotic processes in complex modern ecosystems.

Further insight can be provided by the carbon isotopic compositions ($\delta^{13}C$ values) of lipids as determined by compound-specific isotope analyses. The controls on the carbon isotopic compositions of individual prokaryotic lipids are diverse and include the source of substrate carbon, the biological mechanism of carbon assimilation (Sirevag et al., 1977; van der Meer et al., 2001a,b), and pathways of lipid biosynthesis (Teece et al., 1999; Summons et al., 1998; Jahnke et al., 1999; Sakata et al., 1997). The diversity of these controls complicates interpretation of prokaryotic lipid $\delta^{13}C$ values, but nonetheless provides opportunities to gain further insight into the biology and chemistry of sedimentary systems.

In this paper, we provide a brief overview of prokaryotic biomarkers and the controls on their $\delta^{13}C$ values and briefly discuss examples in which prokaryotic biomarker $\delta^{13}C$ values have been used to evaluate carbon dynamics in ancient environments. We then present two case studies. The first is an investigation of carbon cycling in ancient peat deposits and is an expansion of work previously published by our group (Pancost et al., 2000b). The second is an overview of recent work by several groups, including our own, in which archaeal and bacterial lipid $\delta^{13}C$ values have been used to identify the microorganisms involved with anaerobic methane oxidation (e.g. Pancost et al., 2000a). In both cases we present new data that further illustrate how molecular and isotopic techniques can be used to gain insight into carbon cycling in the respective settings.

2. Methods

The methods used are well established and have been discussed in detail elsewhere. Nonetheless, it is useful to briefly discuss the most commonly employed preparative and analytical techniques and how they are used to obtain $\delta^{13}C$ values for compounds of interest. Samples are typically freeze-dried, if necessary, and then extracted via one of a variety of techniques, including modified Bligh–Dyer extractions, extractions utilizing a Soxhlet apparatus, or sonic extraction. Typically, such extractions employ polar solvents including dichloromethane (DCM), methanol, or chloroform. However, it has been shown (Nishihara and Koga, 1987) that addition of acid increases the efficiency with which highly polar archaeal tetraether lipids are extracted.

Resulting total lipid extracts can be analysed as such, but typically the compound mixture is too complex—particularly for compound-specific isotopic analyses—and subsequent separation techniques are necessary. The variety of fractionation schemes is large, but in the two case studies described here, relatively simple separations of the total extract into apolar and polar fractions were obtained by column chromatography in which the columns had been packed with activated alumina and the eluents were hexane/DCM (9:1 v/v) and methanol/DCM (1:1 v/v) (Pancost et al., 2000a). After appropriate compound derivatisation, sufficiently volatile compounds can then be analysed by gas chromatography (GC) and mass spectrometry (GC-MS) (for specific methods used by our lab, see Schouten et al., 1998).

However, many compounds of geomicrobiological interest are often either too large or too polar to be volatile under GC conditions. In such cases, compounds can be quantified and identified using high pressure liquid chromatography-mass spectrometry (HPLC-MS) as Hopmans et al. (2000) described for archaeal tetraether lipids and Fox et al. (1998) described for bacteriohopanpolyols. However, HPLC instrumentation has yet to be interfaced to an isotope ratio monitoring-mass spectrometer, and chemical degradation is required to determine the isotopic compositions of representative components of these compounds. Such techniques are numerous and a complete description is beyond the scope of this paper. The types of compounds of geomicrobiological interest that are not GC-amenable typically include bacterial phospholipid fatty acids and bacteriohopanpolyols and archaeal ether lipids. For phospholipids, the fatty acid is typically released from the glycerol moiety via

either saponification or transmethylation. Cleavage of the first vicinal dihydroxy moiety in the side-chain of bacteriohopanpolyols by periodic acid (H_5IO_6)/sodium borohydride treatment will generate primary hopanoid alcohols (Rohmer et al., 1984). Cleavage of ether bonds in glycerol di- and tetraether lipids is accomplished using HI with subsequent hydrogenation by $LiAlH_4$ (Schouten et al., 1998). The latter technique can be particularly important even in ancient samples because ether bonds can be preserved deep into the geologic record (Schouten et al., 1998).

Upon appropriate derivatisation of polar compounds, compound fractions can be analysed by gas chromatography/isotope ratio monitoring mass spectrometry (GC-IRMS), allowing the carbon isotopic compositions of individual compounds to be determined (Hayes et al., 1990). The specific operating conditions typically employed in our lab and used in the two case studies described here are described in detail in Schouten et al. (1998), but the general operating principles are the same for all GC-IRMS systems. Sample aliquots are injected into the GC and the compounds are partitioned on a capillary column as in normal GC or GC-MS operation; however, eluting compounds are passed through an oxidizing furnace, which converts organic compounds into CO_2 and H_2O. The H_2O is subsequently removed, and CO_2 enters the mass spectrometer through a continuous flow interface. A typical GC-IRMS trace is shown in Fig. 1 along with the characteristic 'swing' in the 45:44 ratio due to chromatographic separation of isotopically distinct species. Isotopic compositions are expressed relative to the primary standard Pee Dee Belemnite (VPDB): $\delta^{13}C=(^{13}R_{SA}/^{13}R_{PDB}-1)\times 10^3$ where $^{13}R_{SA}$ and $^{13}R_{PDB}$ represent the $^{13}C/^{12}C$ abundance ratios for the sample and PDB, respectively. Established over a decade ago, GC-IRMS has since become an invaluable tool in the investigation of modern ecosystems and ancient sediments.

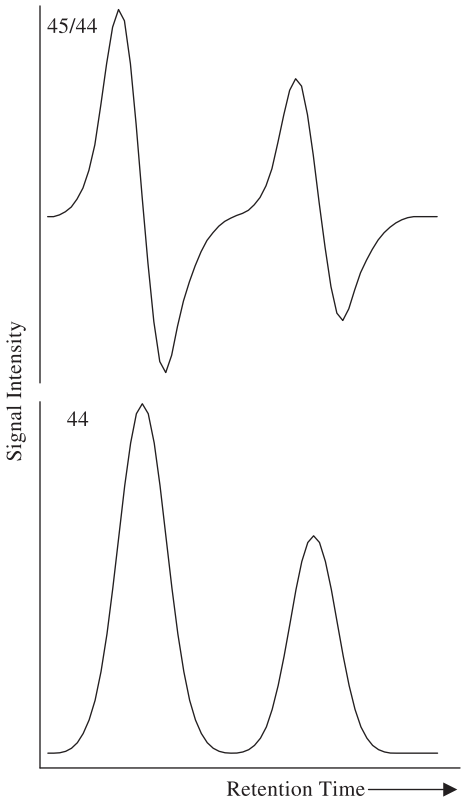


Fig. 1. A characteristic GC-IRMS trace. The lower chromatogram shows the mass 44 signal, which is analogous to a gas chromatogram. The upper trace shows the 45:44 mass ratio which reflects the isotopic composition of CO_2 entering the mass spectrometer source. Note the characteristic 'swings' in the 45:44 ratio trace which reflects the fact that compounds containing greater quantities of ^{13}C elute earlier.

3. Diversity of prokaryotic lipids

The capacity of prokaryotic lipids to provide useful information in either modern or ancient investigations depends on whether a given lipid is diagnostic for a specific organism. Examples of highly diagnostic biomarkers include okenone and isorenieratene, carotenoids synthesized by the purple sulphur bacteria, and the brown strain of green sulphur bacteria, respectively (Liaaen-Jensen, 1978). However, few compounds are organism-specific and sources must be more broadly defined (e.g. hopanoids derived from aerobic bacteria) or interpreted in the context of environmental conditions (e.g. branched fatty acids as tracers of sulfate-reducing bacteria). In the following sections, we briefly describe some of the major compound classes of interest to geomicrobiologists.

3.1. Hopanoids

The hopanoids are pentacyclic triterpenoids and are membrane components of many bacteria, including

cyanobacteria, methanotrophs, and aerobic heterotrophic bacteria (Ourisson et al., 1987; Rohmer et al., 1992). Hopanoid structures have not been found in cultures of anaerobic bacteria and may be diagnostic for aerobic organisms (Ourisson et al., 1987), although the universality of this assertion remains to be confirmed (Pancost et al., 2000a). The most commonly observed hopanoids are diplopterol and bacteriohopanpolyol derivatives. Diplopterol is composed of a C_{30} hopanoid skeleton to which a hydroxy group is attached at the C_{22} position (Fig. 2a). Bacteriohopanoids are comprised of a basic C_{35} skeleton in which an *n*-pentyl group is attached to the hopanoid carbon skeleton at the C_{30} position. A diverse range of functional groups can be attached to the *n*-pentyl group; hydroxy groups are attached to 3 to 5 of the carbon atoms but amino and sugar groups are also commonly attached to the terminal carbon (Rohmer, 1993). It seems unlikely that detailed source-diagnostic information can be gleaned from either diplopterol or bacteriohopanoids because they are common in diverse groups of aerobic bacteria. Farrimond et al. (2000) proposed that bacteriohopanoid functionality could contain useful source-diagnostic information and suggested that hexafunctionalised bacteriohopanoids in sediment samples derive largely from Type I methanotrophs. However, the widespread applicability of this assertion has yet to be tested.

In contrast, the methylhopanoids appear to be highly source-diagnostic. In particular, it appears that 3β-methylbacteriohopanoids are relatively specific to some methane-oxidizing bacteria, although they have also been found in a cyanobacterium (Zundel and Rohmer, 1985). 2β-Methylbacteriohopanoids are common in and largely restricted to cyanobacteria (Summons et al., 1999), although Bisseret et al.

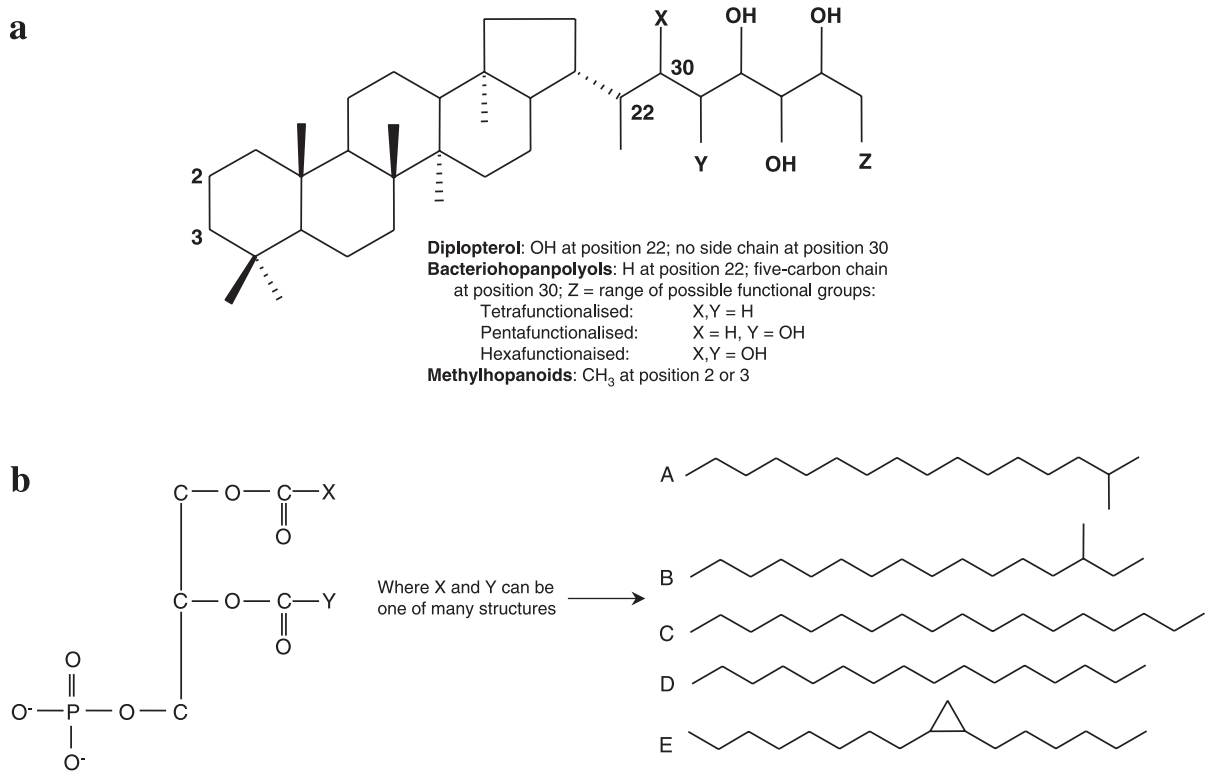


Fig. 2. Chemical structures of biomarkers for bacteria. Hopanoids are shown in (a) with selected carbon numbers indicated and is adapted from Rohmer et al. (1993); the accompanying text describes the differences between diplopterol, bacteriohopanpolyols, and methylhopanoids. For fatty acids (b) a generalised bacterial phospholipid is shown along with a few possible fatty acid carbon skeletons chosen to be representative of the types of functional groups commonly encountered in bacteria.

(1985) reported 2β-methyldiplopterol in both cyanobacteria and methanotrophs.

3.2. Fatty acids

Both free and bound (phospholipid) fatty acids (Fig. 2b) are common in the bacteria and eukarya and are not diagnostic as a class. The most common, such as saturated C_{16} and C_{18} fatty acids (e.g. C and D in Fig. 2b), are particularly widespread and appear to have little utility as tracers of explicit prokaryotic processes. However, some fatty acids, characterized by site-specific methyl groups, double bonds, or cyclic moieties are less common. Many of these are diagnostic for specific groups of organisms—for example, fatty acids containing cyclopropyl units (e.g. E in Fig. 2b) appear to be relatively more abundant in anaerobic bacteria (Fang and Barcelona, 1998 and references therein), possibly sulfate reducers. Branched fatty acids (e.g. A and B in Fig. 2b) are common in diverse bacteria (Kaneda, 1991), including sulfate reducers (Kaneda, 1991), actinomycetes (Kroppenstedt and Kutzner, 1978), and alkane-utilizing bacteria (Ringelberg et al., 1989); however, coupled to the presence of other fatty acids and evaluation within the context of sedimentary conditions more specific sources can be assigned. Among the most specific fatty acids are those for methanotrophic bacteria: monounsaturated C_{16} phospholipid fatty acids with the double bond (*cis* and *trans*) at the Δ8,9,10,11 positions are common in type I and type X methanotrophs, and monounsaturated C_{18} phospholipid fatty acids with the double bond (*cis* and *trans*) at the Δ10,11,12 positions are common in type II methanotrophs (Makula, 1978; Nichols et al., 1985; Jahnke and Diggs, 1989; Bowman et al., 1991).

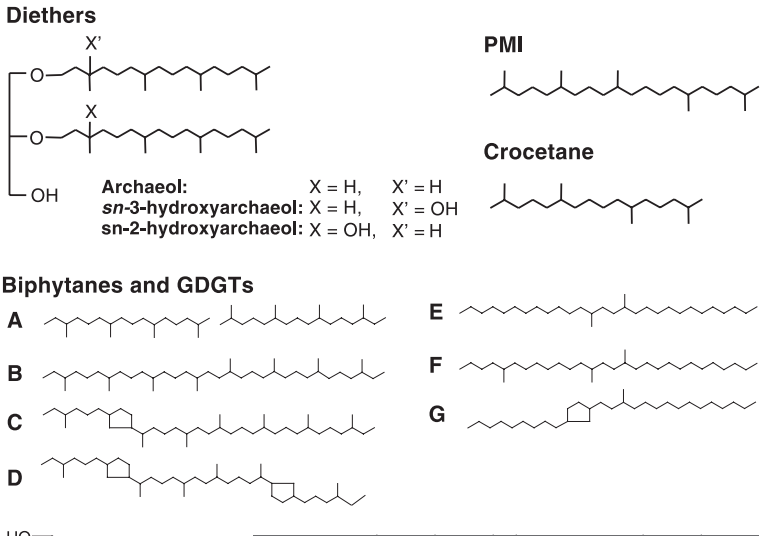
3.3. Other bacterial biomarkers

The previous discussions emphasized the fatty acids and hopanoids because they are common in bacteria involved with sedimentary cycling of carbon. In addition, a wide variety of compounds have been proposed as biomarkers for photosynthetic bacteria. These include biomarkers for cyanobacteria, including monomethyl alkanes (Gelpi et al., 1970; Shiea et al., 1990); a triunsaturated alkane apparently diagnostic for some *Chloroflexus* species (van der Meer et al., 1999); and diverse pigments, including isorenieratene (Liaaen-Jensen, 1978), chlorobactene (Liaaen-Jensen, 1978), and bacteriochlorophylls *d* and *e*, which are diagnostic for green sulphur bacteria. Also, although abundant and ubiquitous in eukaryotes (Huang and Meinschein, 1979) and extremely uncommon in bacteria, steroids have been found in some bacterial species. Specifically, certain 4-methyl- and 4,4-dimethyl steroids have been reported in methanotrophs belonging to the family Methylococcaceae (Bird et al., 1971; Bouvier et al., 1976; Jahnke and Nichols, 1986; Schouten et al., 2000).

3.4. Ether lipids

Archaea are partly distinguished from the bacteria and eukarya because of the unique *sn*-2,3 rather than *sn*-1,2 stereochemistry of the glycerol moieties (Koga et al., 1998a,b) and because they contain ether-bound membrane lipids with isoprenoidal carbon skeletons rather than ester-linked alkyl lipids (Fig. 3; De Rosa and Gambacorta, 1988). Such compounds or their degradation products have been found in hypersaline environments (Teixidor et al., 1993), anoxic swamp sediments (Pauly and van Fleet, 1986; Pancost et al., 2000b), and diverse pelagic settings (Hoefs et al., 1997; DeLong et al., 1998; King et al., 1998; Schouten et al., 1998). Archaeol (Fig. 3) has been identified in halophiles, thermophiles, and methanogens and is the most common of the archaeal diethers (for a review see Koga et al., 1998a,b). Hydroxyarchaeol has the same core structure as archaeol but contains an additional hydroxyl group on the third carbon of the phytanyl moiety ether-linked to either the third (*sn*-3-hydroxyarchaeol) or second (*sn*-2-hydroxyarchaeol) glycerol carbon. This compound has been observed almost exclusively in cultures of methanogenic archaea of the orders Methanococcales and Methanosarcinales. Other occurrences among the archaea include the methanogens of the *Methanosphaera* genus (Koga et al., 1998a,b) 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).

Like diethers, glycerol tetraethers (glycerol dialkyl glycerol tetraether; GDGT) are diagnostic for and common in archaea. The distributions of GDGTs



GDGT no.	X	Y	GDGT no.	X	Y
0-0'	A	B	**Alky Tetraether**	E	E
0-0	B	B		E	F
0-1	A	C		F	F
0-2	A	D		E	G
				G	G

Fig. 3. Chemical structures of selected biomarkers for archaea (and some tetraethers inferred to derive from bacteria). Shown are: glycerol diethers, including archaeol and hydroxyarchaeol; the irregular isoprenoids, PMI and crocetane; and a selection of the carbon skeletons commonly associated with glycerol dialkyl glycerol tetraethers that are discussed in the text (tetraethers comprised of biphytane chains containing three or four pentacyclic groups are found in hyperthermophilic archaea but are not shown).

appear to vary significantly among different archaea (de Rosa et al., 1986), but the source-specificity of such variations remains unclear and perhaps reflects environmental conditions such as pressure or temperature (De Rosa et al., 1980). In the majority of archaeal tetraethers, the alkyl groups are biphytane units with zero to four cyclopentane rings. Caldarchaeol (0-0, Fig. 3; a GDGT composed of two acyclic biphytanes) is apparently widespread in archaea (Schouten et al., 2000a), while GDGTs with cyclopentane units were previously thought to be present only in thermophilic archaea (de Rosa and Gambacorta, 1988). That latter conclusion has been significantly modified by recent work clearly illustrating the presence of biphytanes with one or two cyclopentane rings in GDGTs from non-extreme settings (Hoefs et al., 1997; Delong et al., 1998; King et al., 1998; Schouten et al., 1998, 2000a; Pancost et al., 2000b, 2001b). We have also confirmed in pelagic settings the presence of a potentially highly diagnostic GDGT composed of a dicyclic biphytane and a tricyclic biphytane in which one of the rings consists of six carbon atoms (Schouten et al., 2000a and unpublished results).

Although the vast majority of ether lipids in nature have isoprenoid carbon skeletons and are attributed to archaea, non-isoprenoid alkyl diethers have been found in thermophilic bacteria (Langworthy et al., 1983; Huber et al., 1992, 1996) and diverse settings (Pancost et al., 2001a). Moreover, we recently reported the discovery of non-isoprenoid alkyl tetraether lipids (with hydrocarbon skeletons E–G, Fig. 3; Sinninghe Damsté et al., 2000), although the origin of this compound remains unknown. Thus, it appears that ether lipids are more diverse than previously expected, and can derive in some cases from bacterial sources.

3.5. Other archaeal lipids

The irregular isoprenoid 2,6,10,15,19-pentamethylicosane (PMI) has been shown to be 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 in cultures of *Methanolobus bombayensis* and *Methanosarcina mazei*, further illustrating the utility of these compounds as biomarkers for methanogenic archaea. 2,6,11,15-Tetramethylhexadecane (crocetane) has not been observed in cultured organisms but is analogous to PMI in that it is a C_{20} as opposed to C_{25} irregular isoprenoid. Consequently, and because of isotopic considerations, it also has been invoked as a biomarker for archaea operating as methane consumers (Bian, 1994; Elvert et al., 1999; Thiel et al., 1999; Bian et al., 2001).

4. Controls on prokaryotic lipid $\delta^{13}C$ values

4.1. Mechanisms of carbon assimilation

Although the controls on prokaryotic biomass and lipid $\delta^{13}C$ values remain incompletely understood, the underpinning principles are the same as for other organisms like algae and higher plants. Specifically, the carbon isotopic composition of an organism is dictated primarily by the isotopic composition of the carbon source and the mechanism by which that carbon is assimilated. These in turn are fundamentally related to the ecology of the organism: autotrophs, including photoautotrophs, chemoautotrophs, and autotrophic methanogens, assimilate either $CO_{2(aq)}$ or HCO_3^- (Goericke et al., 1994); heterotrophic organisms assimilate organic substrates, including simple carbohydrates (e.g. glucose) and short-chain acids (e.g. acetate); and methanotrophic organisms assimilate methane.

Dissolved HCO_3^- and $CO_{2(aq)}$ $\delta^{13}C$ values typically range from −1‰ to 1‰ and from −10‰ to −6‰, respectively. However, near cold seeps or in organic-rich sediments, where a significant quantity of dissolved inorganic carbon (DIC) is ultimately derived from oxidation of methane or organic matter, significantly more ^{13}C-depleted values can occur (Whiticar, 1999). In contrast, in closed or semi-closed settings, autotrophic—and particularly methanogenic—assimilation of DIC can cause the residual pool to become significantly enriched in ^{13}C (Whiticar, 1999; Irwin et al., 1977). Significant kinetic isotope effects are also associated with assimilation of DIC. Many chemoautotrophs (Ruby et al., 1987) utilize the Calvin cycle, during which organic carbon can be further depleted by as much as 27‰ (the commonly accepted maximum isotope effect for the enzyme ribulose bisphosphate carboxylase; Goericke et al., 1994), although analyses of diverse phytoplankton species indicate that the actual value is dependant on a variety of environmental and physiological factors (Goericke et al., 1994; Rau et al., 1996). Other pathways, apparently restricted to the bacteria and archaea, are the reverse tricarboxylic acid cycle and the 3-hydroxypropionate pathway, both of which are characterized by significantly smaller isotope effects than the Calvin cycle (Sirevag et al., 1977; Quandt et al., 1977; van der Meer et al., 2001a,b).

Methanotroph $\delta^{13}C$ values can be similarly variable. Methane $\delta^{13}C$ values in nature vary by over 100‰ depending on (1) whether the methane is thermogenic or biogenic; (2) if biogenic, whether methane was generated autotrophically or heterotrophically and, correspondingly, the carbon isotopic compositions of DIC or acetate (Whiticar, 1999). As with autotrophs, significant but variable kinetic carbon isotope effects are associated with methane assimilation. Jahnke et al. (1999) showed that methanotroph biomass $\delta^{13}C$ values can be as much as 24.8‰ depleted relative to substrate methane, but also observed 30‰ variability depending on whether the methanotrophs utilized the ribulose monophosphate or serine pathways and whether they used the membrane-bound particulate monooxygenase enzyme or the soluble isozyme. Unfortunately, anaerobic methane-oxidizing archaea (cf. Hinrichs et al., 1999; Elvert et al., 1999; Pancost et al., 2000b) have not yet been isolated and the mechanism for methane uptake by such organisms is unknown.

In contrast to multicellular heterotrophs in which respiratory loss of carbon can be significant, prokaryotic heterotroph carbon isotopic compositions are generally thought to be similar to that of the food source (Hayes, 1993). However, laboratory incubations of *Shewanella putrefaciens* by Teece et al. (1999) reveal that small differences (<2.5‰) between sub-

strate and biomass $\delta^{13}C$ values can occur and are dependant on growth conditions. More strikingly, recent work on the iron-reducing bacteria, *Geobacter metallireducens* and *Shewanella algae*, indicates that these heterotroph's biomass can be as much as 7‰ depleted relative to substrate acetate and lactate, respectively (Zhang et al., this volume). Thus, assumptions that bacterial heterotroph biomass will always be the same as that of substrate carbon need to be more thoroughly tested. Nonetheless, it is likely that the primary control on heterotroph biomass $\delta^{13}C$ values is the isotopic composition of substrate carbon and, primarily, this should reflect the ecology or physiology of the organism from which such substrates are derived (higher plant vs. algae, photoautotroph vs. methanotroph, etc.). In addition, different substrates (glucose, acetate) derive from different biosynthetic pathways in the source organism and this can impart different carbon isotopic compositions to the consumer. For example, carbohydrates are typically enriched in ^{13}C relative to lipids (Abelson and Hoering, 1961; Deines, 1980; van der Meer et al., 2001a; van Dongen et al., 2001 and unpublished results). The $\delta^{13}C$ values of substrates generated from the degradation of these different compound classes, and thus, heterotrophic microorganisms will reflect those differences.

4.2. Biosynthetic controls on lipid $\delta^{13}C$ values

In addition, further isotope effects during biosynthesis can affect the $\delta^{13}C$ values of specific lipids within an organism. This can result not only in differences among compound classes (e.g. lipids vs. carbohydrates as described above) but also between individual lipids within a structural class. The long-standing paradigm regarding biosynthetic isotope effects is that fractionation occurs during formation of acetyl-CoA with the strongest depletion occurring at the carboxyl carbon (Monson and Hayes, 1982); presumably this fractionation is associated with either the oxidation of pyruvate to acetyl CoA by pyruvate dehydrogenase (DeNiro and Epstein, 1977) or conversion of acetyl phosphate to acetyl-CoA by phosphotransacetylase (Blair et al., 1985). Monson and Hayes (1982) determined that the kinetic isotope effect associated with pyruvate dehydrogenase in *E. coli* was 23‰ (ε_{PDH}); however, this full effect is rarely expressed because of Rayleigh distallations (such that the residual pyruvate carbon pool becomes progressively enriched in ^{13}C as the reaction progresses). Instead, the fractionation expressed during decarboxylation is $(1-f)\varepsilon_{PDH}$ (Monson and Hayes, 1982), where f is the fraction of pyruvate flowing to acetyl-CoA. Since these reactions occur during both photoautotrophic and heterotrophic lipid synthesis, fatty acids and other acetogenic (straight-chain) lipids in most organisms are expected to be ca. 4‰ depleted relative to biomass (Hayes, 1993); isoprenoids synthesized by the mevalonic acid pathway—during which decarboxylation of mevalonic acid-5 pyrophosphate results in a 3:2 ratio of methyl to carboxyl atoms in the resultant isoprene unit—are expected to be depleted relative to biomass by only ca. 2‰ (Hayes, 1993).

In general, a 2‰ to 4‰ offset between fatty acids and biomass is indeed observed for bacteria grown under aerobic conditions, including *E. coli* grown on glucose (Monson and Hayes, 1982), *S. putrefaciens* (Teece et al., 1999), and Type I and Type X methanotrophs using the ribulose monophosphate pathway (RuMP; Summons et al., 1994; Jahnke et al., 1999). However, the fatty acids of methanotrophs utilizing the serine pathway were reported to be 10‰ to 15‰ depleted relative to biomass (Jahnke et al., 1999). The acetogenic lipids of heterotrophic bacteria are also more depleted than expected when grown under anaerobic conditions, with fatty acid $\delta^{13}C$ values being: 7.3‰ to 10.7‰ more depleted than biomass in *S. putrefaciens* grown on lactate and with NO_3 as the oxidant (Teece et al., 1999); 4.5‰ to 8.6‰ more depleted than biomass in *G. metallireducens* grown on acetate and with ferric citrate as the electron acceptor (Zhang et al., this volume); and 10.4‰ to 14.7‰ more depleted than biomass in *S. algae* grown on acetate and with ferric citrate as the oxidant (Zhang et al., this volume). Based on these results, Zhang et al. (this volume) have proposed that the lipids of organisms using the serine pathway, and thus malyl-CoA-lyase, to form acetyl-CoA are more depleted relative to biomass than the lipids of organisms using the TCA cycle or the RuMP pathway.

The isotopic relationships between lipids and biomass can also vary in autotrophic organisms using carbon assimilation pathways other than the Calvin Cycle. Theoretical considerations and culture studies indicate that in organisms using the reverse TCA cycle, such as green sulphur bacteria, acetogenic lipids

are ca. 4‰ enriched in ^{13}C relative to biomass (van der Meer et al., 1998). Studies of *Chloroflexus aurantiacus* grown photoautotrophically indicate that alkyl lipids of organisms using the 3-hydroxypropionate pathway are 1‰ to 2‰ depleted in ^{13}C relative to biomass (van der Meer et al., 2001a,b); this is expected because these organisms also use the TCA cycle to generate ATP and during lipid synthesis, such that the isotopic difference between biomass and lipids should be the same as in photoautotrophs using the Calvin Cycle.

As mentioned earlier, isotope fractionations are also dependant on partitioning of carbon at branch points in carbon metabolic pathways (Hayes, 1993). This is particularly well illustrated by culture studies of the cyanobacterium *Synechocystis* (Sakata et al., 1997). This organism uses the TCA cycle and lipids are biosynthesised from acetyl-CoA generated by decarboxylation of pyruvate; thus, acetogenic lipids are expected to be ca. 4‰ depleted relative to biomass. Instead, Sakata et al. (1997) observed that fatty acids were depleted by 9.1‰. This additional depletion suggests that the fraction of pyruvate flowing to acetyl-CoA is small, which is consistent with the relatively low abundance of lipids in cyanobacteria.

Different carbon assimilation pathways and mechanisms of acetyl-CoA formation will also affect isoprenoid lipid $\delta^{13}C$ values; for example, in the study by Jahnke et al. (1999), hopanoids in methanotrophs utilising the serine pathway had similarly low $\delta^{13}C$ values as the fatty acids. An additional complication with interpreting isoprenoid $\delta^{13}C$ values arises from the recent discovery that isoprenoids are not synthesised via the mevalonic acid pathway in most organisms; specifically, incorporation of ^{13}C-labeled acetate and glucose into bacterial hopanoids indicates that the C_5 isoprene unit can be formed by the condensation of a C_2 subunit derived from pyruvate decarboxylation and a C_3 subunit derived from triose phosphate (Rohmer et al., 1989; Rohmer et al., 1993, 1996; Horbach et al., 1993). This pathway actually appears to be predominant in bacteria and has also been identified in higher plants (Lichtenthaler et al., 1997), unicellular algae, and cyanobacteria (Disch et al., 1998). More significantly, the effect of the glyceraldehyde phosphate/pyruvate pathway on the carbon isotopic composition of isoprenoids remains unclear; although isoprenoids derived from this pathway could be enriched in ^{13}C relative to mevalonate-derived isoprenoids because the former contain only one ^{13}C-depleted carboxyl carbon, the current lack of understanding demands caution. Thus, although paradigms for the biosynthetic controls on lipid $\delta^{13}C$ values can provide a first approximation of expected biomarker $\delta^{13}C$ values in some situations, unresolved issues regarding the isotopic effects, influence of growth conditions, and biological distribution of different metabolic pathways remain a limitation in interpreting sedimentary lipid $\delta^{13}C$ values.

5. Previous studies

Many studies have utilized the unique structures and $\delta^{13}C$ values of prokaryotic lipids to elucidate ancient sedimentary processes or to study modern ecosystems. These include the diagnostic high $\delta^{13}C$ values of biomarkers for green sulphur bacteria (Sinninghe Damsté et al., 1993; Koopmans et al., 1996) and *Chloroflexus* (van der Meer et al., 2001a,b). Lipids of both halophilic (Grice et al., 1998) and non-extremophilic archaea of unknown ecology (Hoefs et al., 1997; Schouten et al., 1998; Kuypers et al., 2001) also tend to be enriched in ^{13}C relative to photoautotroph biomass.

Among the most diagnostic molecular isotopic indicators are highly ^{13}C-depleted bacterial lipids used as indicators for aerobic methanotrophy. ^{13}C-depleted hopanoids have been found in ancient anoxic lacustrine sediments such as the Green River Formation (Collister et al., 1992) and the Messel Shale (Freeman et al., 1990) and in ancient marine settings (Yamada et al., 1997; Köster et al., 1998) and provided strong evidence that methane had been generated and subsequently consumed either in the sediments or water-column. ^{13}C-depleted bacterial lipids have also been used as tracers for methanotrophy in modern samples, including lake sediments (Spooner et al., 1994; Schouten et al., 2001b) and in the tissues of a cold seep mussel (Jahnke et al., 1995).

Similarly, ^{13}C-depleted archaeal biomarkers have been used as indicators of methanogenesis. Like methanotrophs, autotrophic and methylotrophic methanogen biomass can be strongly depleted in ^{13}C relative to their carbon source (Summons et al., 1998). However, methanogens are archaea and produce compounds

distinct from those produced by aerobic methanotrophs, a distinction that allows the processes of methanogenesis and methanotrophy to be independently assessed (however, see below for a discussion of archaeal biomarkers as indicators for anaerobic methane oxidation). ^{13}C-depleted compounds of inferred methanogen origin have been identified in both ancient (Freeman et al., 1990) and modern (Freeman et al., 1994) settings. However, because the isotopic compositions of methanogens are governed by diverse variables that remain incompletely understood (Summons et al., 1998 and references therein), ^{13}C-depletion might not be expected in all cases. For example, in a recent investigation of Ace Lake (Antarctica), Schouten et al. (2001) observed methanogen biomarkers as much as 10‰ enriched relative to photoautotroph biomarkers. Ether lipids supposed to derive from methanogens in the Eocene Messel Shale have δ^{13}C values ranging from −27‰ to −30‰, which is believed to reflect a pronounced enrichment of the DIC pool (Hayes et al., 1987). We have suggested that methanogens growing heterotrophically on acetate or other multi-carbon substrates could have δ^{13}C values similar to those of the source carbon (Pancost et al., 2000b).

Despite these uncertainties, the carbon isotopic compositions of prokaryotic lipids can be used to trace carbon cycling. The wide range of values coupled to the fact that certain prokaryotic lipids can be highly diagnostic for certain organisms and processes allows information to be gleaned from modern and ancient sediments. In many ancient sediments, this is the only method by which such insight can be gained. In modern settings, when some variables can be constrained, prokaryotic lipid δ^{13}C values can be highly useful in ascertaining the link between microorganisms and how they process organic matter. Below we discuss two case studies that illustrate the insights provided by this approach and highlight directions for future research.

6. Carbon cycling in an ancient peat deposit

Peat bogs are an important component of the global carbon cycle, particularly with regard to organic carbon storage and the generation of atmospheric methane (Khalil and Rasmussen, 1990). Bog plants assimilate atmospheric carbon dioxide during growth and much of this carbon is sequestered in the peat deposit due to the relatively low rates of degradation in water saturated and oxygen depleted bogs. However, prokaryotic processes can degrade bog plant organic matter resulting in the ultimate re-release of carbon into the atmosphere. Moreover, because anoxic conditions are achieved at shallow depths in most bogs, much of the mineralised organic matter is released as methane, which can exert a much more significant greenhouse influence than carbon dioxide. Consequently, considerable attention has been devoted to understanding the controls on methane release from a variety of modern bogs. Of particular interest are the relative roles of methanogen communities utilizing either acetate or CO_2-H_2 (Goodwin and Zeikus, 1987; Avery et al., 1999). However, genetic techniques or those dependant on the isotopic composition of substrates (Hornibrook et al., 1997; Avery et al., 1999; Waldron et al., 1999) fail to provide insight into pathways of carbon degradation in ancient settings; instead, recalcitrant indicators such as prokaryotic lipids are needed.

A molecular and carbon isotopic investigation of a late subboreal peat deposit (Bargerveen peat, the Netherlands; Pancost et al., 2000b) was used to investigate aspects of methane cycling in bog systems. Specifically, hopanoids are used as tracers of aerobic bacteria, and ether-bound isoprenoid lipids are used as tracers of methanogenic archaea (cf. Pauly and van Fleet, 1986). The δ^{13}C values of these biomarkers are used to identify links in the pathways of carbon degradation between these two populations and to identify lipids derived from other microorganisms that may be involved with organic matter degradation. These shifts are then tied to changes in climate inferred from macrofauna analyses (Pancost et al., 2000b); specifically, warm and dry local conditions (Ericacaea rootlet zone in Fig. 4) were replaced by a cold and wet oceanic climate (*S. imbicatum* zone) with a particularly wet interval occurring at the transition (high water table associated with *S. cuspidatum*).

6.1. Hopanoids

Previous work (Pancost et al., 2000b) clearly indicates that hopanoids in the Bargerveen peat derive predominantly from heterotrophic bacteria. δ^{13}C values range from −27‰ to −30‰ for 17β, 21β(*H*)-bishomohopanol (data not shown) and from −22.5‰

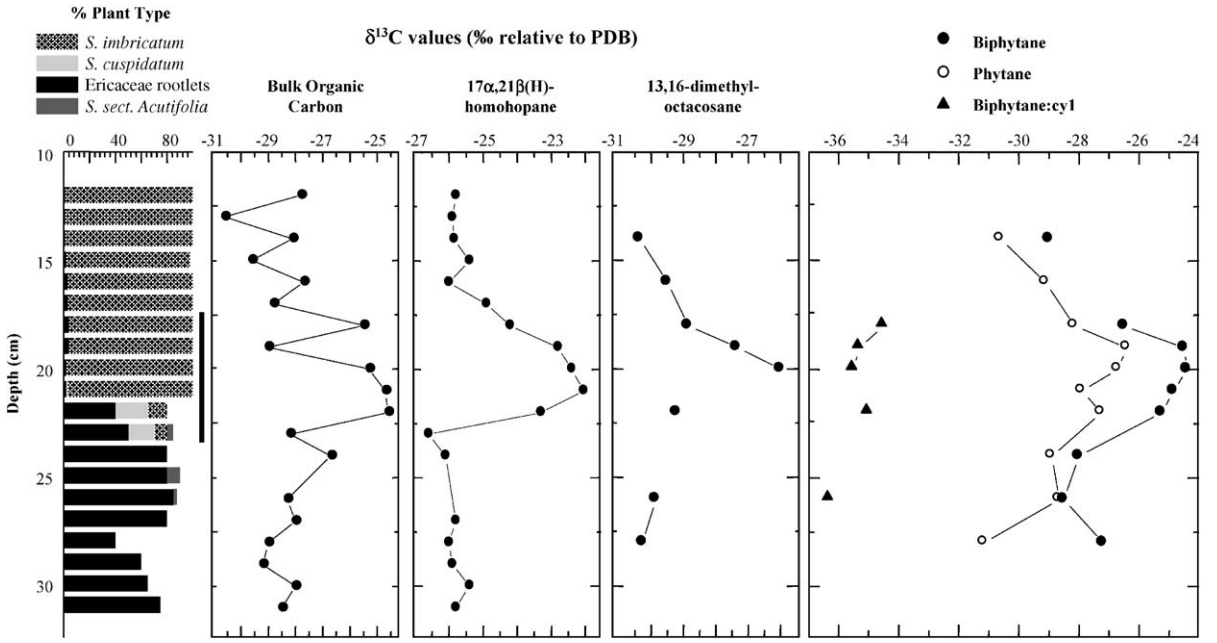


Fig. 4. Profiles of plant vegetation changes and organic matter $\delta^{13}C$ values in the Bargerveen peat sequence. The solid vertical line denotes the period of wettest climate inferred from plant macrofossils and coincides with a positive shift in bulk plant and bacterial lipid $\delta^{13}C$ values.

to −26.5‰ for 17α, 21β(*H*)-homohopane (Fig. 4). These values are significantly enriched in ^{13}C relative to previously reported values for methanotroph biomarkers (~ −50‰ to −120‰ in sediments, field, and culture samples; Jahnke et al., 1999 and references therein), precluding that as a significant source. Excluding methanotrophs, aerobic heterotrophs are the most likely source for hopanoids in these settings. However, the observed values, particularly those of 17α,21β(*H*)-homohopane, are also enriched in ^{13}C relative to a variety of co-occurring higher plant biomarkers (values range from −28‰ to −34‰ for a variety of *n*-alkanes, *n*-alcohols, triterpenoids, and steroids), as well as those of bulk organic matter (−23‰ to −27.5‰). Because lipids tend to be depleted relative to biomass (Hayes, 1993; and see earlier discussion), bacterial biomass is probably even more ^{13}C-enriched than the hopanes and could be strongly enriched relative to bulk plant organic matter; this implies that these organisms were utilizing more abundant and labile ^{13}C-enriched carbon, possibly carbohydrates (cellulose). Indeed, carbohydrates are enriched in ^{13}C by ca. 10‰ relative to lipids in living plants collected from the Bargerveen bog (van Dongen et al., 2001)—closely matching predicted bacterial biomass $\delta^{13}C$ values (Fig. 5).

Variations in hopanoid $\delta^{13}C$ values throughout the section provide further evidence for this conclusion (Fig. 4). During the interval in which wettest conditions are believed to have prevailed (24 to 17 cm, Pancost et al., 2002), hopanoid $\delta^{13}C$ values exhibit a +4.5‰ shift. This directly parallels a shift in the carbon isotopic composition of bulk organic matter, but is much larger than the shifts observed for higher plant lipids (ca. 2.5‰). This is consistent with our assertion that the source of the hopanoids is a heterotrophic bacterium and provides further evidence for a decoupling between hopanoid and higher plant lipid $\delta^{13}C$ values. Thus, both secular shifts and the absolute values of hopanoid $\delta^{13}C$ values suggest that these compounds are recording the initial aerobic degradation of predominantly the carbohydrate component of organic matter.

6.2. Archaeal lipids

We previously reported the presence of three archaea-derived tetraether lipids in the Bargerveen peat and

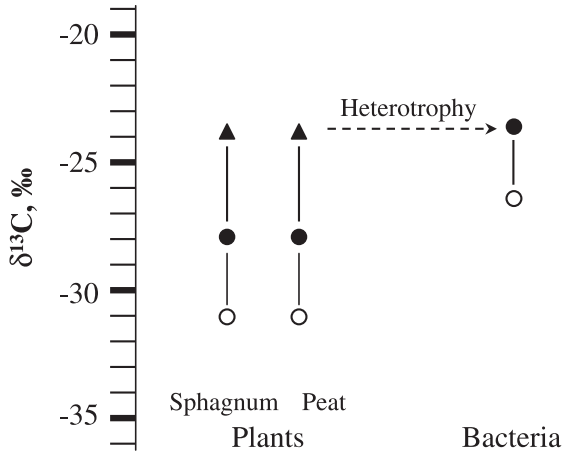


Fig. 5. Inferred relationship between organic carbon constituents of plants and heterotrophic bacteria in the Bargerveen peat. Solid points represent measured $\delta^{13}C$ value of peat biomass and the inferred $\delta^{13}C$ values of bacterial biomass in peat and living sphagnum biomass (assuming that lipids of aerobic heterotrophic bacteria are only 1–2‰ depleted relative to biomass; limitations of this assumption are discussed in the text). Solid triangle denotes measured $\delta^{13}C$ value of carbohydrates in living sphagnum biomass. Open points represent measured $\delta^{13}C$ values of lipids in peat and plants: 17α,21β(*H*)-homohopane, a bacterial lipid in peat; *n*-C_{23}, a *Sphagnum* lipid in peat; and phytol in a modern *Sphagnum* plant.

attributed them to methanogens (Pancost et al., 2000b). The most abundant compound is caldarchaeol (GDGT 0-0, Fig. 3; 50–330 μg/g peat based on abundance of acyclic biphytane generated by ether cleavage reactions). This compound is present in abundances comparable to higher plant lipids and occurs in all samples regardless of vegetation type or inferred climatic conditions. GDGTs comprised of a single biphytane paired with either a biphytane with a single cyclopentane moiety or a biphytane with two cyclopentane moieties (0-1 and 0-2, Fig. 3) were also present in all samples, although in much lower concentrations (based on abundances of monocyclic and dicyclic biphytanes released by ether cleavage reactions: 4 to 40 μg/g). In addition to biphytanes, ether cleavage reactions released 5 to 80 μg of phytane per gram of peat. Likely sources of phytane include phytol (the ester-linked side chain of most chlorophylls) and archaeol. However, neither phytol nor archaeol is present in the pre-treated fraction, suggesting that phytane is released by ether cleavage of an unknown polar compound. Potentially, this could be an archaeol analogue with a branched nonitol (calditol) substituted for the glycerol unit; calditol replacement of one of the glycerols in tetraethers does occur in some hyperthermophilic archaea (de Rosa and Gambacorta, 1986), but has not been reported for diethers. Although we have not identified this polar compound, the fact that phytane is an isoprenoid and was bound to the source molecule via an ether bond suggests that the HI-released phytane is of archaeal origin.

Based on the isotopic compositions of biphytane units released from GDGTs by ether-bond cleavage (−24‰ to −31‰ for acyclic biphytane and phytane and ~ −35‰ to −36‰ for biphytane with a single cyclopentane moiety), we suggested that caldarchaeol was predominantly derived from acetotrophic methanogens and that GDGT 0-1 was derived in part from CO_2/H_2 utilizing methanogens (Pancost et al., 2000b). The reason for these tentative assignments was that $\delta^{13}C$ values of the acyclic biphytane are more enriched in ^{13}C than expected for an autotrophic methanogen (see above discussion) but could be consistent with utilization of acetate derived from plant material; however, this interpretation is limited by the fact that in methanogens, the isotope effects associated with growth on acetate and lipid biosynthesis are unknown. The more ^{13}C-depleted values for the monocyclic biphytanes suggested an additional or different carbon source for those organisms.

Comparison of ether lipid depth profiles with those derived from bacterial biomarkers provides additional insights. $\delta^{13}C$ values for acyclic biphytane and phytane exhibit a ca. 4‰ shift coincident with the shift in hopane and bulk organic carbon $\delta^{13}C$ values. This is not expected for CO_2 utilizing methanogens. CO_2 $\delta^{13}C$ values are probably governed only to a small degree by stratigraphic variations in the isotopic composition of organic matter because CO_2, once generated, can readily migrate vertically through the peat column; thus, CO_2 $\delta^{13}C$ values are instead dictated by production (organic matter degradation) and consumption (methanogenesis) processes that typically impose much greater variability than observed here (Hornibrook et al., 1997; Whiticar, 1999). In contrast, isotopic variability coincident with specific stratigraphic horizons is plausible for methanogens using acetate as a substrate. Although the controls on acetate $\delta^{13}C$ values are also complex, a primary one must be the carbon source. Thus, the isotope profiles shown in Fig. 4 suggest that the climatically induced changes in

biomass $\delta^{13}C$ values have been sequentially inherited by aerobic bacteria utilizing carbohydrates and then by anaerobic methanogens utilizing acetate.

6.3. Novel dialkyl tetraether lipids

In addition to archaeal and bacterial biomarkers, we also discovered a non-isoprenoid dialkyl tetraether lipid in all of our peat samples (alkyl tetraethers shown in Fig. 3; Sinninghe Damsté et al., 2000). A survey of modern and ancient sediments (Schouten et al., 2000) clearly suggests that these compounds are derived from a terrestrial organism. Alkyl ether lipids have not been previously reported for any species of archaea, and tetraether lipids have not been previously observed in any bacteria. Alkyl diether lipids have been reported for some bacteria, and based on this, we tentatively suggest that bacteria are also the source of these novel compounds.

Stable carbon isotopes of the 13,16-dimethyloctacosane carbon skeleton released by ether-bond cleavage provide insight into the ecology of the source organism for the non-isoprenoidal GDGTs. Although the $\delta^{13}C$ values are not diagnostic (-25‰ to -29‰), they are similar to those obtained for bacterial and methanogen biomarkers. More significantly, these values exhibit the same stratigraphic shift as the hopanes and biphytanes indicating that the source organism is likely also involved with heterotrophic processing of peat biomass. The exact ecology of the source organism is unclear based on our data, but given the apparent restriction of alkyl diethers to anaerobic bacteria, it is tempting to speculate that the source organism was a fermentative bacterium and served as an intermediate between the aerobic heterotrophs and the methanogens. Alternatively, the compound could be derived from still uncharacterised methanogenic archaea. Indeed, 16S rRNA analyses of a peat core from the Moorhouse Natural Reserve (UK) revealed that no known methanogens were present at the depth of maximum methane generation (McDonald et al., 1999), indicating that the methanogens in peat bogs are certainly not fully characterised.

6.4. Integration of prokaryote lipid data

Although the prokaryote population in peat bogs is probably more complex than suggested by our biomarker analyses, molecular and isotopic analyses suggest the presence of at least two bacterial and two archaeal populations. These apparently represent the flow of carbon from higher plant biomass to aerobic heterotrophs to acetate-utilizing methanogens to autotrophic methanogens. The non-isoprenoidal ether lipid might also derive from an organism mediating organic matter mineralisation, but inferences that it is an anaerobic heterotrophic bacterium are speculative. Caution in interpretation of stratigraphic variability is also necessary because the depths at which these compounds are generated are also unknown; a change of climate during deposition at 14 cm likely affects the bacterial assemblages in all of the underlying sediments. Thus, inferred changes in prokaryotic activity—although useful indicators of carbon cycling—will not be as time-constrained as changes in plant-based proxies.

The abundances of 17α,21β(*H*)-homohopane decrease from an average value of 300 μg per gram of peat in the lower unit (peaking at 600 μg per gram of peat at 26 cm) to 200 μg/g in the upper unit (reaching a minimum at 14 cm; Fig. 6). There is considerably greater variability in the biphytane and 13,16-dimethyloctacosane abundances data; nonetheless, with the exception of the sample at 19 cm, the abundances of these compounds also generally decrease upsection. This decrease in prokaryote lipid abundances—and presumed biomass assuming no changes in the degree of lipid preservation—is coincident with a decrease in the proportion of degraded plant material as reflected by the number of identifiable plant fragments (Fig. 6). These trends are co-occur with the evolution of the peat from relatively dry and warm (Ericacaea) to relatively wet and cold (*S. imbricatum*) local climate conditions. The wet climate, likely associated with a shallowing of the anoxic water table, could have resulted in decreased aerobic bacterial remineralisation and thus an increase in the proportion of identifiable plant remains and a decrease in the abundances of aerobic bacterial biomass. Moreover, because the bacterial and/or archaeal sources of the biphytane and 13,16-dimethyloctacosane are apparently dependant on substrates generated by initial aerobic processes, the abundances of those compounds might also be expected to decrease with shoaling of anoxic waters. In contrast, the relative abundances of biomarkers for CO_2/H_2-utilising metha-

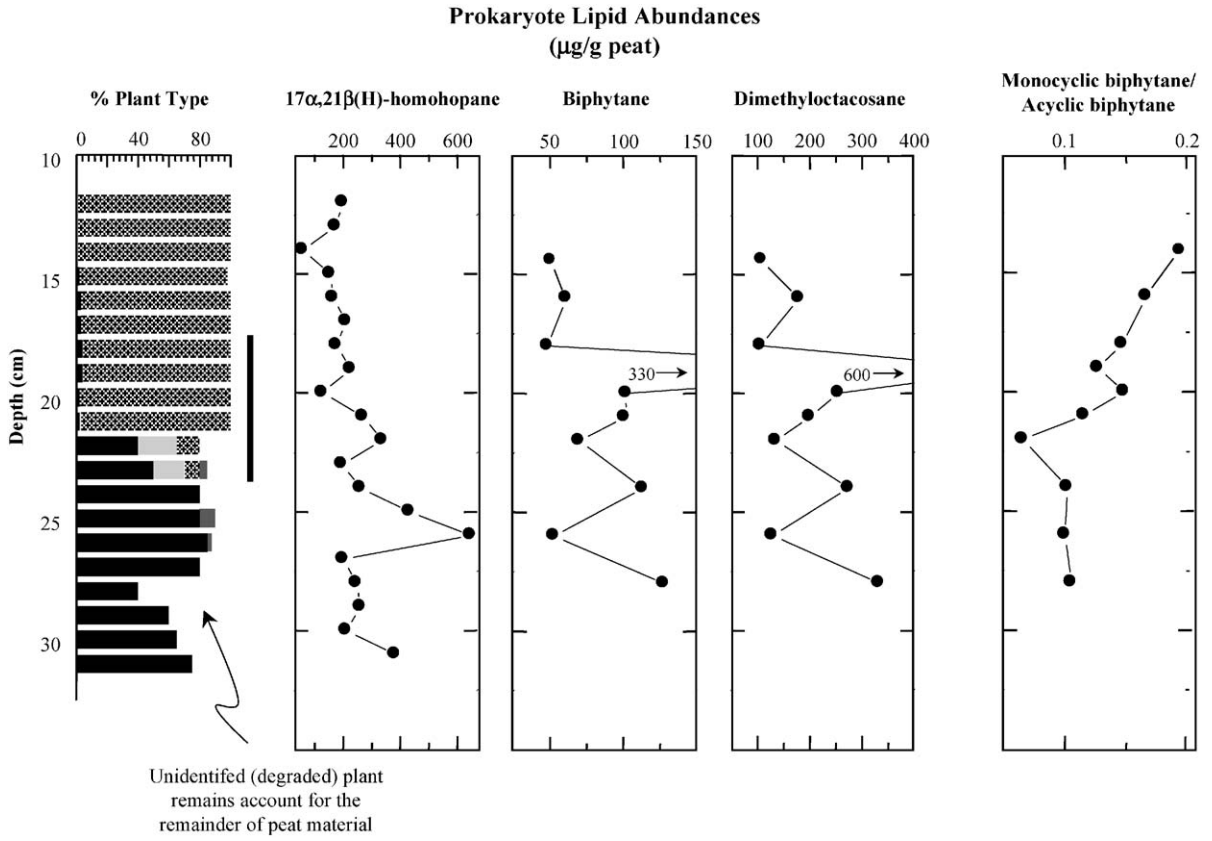


Fig. 6. Profiles of plant vegetation changes and abundances of selected lipids in the Bargerveen peat sequence. The solid vertical line denotes the period of wettest climate inferred from plant macrofossils and biomarkers.

nogens are expected to increase, and indeed the ratio of cyclic to acyclic biphytane doubles in the sample interval (Fig. 6).

Thus, despite the caveats mentioned above, carbon isotopes of specific compounds helped constrain those compounds' sources and pathways of carbon flow. Specifically, overall degradation of organic matter and inferred methanogen biomass decreased upward through the sample interval in response to climatic change. Although we have no record of methanotrophic organisms or other processes that might have consumed methane (Pancost et al., 2000b) nor any means to relate methanogen biomass to rates of methanogenesis, these results suggest that cooling and inferred shoaling of the water table could have resulted in a decrease in methane flux from the bog. Such results conflict with studies of some modern peats, which generally indicate that methane flux from peatlands increases as the depth of the water table shallows (e.g. Bubier et al., 1995). However, this could be due to the fact that methane flux is dependant on a variety of other factors including substrate availability, temperature, plant productivity, and degree of bacterial methane oxidation. Some of these variables have not been constrained during our study (specifically, the degree of methane oxidation, see Pancost et al., 2000b), but our work suggests that the decrease in substrate availability associated with shallowing of the water table was the dominant control on methanogen biomass and possibly methanogenesis. Such a model is in strong agreement with recent work (Bellisario et al., 1999), in which near-surface water tables are associated with low levels of methane production and a decreased contribution from acetoclastic methanogenesis.

7. Anaerobic methane oxidation at cold seeps

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 from marine sediments to the atmosphere is small compared to other sources (Reeburgh, 1996). 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 organisms responsible for this process. Multiple lines of evidence show that in such diverse settings as Cape Lookout Bight (Hoehler et al., 1994), Hydrate Ridge (Elvert et al., 1999; Boetius et al., 2000), the California Margin (Hinrichs et al., 1999), and Mediterranean mud volcanoes (Pancost et al., 2000a), methane is consumed anaerobically in a process mediated by archaea. The latter three research groups identified the role of archaea by showing that archaeal lipids (i.e. archaeol, hydroxyarcheaol, GDGTs, PMI and unsaturated counterparts, crocetane and unsaturated counterparts) were among the most abundant compounds at methane seeps and gas hydrates and that ^{13}C-depleted methane had been incorporated into archaeal biomass.

In addition, the sum of these different studies as well as a site-specific comparison of different mud volcano settings have revealed a pronounced heterogeneity of archaeal lipids at cold seeps, suggesting that diverse archaeal consortia are involved with anaerobic methane oxidation (Hinrichs et al., 1999; Elvert et al., 2001; Pancost et al., 2001b) (Table 1). Some of this lipid variability is likely due to differences in archaeal assemblages performing methane oxidation. Initially, it was proposed that the methanotrophs were methanogens "operating in reverse" in a syntrophic relation with sulfate-reducing bacteria (i.e. H_2O serves as the immediate electron acceptor; Hoehler et al., 1994). Other workers have proposed that while the reverse methanogenesis pathway correctly represents the chemical reactions occurring in the sediments, the mediating archaea are novel organisms and perhaps not capable of methanogenesis (i.e. obligate methanotrophs; Hinrichs et al., 1999). It is unclear which of these hypotheses is correct; given the diversity of archaeal lipids and archaeal gene clusters revealed by 16S rRNA analyses (Hinrichs et al., 1999; Orphan et al., 2001a,b), it is certainly possible that both types of organisms exist and that their relative abundances vary among different settings in response to unknown environmental variables (Pancost et al., 2000a).

In addition to archaea, increasing evidence reveals the importance of the co-existing bacterial community (Hoehler et al., 1994; Pancost et al., 2000a,b; Hinrichs et al., 2000; Boetius et al., 2000; Orphan et al., 2001a,b). It is well established that the energy yield of reverse methanogenesis is relatively low, particularly if hydrogen, a product of the reaction, accumulates in the sediment. Based on this and other evidence, previous workers (Hoehler et al., 1994; Pancost et al., 2000a; Boetius et al., 2000) have suggested that bacterial utilisation of hydrogen via sulfate reduction is vital to maintain methane oxidation under anaerobic conditions (Fig. 7a). In addition or alternatively, Valentine and Reeburgh (2000) have proposed that acetate could be an important intermediate in carbon and electron flow between methanotrophs and sulfate reducers (Fig. 8a–b). Here we expand on our previous findings by analysing the distributions and δ^{13}C values of bacterial biomarkers co-occurring with archaeal lipids in mud volcano sediments; with these data, we speculate on the flow of carbon from methane into the biomass of different microorganisms in mud volcano cold seeps.

7.1. East Mediterranean mud volcanoes

Mud volcanoes result when tectonically over-pressured and methane-charged mud is extruded along fault planes to the sea floor. The resulting mud breccias are rich in methane (Emeis et al., 1996), which then diffuses to the surface where it is oxidised in a reaction presumably coupled to sulfate reduction (de Lange and Brumsack, 1998). However, specific sites on mud volcano surfaces apparently represent areas of more active fluid flow as indicated by observations of brine seepage, abundant macrofauna, and elevated bottom water methane concentrations (The Medinaut Shipboard Scientific Party, 2000). This results in profound spatial variability, which allows the archaeal-bacterial consortium to be compared among diverse settings (for further discussion of the geology of Mediterranean mud volcanoes, see Limonov et al., 1996; Woodside et al., 1998; Emeis et al., 1996).

7.2. Sulfate-reducing bacterial biomarkers

Although archaeal biomarkers are the most abundant compounds in seep samples (Pancost et al., 2001b; excepting some cases in which organic matter indigenous to the extruded mud breccia is predominant), diverse bacterial biomarkers are also abundant (Table 2). In a previous publication (Pancost et al., 2000a), we reported the presence and $\delta^{13}C$ values of fatty acids and some hopanoids in a seep sample from

Table 1
Overview of variations in archaeal lipids among sites at which methane oxidation occurs

Site	Depth (cm)	Archaeol[a]	Hydroxyarchaeol		PMI unsaturations			Crocetane unsaturations			GDGT[c]
			sn-2[b]	*sn*-3	0	1-3	4-5	0	1	>1	
E. Mediterranean Mud Volcanoes											
Napoli Breccia	0-2	☯	-	-	☯	-	-	-	-	-	-
	2-5	☯	-	☯	☯	-	-	-	-	-	-
	10-12	☯	-	☯	☯	-	-	-	-	-	-
	17-20	☯	-	☯	☯	-	-	-	-	-	☯
Napoli Seep	s[d]	☯	-	☯	☯	☯	☯	☯	☯	Tr	☯
Napoli Crust	s	☯	☯	-	☯		-	☯	-	-	☯
Napoli Brine Pool	s	☯	☯	-	☯	☯	☯	☯	☯	-	☯
Napoli Mat	s	☯	☯	-	☯	-	-	☯	-	-	☯
Milano Breccia	s	-	-	-	-	-	-	-	-	-	-
	24-27	☯	-	-	-	-	-	-	-	-	☯
Milano Seep	s	☯	-	-	☯	☯	☯	☯	-	-	☯
Amsterdam Seep	s	☯	☯	☯	☯	☯	☯	☯	-	-	☯
Eel River[e]											
PC26	-	☯	☯	-	-	-	-	-	-	-	n.d.[f]
HPC4	-	☯	☯	-	☯	-	-	-	-	-	n.d.
PC36	0-3	☯	☯	-	n.d.	-	-	-	-	-	n.d.
	3-6	☯	☯	-	n.d.	-	-	☯	-	-	n.d.
	6-9	☯	☯	-	n.d.	-	-	-	-	-	n.d.
	9-12	☯	☯	-	n.d.	-	-	-	-	-	n.d.
SBB[g]	-	☯	☯	-	☯	-	-	☯	-	-	n.d.
Hydrate Ridge[h]											
SO109 TVG 43-1	-	n.d.	n.d.	n.d.	☯	☯	☯	☯	☯	☯	n.d.
SO109 TVG 43-2	-	n.d.	n.d.	n.d.	☯	☯	☯	☯	☯	☯	n.d.
SO109 TVG 41-1	-	n.d.	n.d.	n.d.	☯	☯	☯	☯	☯	☯	n.d.
Beggiatoa Seds.[i]	-	☯	☯	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aleutian Ridge[i]											
TV-GKG 40	-	-	-	-	☯	☯	☯	☯	☯	☯	n.d.
TV-G 48	-	☯	-	-	☯	-	☯	☯	☯	☯	n.d.
TV-G 97	-	☯	-	-	☯	-	☯	☯	☯	☯	n.d.
Black Sea[j]											
Carbonate Crust	-	☯[k]	n.d.	n.d.	☯	☯	-	☯	-	-	☯
Microbial Mat	-	-	n.d.	n.d.	☯	☯	-	-	-	-	n.d.
Ancient Limestones											
Marmorito Ls.[l]	-	☯[m]	-	-	☯	-	-	☯	-	-	n.d.
Beauvoisin Ls.[n]	-	-	-	-	☯	-	-	-	-	-	n.d.
Lincoln Creek Fm.[o]	-	-	-	-	☯	-	-	☯	-	-	n.d.

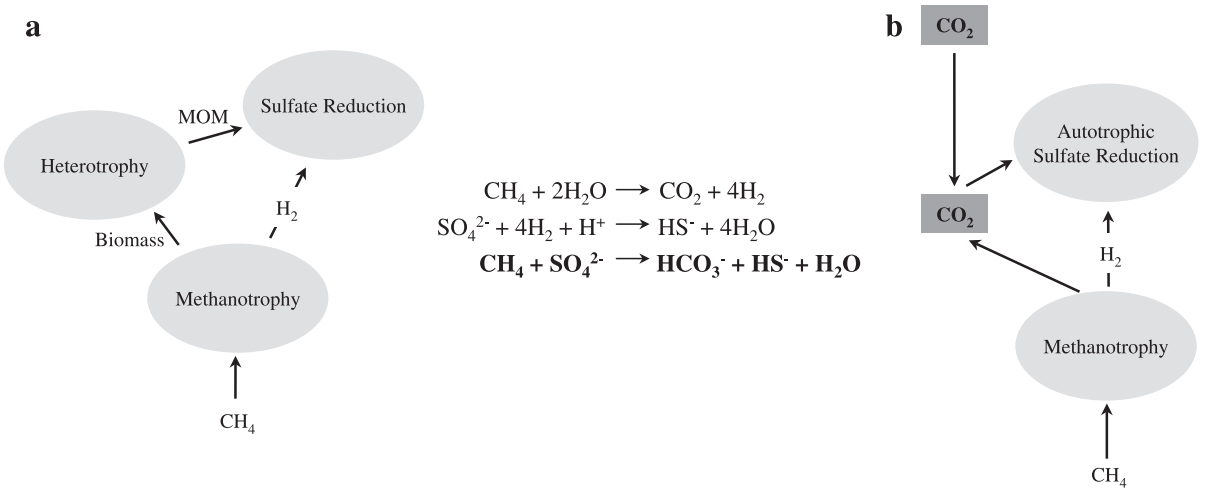


Fig. 7. Postulated reverse methanogenesis reactions illustrating the anaerobic oxidation of methane as mediated by a consortium of organisms including archaea and sulfate-reducing bacteria (Hoehler et al., 1994). (a) and (b) show two possible pathways of carbon flow consistent with reverse methanogenesis and by which sulfate-reducers could obtain methane-derived ^{13}C-depleted carbon through either heterotrophy (a) or autotrophy (b).

Napoli mud volcano. In general, bacterial lipids are depleted in ^{13}C but not as depleted as archaeal lipids. The same patterns prevail throughout our sample set (Fig. 9) and have also been reported for California Margin sites (Hinrichs et al., 2000); reasons for this isotopic relationship are discussed here.

The most diagnostic bacterial lipids in our samples are C_{15} and C_{17} branched (*iso*- and *anteiso*-) fatty acids. Although these compounds are not exclusive to sulfate-reducing bacteria, those organisms are a likely source given pore-water profiles clearly showing that sulfate reduction is occurring (de Lange and Brumsack, 1998) and previous reports identifying abundant sulfate-reducers in sediments at Hydrate Ridge (relatives of *Desulfosarcina* species; Boetius et al., 2000) and the California margin (both *Desulfosarcina* and *Desulfococcus* species; Orphan et al., 2001a), where methane oxidation occurs. Other workers have identified the same compounds in methane-oxidising sediments of the California margin (Hinrichs et al., 2000)

Notes to Table 1:

[a] With the exception of hydroxyarchaeol, a ☯ denotes the observed presence or absence of the given compound; a—denotes its absence.

[b] Indicates the relative predominance of a given hydroxyarchaeol. Thus, for the Amsterdam seep, in which both are present in similar abundances, a ☯ is placed in both boxes.

[c] For GDGTs, a ☯ denotes the clear presence of tetraether lipids other than those derived from pelagic archaea.

[d] s = surface sediment sample.

[e] (Hinrichs et al., 1999, 2000; Orphan et al., 2001a,b).

[f] n.d. = not determined or not reported.

[g] Santa Barbara Basin; (Hinrichs et al., 2000).

[h] Elvert et al. (1999).

[i] Boetius et al. (2000). Boetius et al. only note the additional observation of archaeol and hydroxyarchaeol in sediments underlying *Beggiato* materials. It is unclear if unsaturated crocetenes and PMIs are also present in those samples or if diether lipids are present in the Elvert et al. (1999) samples.

[j] Elvert et al. (2001).

[k] Presence of archaeol and/or hydroxyarchaeol possibly implied by the release of phytane by ether cleavage reactions.

[l] Miocene; Thiel et al. (1999); Peckmann et al. (1999).

[m] Thiel et al. did not observe archaeol but did observe a novel ether lipid they tentatively identified as 1-*O*-hexadecyl-2-*O*-phytanylglycerol.

[n] Oxfordian; Peckmann et al. (1999). [o]Oligocene; Thiel et al. (2001).

[o] Oligocene; Thiel et al. (2001).

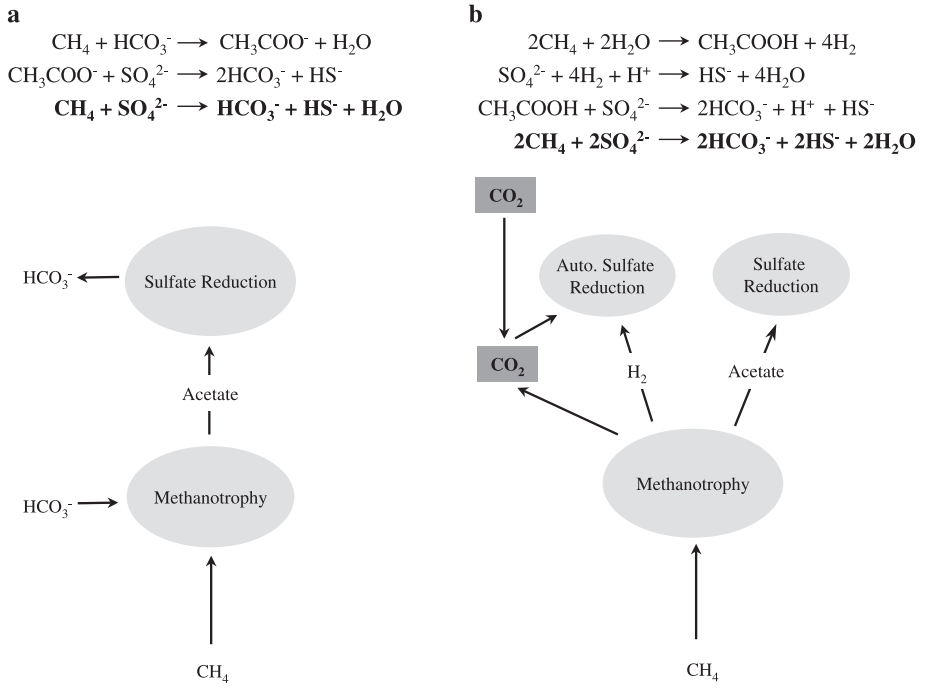


Fig. 8. Possible reaction schemes in which two-carbon compounds serve as redox intermediates during anaerobic methane oxidation by a consortium of microorganisms; (a) and (b) illustrate how acetate or acetic acid can also transfer carbon from putative anaerobic methane oxidising archaea to sulfate reducers (Valentine and Reeburgh, 2000).

and Hydrate Ridge (Boetius et al., 2000) and also attributed them to sulfate-reducing bacteria (see also Table 2). Low abundances of branched fatty acids in the mud volcano samples preclude accurate quantification (concentrations from ca. 1 to 15 μg/g of dry sediment), but comparisons to the trace abundances of these compounds in nearby inactive mud breccias suggest that there is minimal contamination from allochthonous sources. Thus, $\delta^{13}C$ values of branched fatty acids likely represent only indigenous organisms associated with the methane-oxidising community. Where determined, both C_{15} and C_{17} *iso*- and *anteiso*-fatty acids are strongly depleted in ^{13}C relative to typical marine organic matter (−20 to −30‰) but 0‰ to 10‰ enriched relative to the dominant archaeal biomarkers.

Other compounds apparently produced by sulfate-reducing bacteria include at least two newly characterised groups of non-isoprenoidal alkyl diethers observed primarily in carbonate crust samples (Pan-

Notes to Table 2:

[a] A ☯ denotes the observed presence of the given compound as well as isotopic evidence that it is at least partially derived from indigenous bacteria in a methane-oxidizing community (i.e. $\delta^{13}C$ values ≤ 40‰); a—denotes the lack of such evidence but could simply reflect the compound's abundance was too low for isotopic analysis.

[b] s = surface sediment sample.

[c] All site names are from the same references as in Table 1.

[d] n.d. = not determined or not reported.

[e] Presence implied by *iso*-C_{15} released by ether bond cleavage.

[f] Not found; but *iso*-C_{18} released by ether bond cleavage.

[g] The authors (Peckmann et al., 1999) report the occurrence of 4-methylbishomohopanol, attributed to aerobic methane-oxidizers.

[h] Diploptene is not reported but the isomer hop-17(21)-ene is reported.

[i] Thiel et al. (1999) did not report fatty acid distributions but did report the presence of *iso* and *anteiso*-branched alcohols.

cost et al., 2001a). In the first series of diethers, an 11,12-methylenehexadecyl moiety is ether bound at the *sn*-2 position of the glycerol group, and in diethers from the second series, an *anteiso*-pentadecyl group is ether bound to the *sn*-2 position (Fig. 10). In both series, C_{14}–C_{17} alkyl units (either *n*-alkyl or alkylcyclohexyl) are connected to the first glycerol moiety. Series II compounds are always 10‰ to 15‰ enriched relative to archaeal biomarkers and thus have similar $\delta^{13}C$ values as the *iso*- and *anteiso*-fatty acids.

Table 2
Overview of variations in bacterial lipids among sites at which methane oxidation occurs

Site	Depth (cm)	Hopanoids		Fatty Acids		Non-isoprenoidal Ether Lipids	
		Bishomohopanol[a]	Diploptene	C_{15} branched	C_{17} branched	Monoethers	Diethers
E. Mediterranean Mud Volcanoes							
Napoli Breccia	0-2	☯	-	-	-	-	-
	2-5	☯	-	-	-	-	-
	10-12	☯	-	-	-	-	-
	17-20	☯	-	-	-	-	-
Napoli Seep	s[b]	☯	☯	☯	☯	-	-
Napoli Crust	s	☯	☯	☯	☯	-	☯
Napoli Brine Pool	s	☯	-	☯	☯	-	-
Napoli Mat	s	☯	-	☯	☯	-	-
Milano Breccia	s	☯	-	☯	☯	-	-
	24-27	☯	-	-	-	-	-
Milano Seep	s	☯	-	☯	☯	-	-
Amsterdam Seep	s	☯	-	☯	☯	-	☯
Eel River[c]							
PC26	-	n.d.[d]	n.d.	☯	☯	☯	☯
HPC4	-	n.d.	n.d.	☯	-	-	-
PC36	0-3	n.d.	n.d.	☯	☯	☯	☯
	3-6	n.d.	n.d.	☯	☯	☯	☯
	6-9	n.d.	n.d.	☯	☯	☯	☯
	9-12	n.d.	n.d.	☯	☯	☯	☯
SBB	-	n.d.	n.d.	☯	☯	☯	☯
Hydrate Ridge							
SO109 TVG 43-1	-	n.d.	n.d.	☯	n.d.	n.d.	n.d.
SO109 TVG 43-2	-	n.d.	n.d.	☯	n.d.	n.d.	n.d.
SO109 TVG 41-1	-	n.d.	n.d.	☯	n.d.	n.d.	n.d.
Beggiatoa Seds.	-	n.d.	n.d.	☯	n.d.	n.d.	n.d.
Aleutian Ridge							
TV-GKG 40	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TV-G 48	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TV-G 97	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Black Sea							
Carbonate Crust	-	-	-	☯	-	☯[e]	-
Microbial Mat	-	-	☯	-	-	*[f]	-
Ancient Limestones							
Marmorito Ls.	-	☯[g]	☯[h]	☯[i]	-	n.d.	n.d.
Beauvoisin Ls.	-	-	-	-	-	-	-
Lincoln Creek Fm.	-	-	☯[h]	-	-	-	-

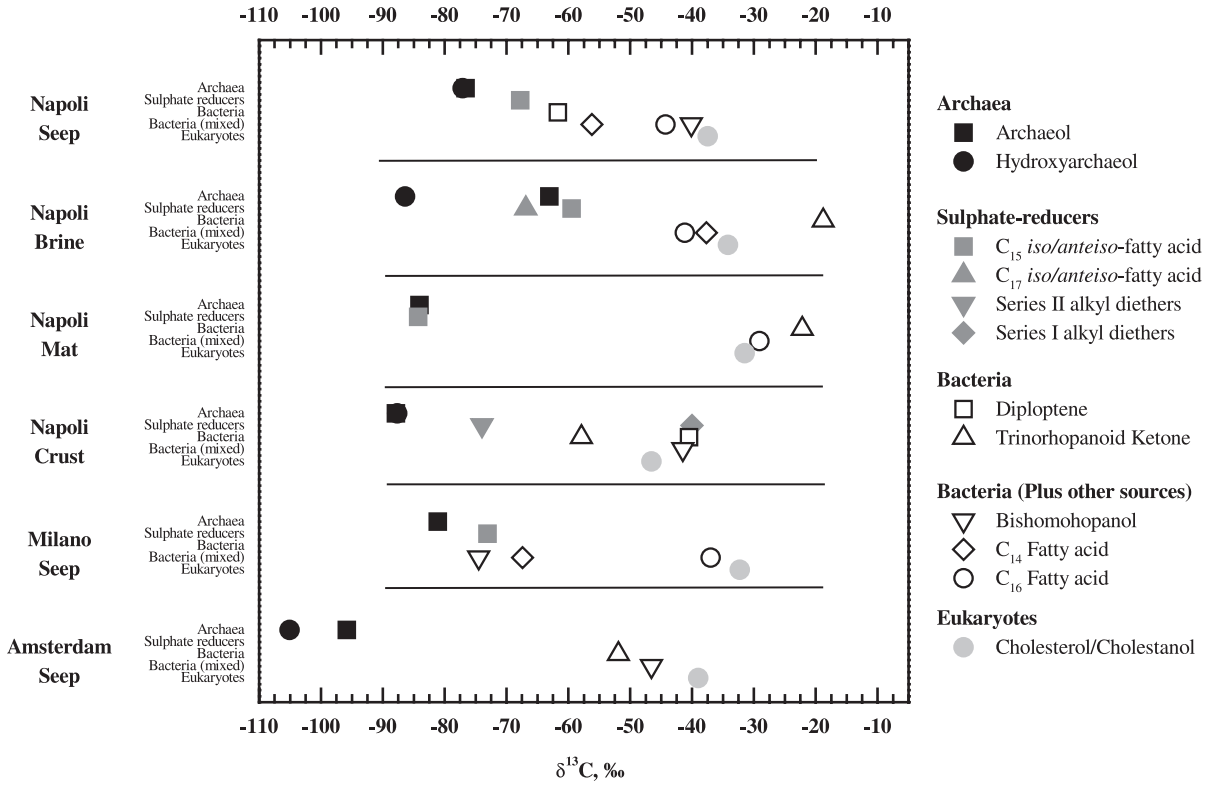


Fig. 9. Relationships among archaeal, bacterial, and eukaryotic biomarker $\delta^{13}C$ values at six mud volcano sites of the Eastern Mediterranean Ridge.

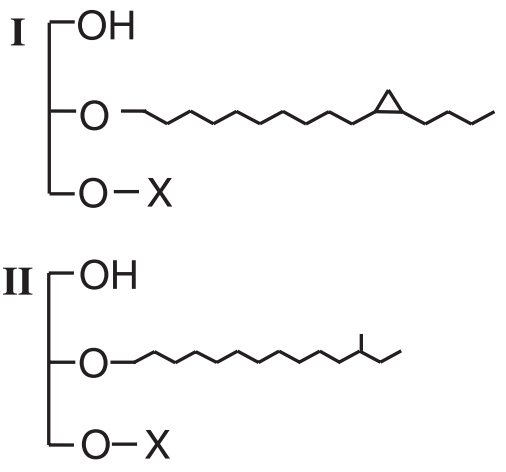


Fig. 10. Structures of Series I and Series II dialkyl ethers observed in cold seep related carbonate crusts. In both series, X denotes C_{14}–C_{17} alkyl units (either *n*-alkyl or alkylcyclohexyl) that are connected to the first glycerol moiety.

Series I compounds are always 25 ‰ to 35 ‰ enriched relative to archaeal biomarkers. Similar monoalkyl and dialkyl glycerols have been tentatively identified in California margin seeps (Eel River and Santa Barbara basins) and exhibit similar ^{13}C-enrichment relative to archaeal biomass (Hinrichs et al., 2000).

Low ^{13}C contents for sulfate-reducing bacteria biomarkers (*iso*- and *anteiso*-fatty acids and Series II dialkyl diethers) suggest that these organisms have assimilated organic matter derived from archaeal methanotrophs (Pancost et al., 2000a; Boetius et al., 2000). The consistency of the offset between archaeal and sulfate-reducer biomarker $\delta^{13}C$ values (typically 10 ‰ to 15 ‰ for fatty acids and Series II diethers and 30 ‰ for Series I diethers; Fig. 9) provides further evidence for a coupling of carbon flow from archaea to bacteria. However, the magnitude of the ^{13}C enrichment of bacterial relative to archaeal biomarkers is larger than expected. One possible explanation is that $\delta^{13}C$ values for archaeal and sulfate reducer biomass

are the same but the offset between lipid and biomass $\delta^{13}C$ values differs (Fig. 11). Indeed, previous work has shown that this offset can be much larger in archaea (methylotrophic methanogens; Summons et al., 1998) than in bacteria (Summons et al., 1994; Jahnke et al., 1999). However, recent work using an ion probe to directly determine $\delta^{13}C$ values of different components of an archaea-sulfate reducer aggregate indicates that sulfate reducer biomass is also ca. 30‰ enriched relative to archaeal biomass (Orphan et al., 2001b).

Assuming that differences in bacterial and archaeal biomarker $\delta^{13}C$ values ($\Delta^{13}C_{SRB\text{-}A}$) reflect differences in bacterial and archaeal biomass, we can evaluate the proposed mechanisms for anaerobic methane oxidation. The first requirement of any proposed mechanism is that it be able to not only sufficiently explain electron flow and be thermodynamically favourable, but also account for transfer of ^{13}C-depleted carbon to the sulfate reducer community. Hoehler et al. (1994) proposed that anaerobic methane oxidation proceeds via reverse methanogenesis and that generated H_2 is consumed by sulfate reducers (Figs. 7 and 8). In this model, there is no explicit mechanism to account for ^{13}C-depleted sulfate reducer biomass, but in a previous paper, we suggested that such depletion could occur if archaeal biomass was used as a food source for heterotrophic sulfate reducers (Fig. 7a; Pancost et al., 2000a). Sulfate reducers can utilize *n*-alkanes and other lipids as carbon sources (Zengler et al., 1999), but this is a very slow process (Parkes, 1999), which seems inconsistent with the high lipid concentrations here and rapid rates of sulfate reduction in related sediments (Boetius et al., 2000). Thus, it is possible that some other anaerobe mediates conversion of archaeal biomass into substrates such as acetate that are more readily assimilated by sulfate reducers, and possible lipid evidence for such organisms is presented below. However, in such a scenario, it is unclear why sulfate reducing bacterial biomarkers are enriched in ^{13}C relative to archaeal biomarkers or why the magnitude of offset varies by as much as 10‰ among different sites.

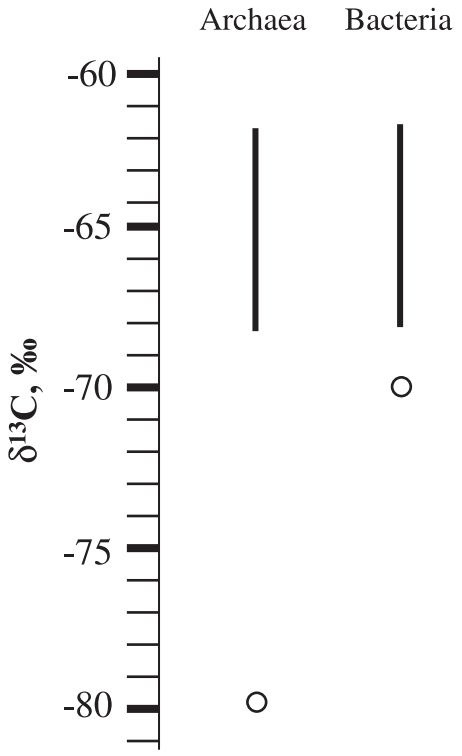


Fig. 11. Possible relationship between lipid and biomass $\delta^{13}C$ values of anaerobic methane-oxidising archaea and heterotrophic bacteria. Open circles represent measured $\delta^{13}C$ values of archaeal and bacterial biomarkers in the Napoli cold seep sample. Solid lines represent inferred $\delta^{13}C$ values of archaeal and bacterial biomass based on lipid $\delta^{13}C$ values and the lipid-biomass offsets observed for methylotrophic archaea (Summons et al., 1998) and bacteria (Summons et al., 1994; Jahnke et al., 1999; Teece et al., 1999).

A possibility consistent with the Hoehler hypothesis is that the sulfate reducing bacteria are autotrophic (Fig. 7b). Indeed, 16S rRNA investigations of seeps at Hydrate Ridge (Boetius et al., 2000) revealed the presence of sulfate-reducers closely related to species known to autotrophically assimilate CO_2. Because methane oxidation causes dissolved inorganic carbon (DIC) to be depleted in these settings—but due to contributions from seawater not as depleted as methane (as reflected by carbonate data published by Aloisi et al., 2001)—autotrophic growth is consistent with the observed $\delta^{13}C$ values. Moreover, because DIC $\delta^{13}C$ values will vary depending on the relative contributions of seawater DIC and methane, an autotrophic mechanism can also explain the variability in $\Delta^{13}C_{SRB\text{-}A}$ values. Additional ^{13}C-depletion could arise from sulfate reducers using the carbon monoxide dehydrogenase pathway, which is present in members of *Desulfosarcina* and characterized by relatively large isotope effects (Preuß et al., 1989).

Alternatively, it has also been proposed that acetate (or acetic acid) is directly generated by methane-oxidising bacteria, and this could serve as the source of ^{13}C-depleted carbon for sulfate reducers (Valentine and Reeburgh, 2000). Valentine and Reeburgh (2000) have offered two possible mechanisms involving acetate as an intermediate. In the first (Fig. 8a), acetate is directly generated from methane and bicarbonate. However, it is unclear why bacteria assimilating such acetate would be enriched in ^{13}C relative to archaea that presumably could use the same acetate pool during biosynthesis; thus, for this mechanism to explain the observed δ^{13}C values, archaea most likely would have to generate two distinct acetate pools. In an alternative mechanism, Valentine and Reeburgh (2000) propose that methane could be oxidized by water yielding both acetic acid and H_2, which could support two distinct sulfate reducing communities (Fig. 8b). Such a mechanism is appealing, because it could explain much of the variability in sulfate reducer biomarker δ^{13}C values and the isotopic differences among bacterial lipids that suggest that at least two sulfate reducer communities are present at some sites.

7.3. Other bacterial biomarkers

Less diagnostic bacterial lipids present in our samples include diploptene, bishomohopanol, and C_{14} and C_{16} fatty acids. In a previous publication (Pancost et al., 2000a), we also reported the presence of diplopterol; however, this compound is actually tetrahymanol that had been misidentified. The generally low δ^{13}C values (≤ 40‰) of bishomohopanol and the C_{14} and C_{16} fatty acids indicate that they are produced in part by bacteria living in the mud breccia. Some insight into the source of bishomohopanol is provided by a depth profile developed for a mud breccia near the active site on Napoli mud volcano. In contrast to diploptene abundances that increase with depth, bishomohopanol abundances decrease from 123 μg/g of sediment in a surface sample to 48 μg/g of sediment at 20-cm depth. Thus, consistent with previous observations (Rohmer et al., 1992), we propose that bishomohopanol derives from an aerobic bacterium, possibly an H_2S-oxidizing chemoautotroph. Such an origin is consistent with the abundance of hydrogen sulphide generated by sulfate reduction and the observation of white, filamentous (possibly *Beggiatoa*) bacterial colonies on the mud volcano surface. It is also consistent with the bishomohopanol δ^{13}C values, which are typically between -40‰ and -50‰, as expected for assimilation of ^{13}C-depleted CO_2 generated during methane oxidation but not with direct consumption of highly depleted archaeal biomass. However, bishomohopanol is commonly observed in marine sediments, and its abundances are sufficiently high in apparently inactive mud breccias to preclude interpretation of δ^{13}C values as solely representative of indigenous bacteria. Moreover, there are insufficient studies of the hopanoids of sulfide oxidizers to confidently attribute bishomohopanol directly to such organisms and other sources such as methanotrophs and heterotrophic bacteria cannot be precluded.

In contrast, diploptene is much more abundant at active sites (as much as 1 μg/g of dry sediment) than in inactive mud breccias where it is present in only trace quantities, indicating that at seeps diploptene δ^{13}C values should primarily reflect the carbon isotopic composition of indigenous bacteria. Diploptene δ^{13}C values range from -41‰ to -61‰ and are 20‰ to 50‰ enriched in ^{13}C relative to archaeal biomarkers. In addition to these compounds, we also observe a C_{27} hopanoid ketone (its exact structure is unknown because low quantities precluded isolation and rigorous characterization). δ^{13}C values for this compound are highly variable, ranging from -19‰ in a bacterial mat and -22‰ in a brine pool to -57‰ in a carbonate crust sample. Because hopanoids are ubiquitous in aerobic bacteria, it is difficult to constrain the sources of diploptene and the C_{27} hopanoid ketone. We previously proposed that diploptene, unexpectedly focused in the anoxic portions of the sediment, could be a biomarker for fermentative organisms mediating degradation of archaeal biomass (Pancost et al., 2000b). While this remains possible, it is unclear why diploptene δ^{13}C values should be so consistently and profoundly enriched relative to archaeal biomarker δ^{13}C values. A possible explanation is that diploptene is present in a variety of seep microorganisms of diverse ecology. Thus, the high variability in δ^{13}C values could reflect contributions from both a heterotroph as well as chemoautotrophs utilizing DIC as a carbon source. Different species of chemoautotrophic bacteria also could be sources of the relatively ^{13}C-enriched (-19‰ to -57‰) trinorhopanoid ketone if the source organism is using

a carbon assimilation pathway other than the Calvin cycle.

The relatively ^{13}C-depleted compositions of all bacterial biomarkers clearly indicate that carbon ultimately derived from methane is transported through and assimilated by some part of the prokaryotic community. Although multiple sources for some compounds complicate interpretation, the ^{13}C-depletion provides insight into the diversity of the bacterial population. First, no sulfate reducers are known to produce hopanoids; thus, the hopanoids likely represent different bacterial populations. Second, isotopic (Pancost et al., 2000b) and abundance trends reveal a decoupling of bishomohopanol and diploptene sources. Third, trinorhopanoid ketone $\delta^{13}C$ values are uncoupled and profoundly different from those of all other biomarkers, indicating a unique but unknown source. Fourth, the two isotopically unique populations of novel dialkyl diethers (Pancost et al., 2001a) are found in carbonate crusts but not in nearby sediments from the same mud volcano, suggesting they are produced by sulfate reducers distinct from those present in the sediment and producing the *iso*- and *anteiso*-fatty acids. Thus, biomarker $\delta^{13}C$ values and abundance trends signal the presence of at least six different bacterial populations. Comparison of our results to the presence of ^{13}C-depleted biomarkers at other sites (Table 2) suggests that similar bacterial populations are commonly associated with anaerobic methane oxidation.

7.4. Eukaryote biomarkers

Although not the focus of this paper, it is worth noting that stable carbon isotopes are also useful in tracking carbon flow among eukaryotic organisms. Tetrahymanol is present in all mud volcano samples, is commonly abundant (ca. 2 μg/g of sediment), and in the microbial mat sample, has a concentration of 140 μg/g of sediment and is one of the most abundant compounds. $\delta^{13}C$ values (-46.4‰ to -70.0‰) of tetrahymanol clearly indicate that this compound derives from organisms utilizing carbon at least partially derived from methane. However, the source of tetrahymanol in these samples is unclear. It is widespread in marine sediments (ten Haven et al., 1989) and has been observed in photosynthetic sulfur bacteria (Kleeman et al., 1990); although such bacteria are unlikely sources for tetrahymanol in our samples, other bacteria cannot be excluded. An alternative source is marine ciliates, which produce tetrahymanol but only when sterols are absent from their diet (Harvey and McManus, 1991; Holler et al., 1993), and thus, only when they rely solely on a prokaryotic food source. Such an origin is consistent with the high abundances of prokaryotes in these settings and the inference that they serve as the base of the cold seep ecosystem. In such a situation, $\delta^{13}C$ values for tetrahymanol should reflect those of the food source (e.g. Sinninghe Damsté et al., 1995), which is consistent with the low $\delta^{13}C$ values observed in our mud volcano samples. Ciliates can live under both anaerobic and aerobic conditions (Fenchel and Finlay, 1991 and references therein) and, thus, the presence of tetrahymanol in our samples—even below the sediment–water interface—is not unexpected. However, the particularly high abundances in the microbial mat suggests that a large ciliate population thrives in the bottom waters overlying active cold seeps and grazes on surface microbial communities.

In addition, cholesterol and cholestanol, steroidal biomarkers for eukaryotic organisms, are present at concentrations above background mud breccia levels. In these situations, steroid $\delta^{13}C$ values are significantly lower with lowest values (-47‰) observed in the carbonate crust. These values certainly reflect a mixture of contributions from organisms currently living at the cold seeps and from the organic matter that has been extruded with the mud breccia; thus, the isotopic compositions of currently living organisms are probably much lower. Nonetheless, this provides direct evidence that carbon ultimately derived from methane is eventually assimilated into eukaryote biomass by some form of trophic transfer.

7.5. Summary

Interpretation of prokaryotic biomarker $\delta^{13}C$ values in cold seep settings is complex due to the diverse community of organisms present. Many of our conclusions are tempered by the fact that not all biomarkers are diagnostic for specific bacteria (hopanoids) or that some biomarkers derive from currently unknown sources (trinorhopanoid ketone). However, compound-specific isotope analyses can still provide powerful insights into prokaryotic processes even at

such complicated settings. A first order contribution of such analyses is the identification of distinct bacterial populations. Although exact source assignments are difficult to make, initial inferences suggest that the bacterial lipids derive from both heterotrophs (branched fatty acids; Series II non-isoprenoidal diethers; diploptene?) and chemoautotrophs (Series I non-isoprenoidal diethers; bishomohopanol; trinorhopanoid ketone).

The range of bacterial and archaeal $\delta^{13}C$ values in our samples suggests that the flow of carbon from methane to CO_2 and biomass is a complex process. Initially methane is oxidized by archaea to CO_2 (as a source of energy) and consumed to form biomass. Because archaea biomarker distributions and $\delta^{13}C$ values are so variable, other species of archaea might also use DIC as a carbon source or act as heterotrophs (Pancost et al., 2001b); however, these different possibilities are difficult to evaluate because the biomarkers of anaerobic methane oxidizing archaea have not been rigorously constrained. Chemoautotrophic bacteria, possibly autotrophic sulfate-reducing bacteria, apparently utilize the resultant CO_2, while heterotrophic sulfate-reducing bacteria could consume archaeal-derived organic carbon. While this latter process almost certainly occurs, the underlying mechanisms are difficult to constrain. It is possible that sulfate-reducing bacteria simply thrive on intermediate metabolites (acetate) generated by the archaea (Valentine and Reeburgh, 2000). If not, it seems unlikely that sulfate reducers consuming larger biological molecules could maintain high sulfate reduction rates, and it is proposed that fermentative bacteria are also present and serve as intermediates in the heterotrophic processing of archaeal biomass. In any case, the consequence of this microbial consortium's activity is a pronounced decrease in the flux of methane to the water-column. Ongoing studies of pore-water geochemistry and methane concentrations in the bottom waters overlying the mud volcanoes will help constrain the magnitude of this effect but previous work (de Lange and Brumsack, 1998) suggests that most of the methane diffusing through the mud breccia is oxidised below the sediment–water interface. Thus, in the absence of catastrophic gas hydrate destabilisation, microbial processes will strongly mitigate the climatic impact of gradual gas hydrate dissolution in the East Mediterranean.

Interpretations of carbon flow also provide insight into the thermodynamics of anaerobic methane oxidation. Both microbiological approaches (Boetius et al., 2000; Orphan et al., 2001a) and lipid data (Pancost et al., 2000a; Hinrichs et al., 2000; Orphan et al., 2001a) reveal an abundant and diverse population of sulfate-reducing bacteria. These organisms likely utilize hydrogen generated by anaerobic methane oxidation and in doing so maintain its thermodynamic favourability (Hoehler et al., 1994). In turn, sulphide generated by sulfate-reducing bacteria diffuses to the mud volcano surface where it could supports sulfide-oxidizing communities as indicated by biomarkers and observations of bacterial communities. Thus, sedimentary anaerobic oxidation of methane is coupled to the flow of electrons to H_2O, SO_4^{2-}, and finally bottom water oxygen and could indirectly have an impact on bottom-water redox conditions.

8. Conclusions and directions for future research

The use of compound-specific carbon isotopic compositions can help resolve pathways of carbon flow in diverse modern and ancient ecosystems. Here we have presented two case studies in which biomarker $\delta^{13}C$ values contributed to the interpretation of sedimentary processes. However, limitations in source assignment can significantly hamper interpretation, and it is vital to continue to identify biomarkers diagnostic of specific organisms and processes. Also, although some settings are characterized by organisms whose biomarker $\delta^{13}C$ values are unique (^{13}C-depleted) and readily identify their participation in carbon cycling, this is not true for all cases and interpretation must sometimes be based on stratigraphic profiles and subtle isotopic relationships. One particularly powerful tool to partially resolve these concerns is the coupling of compound-specific carbon isotope analyses to ^{13}C-labeling (Boschker et al., 1999; Bull et al., 2000). Such techniques, although limited to the study of modern samples, can be used to define the relationships between lipids, organisms, and substrate carbon. Not only will that help elucidate pathways of carbon flow in those settings, but it could constrain the sources of novel biomarkers, contributing to the development of new molecular proxies.

Acknowledgements

We are grateful to a large number of people who have provided samples and expertise during these investigations. For peat samples and analyses, we thank B. van Geel, M. Baas, E. Heijna, and M. Schenck. For mud volcano samples, we thank the Medinaut Shipboard Scientific Party and the captain and crew of the *R/V Nadir* and the submersible *Nautile*. Special thanks also to our collaborators in studying the organic geochemistry of carbonate crusts, I. Bouloubassi, V. Aloisi, and C. Pierre. We are grateful to S. Schouten for considerable assistance in the interpretation of data and comments on the manuscript, and three anonymous reviewers for their helpful suggestions. We also thank E. Hopmans, M. Dekker, W. Pool, and M. Kienhuis for analytical assistance. The Medinaut investigation was supported by grants (NWO 750.1999.01 and ALW 809.63.010) from the Research Council for Earth and Life Sciences (ALW) of the Netherlands Organisation for Scientific Research (NWO). This is NIOZ contribution 3534. **[EO]**

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