

Molecular isotopic tracing of carbon flow and trophic relationships in a methane-supported benthic microbial community

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Abstract

A molecular isotopic study in cold-seep sediments from Kazan mud volcano in the eastern Mediterranean Sea indicates that a significant proportion of methane released in this environment is incorporated into biomass in methane-supported chemosynthetic microbial communities. Furthermore, extremely ¹³C depleted biomarkers (as much as -111‰ Vienna Pee Dee Belemnite (VPDB)) have revealed pathways of methane-derived carbon flow through the microbial community and into eukaryotic biomass. Specifically, we are able to trace the flow of methane-derived carbon through anaerobic methane-oxidizing archaea into sulfate-reducing bacteria, as well as into aerobic methane-oxidizing bacteria. The methane-derived carbon is then incorporated into eukaryotic biomass through heterotrophy by bacterivorous ciliates.

Fluxes of methane to and from the atmosphere are influential in moderating Earth's climate over a variety of time scales (Dickens et al. 1995) due to the strength of methane as a greenhouse gas. However, only small amounts of methane escape the sediments, and even less makes its way to the atmosphere under present-day conditions (Reeburgh et al. 1993). It has been proposed, however, that major climate shifts in Earth's history have been caused by sudden catastrophic methane release from marine sediments, possibly due to destabilization of gas hydrates (Dickens et al. 1995). Thus, the mechanisms of methane scavenging in marine sediments remain subjects of considerable study.

A number of studies have implicated anaerobic methane oxidation as the primary process scavenging methane in methane-rich anaerobic environments and incorporating it into organic matter in marine sediments (Reeburgh 1980). A model has been proposed in which a consortium of methane-oxidizing archaea and sulfate-reducing bacteria could be carrying out the net process of anaerobic methane oxidation, or reverse methanogenesis (Hoehler et al. 1994). This hypothesis has received substantial support from multiple lines of evidence including molecular isotopic studies in modern sediments (Elvert et al. 2001; Pancost et al. 2000; Hinrichs et al. 1999), ancient sediments (Thiel et al. 1999), and the water column (Schouten et al. 2001). A recent study using fluo-

rescence in situ hybridization (FISH; Boetius et al. 2000) identified a structured consortium of archaea and sulfate-reducing bacteria in a methane-rich marine sediment. Other studies using rRNA gene surveys (Hinrichs et al. 1999) and FISH coupled with isotopic analysis (Orphan et al. 2001) have provided additional evidence for the existence of a consortium of methane-oxidizing archaea and sulfate-reducing bacteria carrying out the net process of anaerobic methane oxidation. Despite such intense study, the specific mechanisms and biochemical pathways of methane assimilation in these biological communities remain topics of considerable debate (Hoehler et al. 1994; Valentine and Reeburgh 2000).

Methane is typically depleted in ¹³C relative to other carbon sources, and the carbon-isotopic fractionation associated with methane oxidation generally results in biomass with an extremely depleted carbon-isotope composition (Alperin et al. 1988; Whiticar 1999). Consequently, molecular isotopic techniques are useful in tracing the flow of methane-derived carbon through methane-supported biological communities.

Methane seeps have been identified in mud volcano fields in the eastern Mediterranean Sea (Woodside et al. 1998). They are formed as a result of tectonic compression which leads to the extrusion of fluid-rich mud flows (mud breccias) (Woodside et al. 1998). Methane is typically associated with the ascending fluid and mud flows (Medinaut/Medineth Shipboard Scientific Parties 2000). In fact, methane concentrations up to 1,000 times background values have been measured in the water column overlying mud volcanoes in both the Olimpi and Anaximander mud volcano areas (Medinaut/Medineth Shipboard Scientific Parties 2000); however, the concentration varies significantly between mud domes.

The present study traces methane incorporation into microbial biomass and subsequent cycling into higher trophic levels in the sediments of Kazan mud volcano in the Anaximander Mountains area of the eastern Mediterranean Sea. The methane cold seeps on Kazan and other mud volcanoes stimulate and support a significant biological community in near-surface sediments, including microbes (archaea and bacteria) as well as macrofauna such as clams, mussels, and tube worms (Medinaut/Medineth Shipboard Scientific Par-

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Table 1. Abundances ($\mu\text{g g}^{-1}$ dry sediment) of biomarkers from Kazan mud volcano.

Depth (cm)	<i>sn</i> -3-hydroxy archaeol	Archaeol	Diether 1	Diether 3	Diploptene	Diplopterol	Pristane	<i>n</i> -C ₃₁	Tetrahymanol
0–2					0.08	0.20	0.23	0.30	0.17
2–3	0.19				0.06	0.14	0.36	0.27	0.16
10–12	0.20	0.07	0.01	0.04	0.02	0.001	0.38	0.30	0.06
16–18	0.93	0.32	0.06	0.07	0.01		0.48	0.30	0.09
20–22	0.61	0.16	0.06	0.10			0.36	0.16	0.08
27–29	0.29	0.10	0.03	0.03			0.42	0.21	0.05

ties 2000). This site therefore provides an ideal location in which to trace methane flow through several trophic levels of a chemosynthetically based community. By measuring the carbon-isotopic composition of biomarkers indicative of various organism from sediments on Kazan mud volcano, we are able to trace the flow of methane-derived carbon in this environment through a microbial community consisting of anaerobic methane-oxidizing archaea, aerobic methane-oxidizing bacteria, and (anaerobic) sulfate-reducing bacteria, and into a meiofaunal community of bacterivorous ciliates.

Methods

Samples were taken by box core during the Medineth cruise of the R/V *Professor Logachev* to the eastern Mediterranean in August 1999. Core MNLBC19 covers the upper 30 cm of sediments from the Kazan mud volcano in the Anaximander mountains area (35°25.950'N, 30°33.679'E, water depth 1673 m).

Sediment samples (45 to 175 g) were freeze dried and extracted via sonication in a sequence of solvent mixtures of increasing polarity (methanol 2×, 3:1 methanol/dichloromethane 1×, 1:1 methanol/dichloromethane 2×, 1:3 methanol/dichloromethane 1×, dichloromethane 2×). Elemental sulfur was removed by adding activated copper and stirring ca. 24 h. Aliquots of total extracts were separated into apolar and polar fractions on an alumina column (activated for 2 h at 150°C) using 3× the column volume of hexane/dichloromethane (9:1) followed by 1:1 dichloromethane/methanol. Alcohols in the polar fractions were converted to trimethylsilyl derivatives with 25 μl each of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine heated at 60°C for 30 min. Apolar and polar fractions were analyzed by gas chromatography–mass spectrometry (GC-MS) and GC. GC-MS was conducted with a Hewlett-Packard 5890 GC interfaced with a VG Autospec Ultima Q MS

operated at 70 eV with a mass range m/z of 50–800 and a cycle time of 1.8 s (resolution 1,000). For both GC and GC-MS, a fused-silica CP-Sil 5 capillary column (25 m by 0.32 mm, $d_f = 0.12 \mu\text{m}$) was used with helium as carrier gas. Samples were injected at 70°C, with a temperature ramp of 20°C min^{-1} to 130°C and 4°C min^{-1} to 320°C with a 15 min isothermal period. Compound identification was based on retention time and comparison of mass spectra with the literature. Compound abundances were determined by GC with a flame ionization detector based on comparison of peak areas with standards added to the sample after column chromatography.

Isotope ratio monitoring GC-MS was performed on a Finnigan Delta C isotope ratio MS coupled to a Hewlett-Packard 5890 GC to determine compound-specific $\delta^{13}\text{C}$ values. GC conditions were the same as for GC and GC-MS. Isotope ratios are reported relative to the VPDB (Vienna Pee Dee Belemnite) standard in conventional per mil (‰) notation. $\delta^{13}\text{C}$ values have been corrected for carbon added during derivatization and have an error of less than $\pm 1\%$ unless otherwise noted (based on analytical accuracy and precision of measurements of coinjected standards).

Results and discussion

Geochemical background data—Anaerobic methane oxidation (AMO) is typically evident based on pore-water profiles of sulfate and methane (Reeburgh 1980). Sulfate and methane profiles in pore waters from Kazan mud volcano suggest that AMO may be occurring between 7- and 16-cm depth in the sediments (Fig. 1 data from R. R. Haese pers. comm.). Furthermore, significantly ^{13}C -depleted authigenic carbonates have been identified on Kazan mud volcano (–10 to –40‰; Aloisi et al. 2000), also suggesting the existence of AMO in this environment. It should be noted that pore-water geochemistry presents a view of current conditions in

Table 2. Carbon isotope compositions of biomarkers from Kazan mud volcano ($\delta^{13}\text{C}$, ‰ VPDB).

Depth (cm)	<i>sn</i> -3-hydroxy archaeol	Archaeol	Diether 1	Diether 2	Diploptene	Diplopterol	Pristane	<i>n</i> -C ₃₁	Tetrahymanol
0–2					–54.1	–55.5	–29.1	–31.2	–38.2
2–3	–103.3	–94.0			–59.7	–62.3	–28.7	–30.6	–43.5
10–12	–107.5	–94.8		–85.3			–27.8	–30.9	–64.1
16–18	–111.2	–102.5	–90.8	–90.7			–27.9	–30.4	–75.1
20–22	–111.0	–101.4	–88.4	–73.3			–28.2	–31.0	–80.1
27–29	–101.2	–80.5	–69.9	–61.4			–31.0	–32.6	–68.4

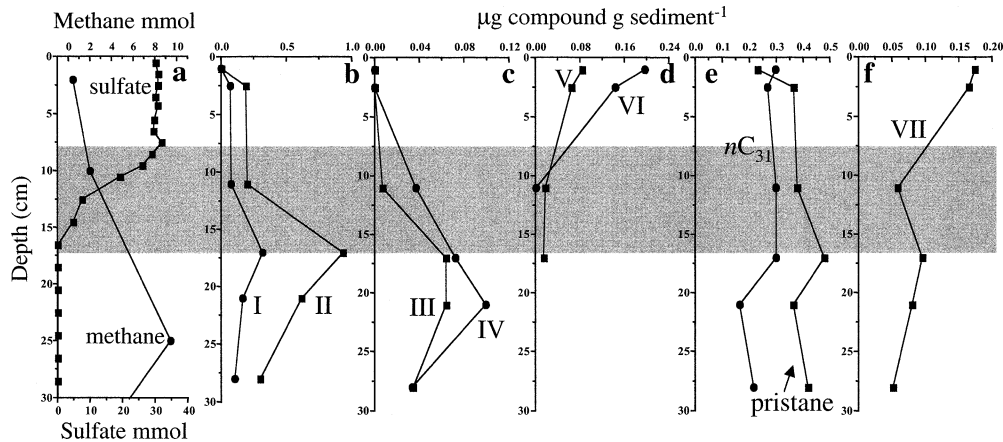


Fig. 1. Depth profiles of pore-water sulfate and methane and biomarker concentrations. (a) Net sulfate reduction is occurring between 7- and 16-cm depth, based on sulfate depletion at 16 cm and oxygen penetration to ~7 cm (R.R. Haese pers. comm.). Assuming a direct coupling of sulfate reduction and methane oxidation, present-day AMO is expected to be occurring in this same interval. Methane data are minima due to degassing during sampling (R.R. Haese et al. pers. comm.). (b)–(f) biomarkers of (b) anaerobic methane-oxidizing archaea: archaeol (I) and hydroxyarchaeol (II), (c) sulfate-reducing bacteria: alkyl diether 1 (III) and alkyl diether 2 (IV), (d) aerobic methane-oxidizing bacteria: diploptene (V) and diplopterol (VI), (e) marine and terrestrial biomarkers indigenous to the ascending mud matrix: pristane and $n\text{-C}_{31}$, and (f) bacterivorous ciliates: tetrahymanol (VII).

the sediments, whereas the existence of carbonates and the sedimentary biomarker record are a more integrated record of processes that have occurred in the sediments over time. For example, it would be possible for AMO to have existed in a particular environment long enough to produce a characteristic carbonate or biomarker signature, but if the process is not currently occurring, it will not be evident in the pore-water profiles. Thus, the indications from both the ^{13}C -depleted carbonates and pore-water sulfate and methane profiles for the existence of AMO on the Kazan mud volcano suggest the process is ongoing and probably has existed for some time.

Organic matter associated with ascending mud flows (indigenous biomarkers)—Ascending mud flows typically bring with them organic matter (OM) that was deposited at

the same time as the sediments, which has nothing to do with the processes currently occurring in the near-surface sediments. This organic matter can serve as a background against which to compare biomarkers related to present-day processes occurring in the sediments of Kazan mud volcano. For this purpose, two biomarkers were quantified: pristane, a general marine algal biomarker, and the straight-chain alkane with 31 carbons ($n\text{-C}_{31}$), a general terrestrial higher plant biomarker. The abundances of these two compounds vary only slightly with depth (Fig. 1). The homogeneity observed is believed to be due to mixing of the mud matrix during extrusion. The carbon-isotope composition of these two compounds is virtually invariant through the interval of study, with values between -28 and -32‰ (Fig. 2). Values in this range are typically related to the photoautotrophic sources of these biomarkers, and certainly not to methane consumption.

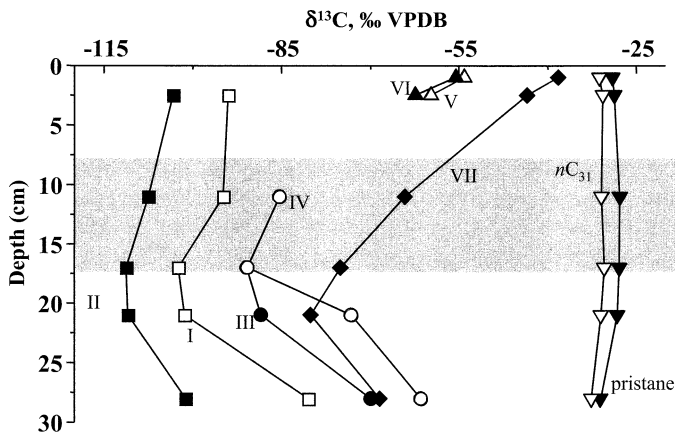


Fig. 2. Depth profiles of biomarker isotopic compositions.

Anaerobic methane oxidation—While the specific organisms responsible for carrying out anaerobic methane oxidation in marine sediments remain uncultured, significant evidence exists implicating a consortium of methane-oxidizing archaea and sulfate-reducing bacteria (Boetius et al. 2000; Pancost et al. 2000). Some of the first evidence implicating archaea in the anaerobic oxidation of methane was the identification of extremely ^{13}C depleted archaeal biomarkers in cold seeps (Thiel et al. 1999; Hinrichs et al. 1999). The ^{13}C depletion results from the light carbon-isotopic composition of methane (Whiticar 1999), which is subsequently transferred to the biomass of methane-consuming organisms (Alperin et al. 1988). Among the biomarkers of anaerobic archaeal methane oxidizers, two stand out as occurring in high abundance in many environments, archaeol (bis-*O*-phytan-

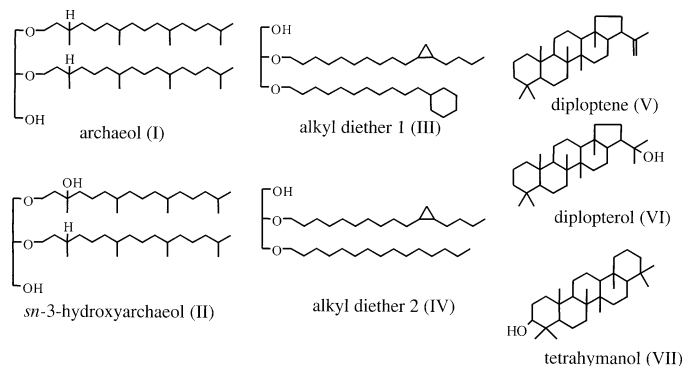


Fig. 3. Structures of relevant biomarkers.

ylglyceroldiether; compound I), (see Fig. 3 for structures) and *sn*-3-hydroxyarchaeol (II) (Pancost et al. 2000). The occurrence of these compounds in archaea has been firmly established (Koga et al. 1998), though these compounds have only been unequivocally identified in thermophiles and methanogens (Sprott et al. 1997). Despite the lack of direct observation of these compounds in methane-oxidizing archaea (due to the current inability to culture these archaea), extreme ^{13}C depletion is a clear indication of a methane-consuming metabolism.

In the sediments of Kazan mud volcano, both archaeol and *sn*-3-hydroxyarchaeol are present in relatively high abundance (Fig. 1). The concentrations of these compounds are greatest within the zone in which AMO is expected based on pore-water profiles and in fact have the greatest concentration of any compound in the extractable organic matter in this interval. The concentration of hydroxyarchaeol is more than twice that of pristane or *n*- C_{31} within this interval. The carbon-isotopic compositions of these compounds in conjunction with their structures confirm that they are derived from archaeal methane consumers: archaeol ranges from -80.5‰ to -102.5‰ and hydroxyarchaeol ranges from -101.2‰ to -111.2‰ (Fig. 2). The most depleted isotopic values of these compounds occur in the same depth interval as the maximum concentrations (16–18 cm, see Figs. 1 and 2).

Sulfate reduction—Sulfate reduction linked to methane oxidation in anoxic sediments is apparent based on pore-water profiles of sulfate and methane (Reeburgh 1980). Confirmation of the linkage between sulfate-reducing bacteria (SRB) and methane-oxidizing archaea comes from a recent study that microscopically identified a structured consortium of these microbes in sediments from the Cascadia margin (Boetius et al. 2000). Although sulfate reducers have been clearly implicated in contributing to the net process of anaerobic methane oxidation, few, if any, biomarkers can be attributed exclusively to SRB. C_{15} and C_{17} iso and anteiso fatty acids are commonly used (Pancost et al. 2000; Boetius et al. 2000), but these compounds are also produced by a host of other bacteria.

A recent study of carbonate crusts from the eastern Mediterranean believed to be formed as a byproduct of anaerobic methane oxidation (Aloisi et al. 2000) identified three novel series of dialkyl glycerol diethers that are attributed to SRB

(Pancost et al. 2001). Bacterial membranes are generally thought to contain alkyl chains ester-linked to glycerol, while archaeal membranes contain exclusively isoprenoidal units ether-linked to glycerol. However, a few bacteria seem to have developed that have a mixed membrane composed of alkyl chains ether-linked to glycerol, primarily in thermophilic settings (Langworthy et al. 1983). In one case, it has even been observed that membrane lipids are a hybrid composed of both isoprenoidal and alkyl chains ether-linked to the same glycerol groups (Schouten et al. 2000), though the organisms synthesizing this compound remain unknown. The attribution of these novel dialkyl glycerol diethers to SRB by Pancost et al. (2001), however, seems solid. In the crust samples studied, the alkyl diethers formed one of two very abundant groups of compounds, the other being isoprenoidal diethers (archaeol and hydroxyarchaeol; Pancost et al. 2001). Parallel 16S rRNA gene surveys on the same crust samples revealed abundant gene sequences from only two groups, both of which were previously unknown. One group was most closely related to known methanogens (and other suspected methane-oxidizing archaea; S.K. Heijs, unpubl. data; Pancost et al. 2001), and one group was closely related to known SRB (S.K. Heijs, unpubl. data; Pancost et al. 2001).

In sediments from Kazan mud volcano, we observe diethers characterized by a cyclopropyl C_{17} alkyl chain and either an *n*-alkyl (compound III) or ω -cyclohexyl (compound IV) moiety (series II compounds of Pancost et al. 2001; see Fig. 3 for structures) that we also attribute to SRB. These two compounds are not detectable in the upper 3 cm of sediment but have concentration maxima in the zone of AMO at the same horizon as the maximum concentrations of archaeol and hydroxyarchaeol (Fig. 1). Their carbon-isotopic compositions indicate the use of methane-derived carbon in their formation, ranging from -61.4‰ to a maximum ^{13}C depletion of -90.8‰ in the zone of AMO (Fig. 2). These biomarkers are always enriched relative to archaeol and hydroxyarchaeol by 10–40‰.

Although the carbon-isotopic composition of sulfate-reducer biomarkers clearly indicates the incorporation of methane-derived carbon, the specific pathway(s) by which they incorporate methane-derived carbon remain(s) unclear (Valentine and Reeburgh 2000; Sørensen et al. 2001). For example, it has been proposed that the syntrophic SRB may incorporate methane-derived carbon heterotrophically (Pancost et al. 2000). Pancost et al. (2000) suggested that biomass of methane-oxidizing archaea could be consumed by chemoorganotrophic bacteria such as acetogens, which would in turn provide a supply of organic substrates such as acetate for the SRB. This mechanism seems unlikely given the extremely slow rate of the process (Zengler et al. 1999) and the high rates of sulfate reduction observed in many sites (Boetius et al. 2000). Furthermore, heterotrophic metabolism is generally not associated with such large isotopic fractionations (10 to 20‰) (Blair et al. 1985).

Alternatively, it has been proposed that the sulfate reducers incorporate methane-derived carbon autotrophically. Reverse methanogenesis, as proposed by Hoehler et al. (1994) yields CO_2 that is extremely ^{13}C depleted compared to normal marine dissolved CO_2 (Whiticar 1999). Assimilation of

this CO₂ produced by the methane-oxidizing archaea could yield ¹³C-depleted sulfate-reducing bacterial biomass. In the sediments of Kazan mud volcano, dissolved inorganic carbon (DIC) isotopic values reach a maximum ¹³C depletion of ~-35‰, which suggests that up to two-thirds of the DIC pool in this interval is methane derived (based in isotopic mass balance; R.R. Haese pers. comm.). Thus, if this pathway is occurring, it appears that a carbon-isotopic fractionation of nearly 30‰ associated with DIC assimilation by sulfate reducers would be required. Carbon-isotopic fractionations of this magnitude have been observed in the laboratory (Preuß et al. 1989), so this pathway is certainly a possibility. Furthermore, the SRB in these settings are living in close physical association with the methane-oxidizing archaea as demonstrated in recent FISH and isotopic studies (Boetius et al. 2000; Orphan et al. 2001). The SRB would therefore likely be exposed to a DIC source more directly related to methane oxidation as opposed to the bulk DIC pool (i.e., exposed to a more ¹³C depleted DIC pool). Thus, a bulk isotopic value of -35‰ for DIC represents a maximum (most positive) value for the carbon isotope composition of the CO₂ being used by the SRB, and consequently a fractionation of 30‰ associated with SRB metabolism is also a maximum value.

A third possibility is that the sulfate reducers incorporate intermediate carbon species such as acetate or acetic acid (Valentine and Reeburgh 2000) produced by the anaerobic methane-oxidizing archaea. These pathways, particularly the acetic acid pathway, provide greater free energy than the mechanisms discussed above (Valentine and Reeburgh 2000), and therefore may be preferable pathways of carbon assimilation to consider. In contrast, a recent thermodynamic and kinetic study by Sørensen et al. (2001) suggests that hydrogen, acetate, and methanol are all excluded as potential electron shuttles in coupled methane oxidation/sulfate reduction. The results of Sørensen et al. (2001) apparently reduce the likelihood that acetate is a significant reactive intermediate in the net anaerobic oxidation of methane. The study by Sørensen et al. (2001) did not consider the possibility of an environment with high methane levels where acetate becomes a favorable intermediate, however, nor did it consider the specific mechanism proposed by Valentine and Reeburgh (2000) involving the transformation of two molecules of methane into acetate and hydrogen. Based on these results, therefore, acetate and acetic acid as reactive intermediates in net AMO remain distinct possibilities that would adequately explain the transfer of ¹³C depleted methane-derived carbon into SRB biomass.

Aerobic methane oxidation—Hopanoid compounds are well-documented biomarkers for bacteria (Ourisson and Rohmer 1992). Among the hopanoids, hop-22(29)-ene (diploptene) and its biosynthetic precursor hopanol (diplopterol; see Fig. 3 for structures) occur ubiquitously in sediments and have been attributed to a variety of different aerobic bacteria, including cyanobacteria, purple nonsulfur bacteria, acetic acid bacteria, ammonia-oxidizing bacteria, methylotrophs, and methanotrophs (Rohmer et al. 1984; Elvert et al. 2001), but have not been identified in anaerobic bacteria. Diploptene has also been identified in terrestrial ferns (Bot-

tari et al. 1972); however, this source is thought to be minimal in the eastern Mediterranean mud volcano sediments studied, based in large part on the carbon-isotope composition.

In sediments from Kazan mud volcano, both diploptene and diplopterol are identified. Their concentration profiles indicate a maximum in the most surficial sediments, with a rapid decrease down core to concentrations almost below the level of detection by 10-cm sediment depth (Fig. 1) and completely below detection levels by 20-cm sediment depth. Thus, they barely overlap with the biomarkers for anaerobic methane oxidizers. The carbon-isotopic compositions of these compounds range from -54.1‰ at the sediment-water interface to -71.2‰ at 12-cm sediment depth (Fig. 2). Carbon-isotope values of this range again suggest the incorporation of methane or methane-derived carbon (Alperin et al. 1988; Whiticar 1999). We attribute these compounds to aerobic methane oxidizers based on concentration and carbon-isotope profiles, which is consistent with the findings of other studies (Elvert et al. 2001). Furthermore, the profiles of various pore-water constituents (e.g., Na, Mg, Ca) suggest that bioirrigation in sediments of Kazan mud volcano mixes the pore waters down to a depth of ~7 cm (R.R. Haese pers. comm.), which suggests that oxygen would be available at depths coincident with these biomarkers.

The isotopic enrichment of these compounds relative to the anaerobic methane-oxidizer biomarkers can be explained in a number of ways. First, it is possible that the hopanoid compounds are derived from multiple sources. A simple mass balance shows that a combination of diploptene from methane oxidizers (¹³C depleted) as well as acetic acid bacteria, ammonia-oxidizing bacteria, or cyanobacteria using relatively ¹³C enriched carbon sources (e.g., dissolved inorganic carbon) would result in carbon-isotope compositions that are ¹³C enriched relative to the anaerobic methane-oxidizer biomarkers. However, this interpretation does not adequately explain the observed concentration trends in diploptene and diplopterol (Fig. 1). Alternatively, it is possible that a Rayleigh distillation effect is being observed (e.g., Pancost et al. 2000). In other words, as the methane ascends through the sediment, the anaerobic microbes living deeper in the sediments have the first opportunity to oxidize the methane, preferentially using ¹²CH₄. The remaining methane in the pore-water pool that reaches the surface community is consequently enriched in ¹³C relative to the deeper methane, which is reflected in the lipid biomarkers of the aerobic methane-oxidizing bacterial community. Such a trend of ¹³C enrichment in pore-water methane profiles upward across the sulfate to methane transition has been observed in Skan Bay (Valentine and Reeburgh 2000). The observed trends in the δ¹³C of the anaerobic methane-oxidizing archaea are also consistent with this interpretation.

A third possible explanation is that the hopanoid compounds are derived from an early colonizing group of aerobic methane-oxidizing bacteria that was subsequently out-competed by the anaerobic methane-oxidizing archaea (i.e., a paleocommunity). A scenario can be envisioned in which the (periodic) oxygenation of near-surface sediments through bioirrigation leads to the development of an aerobic methanotrophic community, which then disappears as the oxygen

is depleted. Such a cyclical process would be preserved in the concentration profiles of the lipid biomarkers.

Therefore, given the data available, we believe that assignment of these hopanoid biomarkers to aerobic methane-oxidizing bacteria is the most valid interpretation. It should be noted that the second and third options are not necessarily mutually exclusive. Either periodic aerobic methanotrophic colonizers associated with bioirrigation induced oxygenation or a community currently living in the most surficial sediments is a possible explanation for the observed trends in diploptene and diplopterol.

Bacterivory—The compound tetrahymanol (Fig. 3) has been identified in many different environments (Venkatesan et al. 1989). Multiple biological sources of tetrahymanol have been identified, including ferns (Zander et al. 1969), ruemen fungus (Kemp et al. 1984), photosynthetic purple sulfur bacteria (Kleemann et al. 1990), and freshwater ciliates (Mallory et al. 1963). Marine ciliates have been shown to produce tetrahymanol when their diet is deprived of sterols (Harvey et al. 1997), i.e., when they use prokaryotes as their sole food source.

Tetrahymanol is a major compound identified in the polar fraction of lipid extracts from Kazan mud volcano. Its concentration profile suggests major inputs from the surface sediments with a smaller contribution at depth (16–22 cm, Fig. 1). The carbon-isotope composition of tetrahymanol ranges from -38.2‰ at the sediment–water interface to -80.1‰ in the zone of AMO (20 cm, Fig. 2). In fact, the depth profile of the carbon-isotope composition of tetrahymanol very closely tracks those of the microbial (bacterial) communities discussed above (i.e., the aerobic methane oxidizers and the sulfate reducers). The carbon-isotope profile of tetrahymanol strongly suggests that it is derived from an organism that is heterotrophically feeding on bacteria, and perhaps archaea, in the sediments of Kazan mud volcano.

Carbon-isotopic evidence indicative of a heterotrophic relationship can be found in other studies. Tetrahymanol that was relatively ^{13}C enriched (compared to primary photosynthetic biomarkers) was identified in systems in which a significant portion of the diet of anaerobic bacterivorous ciliates had been obligately anaerobic photosynthetic green sulfur bacteria (Sinninghe Damsté et al. 1995 and references therein). These bacteria are typically enriched in ^{13}C due to their carbon fixation pathway, the reverse TCA cycle (Quandt et al. 1977). Thus, our interpretation is consistent with an origin of tetrahymanol from bacterivorous marine ciliates (Harvey et al. 1997). Furthermore, ciliates are known to be able to live under both aerobic and anaerobic conditions (Fenchel and Finlay 1991), making them excellent candidates for life from higher trophic levels in the surface sediments of the Kazan mud volcano, where they could graze across the oxic–anoxic interface. It should be noted that ciliates are unable to live in mineral sediments in which the grain size is small ($125\ \mu\text{m}$); however, they can survive in loose organic sediments (such as found in many lakes) (Fenchel and Finlay 1991). The sediments on Kazan mud volcano are composed of a mud breccia, that is, a fine-grained mud matrix containing large (mm to cm sized) mudstone clasts. It would therefore be possible for ciliates to move through the sedi-

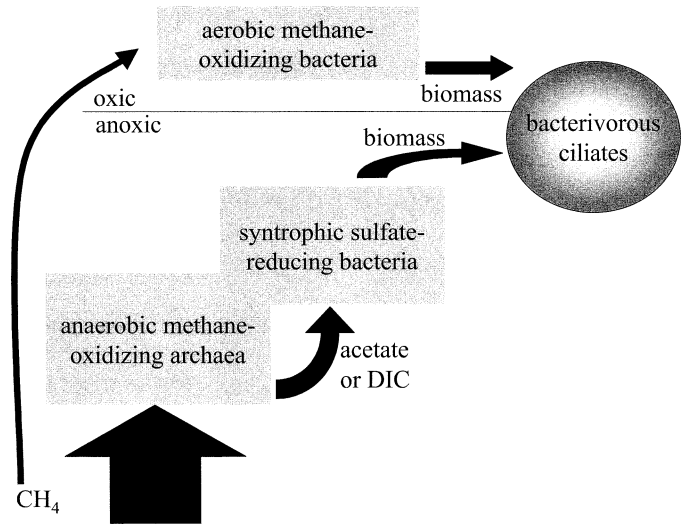


Fig. 4. Graphic representation of methane-derived carbon flow in sediments of the Kazan mud volcano. Methane associated with ascending fluids is incorporated into anaerobic methane-oxidizing archaea and aerobic methane-oxidizing bacteria. Byproducts of the anaerobic methane-oxidation process (e.g., acetate or DIC) are incorporated into sulfate-reducing bacteria. Ciliates then feed on bacteria (and possibly archaea).

ments due to the increased pore spaces associated with the clasts. Thus, it seems that ciliates in the sediments of the Kazan mud volcano are moving across the oxic–anoxic interface and feeding on various bacteria, thereby incorporating methane-derived carbon heterotrophically.

Carbon-isotopic evidence for heterotrophic transfer of methane-derived carbon has been identified in other localities. ^{13}C -depleted tetrahymanol has been identified in other mud volcano settings, including mud breccias, brine lakes, authigenic carbonate crusts, and a bacterial mat, though the source is not clearly identified (R.D. Pancost, pers. comm.). The concentration of tetrahymanol in a bacterial mat sample is 2–3 orders of magnitude greater than in other samples in which the bacteria are not so easily accessible (e.g., the bacterial biomass is “diluted” or “protected” with sediments or carbonate) (R.D. Pancost, pers. comm.). These results strongly support the assignment of tetrahymanol as a biomarker for bacterivorous ciliates in these environments.

Summary—Molecular isotopic evidence has been presented that identifies the incorporation of methane-derived carbon into both prokaryotic microbial biomass as well as eukaryotic biomass. Figure 4 summarizes the pathways of methane-derived carbon flow in cold-seep sediments of Kazan mud volcano. Methane seeping upward through mud volcano sediments is first oxidized and incorporated into biomass by anaerobic methane-oxidizing archaea. Other carbon substrates such as acetate or DIC are released by these archaea, either through decomposition of biomass (e.g., by acetogens) or as waste products. These reactive intermediate substrates are incorporated by sulfate-reducing bacteria that are living syntrophically with the archaea in the anaerobic zone of the sediments. A small proportion of the ascending methane escapes the anaerobic methane-oxidizing archaea and is subsequently incorporated by aerobic methane-oxi-

dizing bacteria in the upper ~10 cm of sediment, which are bioirrigated. Finally, at least bacterial biomass, and possibly archaeal biomass, is incorporated heterotrophically into the biomass of ciliates grazing exclusively on prokaryotes in both the aerobic and anaerobic zones of the sediment. These data suggest that significant amounts of methane can be scavenged by anaerobic and aerobic deep-sea communities and incorporated into biomass, thereby removing it from active cycling. This process significantly reduces the flux of methane into the overlying water column and eventually to the atmosphere, thereby regulating global climate change associated with the flux of methane to the atmosphere.

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