# Lipid and carbon isotopic evidence of methane-oxidizing and sulfate-reducing bacteria in association with gas hydrates from the Gulf of Mexico

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## ABSTRACT

**An integrated lipid biomarker–carbon isotope approach reveals new insight to microbial methane oxidation in the Gulf of Mexico gas-hydrate system. Hydrate-bearing and hydrate-free sediments were collected from the Gulf of Mexico slope using a research submersible. Phospholipid fatty acids consist mainly of $C_{16}$–$C_{18}$ compounds, which are largely derived from bacteria. The phospholipid fatty acids suggest that total biomass is enhanced 11–30-fold in gas-hydrate–bearing sediment compared to hydrate-free sediment. Lipid biomarkers indicative of sulfate-reducing bacteria are strongly depleted in $^{13}C$ ($\delta^{13}C$ = −48‰ to −70‰) in the hydrate-bearing samples, suggesting that they are involved in the oxidation of methane ($\delta^{13}C$ = −47‰ for thermogenic methane and −70‰ for biogenic methane). Isotopic properties of other biomarkers suggest that sulfur-oxidizing bacteria (*Beggiatoa*) may also contribute to the lipid pool in hydrate-bearing samples, which are characterized by less negative $\delta^{13}C$ values (to −11.2‰). In the hydrate-free sample, fatty acid biomarkers have $\delta^{13}C$ values of −27.6‰ to −39.6‰, indicating that crude oil (average ∼−27‰) or terrestrial organic carbon (average ∼−20‰) are the likely carbon sources. Our results provide the first lipid biomarker–stable isotope evidence that sulfate-reducing bacteria play an important role in anaerobic methane oxidation in the Gulf of Mexico gas hydrates. The coupled activities of methane-oxidizing and sulfate-reducing organisms contribute to the development of ecosystems in deep-sea environments and result in sequestration of carbon as buried organic carbon and authigenic carbonates. These have implications for studying climate change based on carbon budgets.**

**Keywords:** Gulf of Mexico, gas hydrates, anaerobic methane oxidation, sulfate-reducing bacteria, lipid biomarkers, carbon isotopes.

## INTRODUCTION

The Gulf of Mexico has abundant gas hydrates and oil seeps (Brooks et al., 1986; Roberts and Carney, 1997). Geochemical evidence indicates that oxidation of hydrocarbons including methane plays an important role in carbon cycling and the development of biological communities in gas-hydrate systems. Methane oxidation affects seafloor geology by production of $CO_2$, which is precipitated as authigenic carbonate rock on the seafloor (e.g., Roberts and Aharon, 1994). Methane oxidation also provides energy necessary for the growth and metabolism of complex chemosynthetic communities (Sassen et al., 1998) associated with gas hydrates.

Specific microorganisms responsible for methane oxidation in the Gulf of Mexico have not been identified. However, chemical analyses indicate a coupling between sulfate reduction and methane oxidation (Aharon and Fu, 2000). In this study we adopt a lipid biomarker and stable carbon isotope approach to assess the types of microorganisms involved in carbon cycling in the Gulf of Mexico gas-hydrate system. Our results suggest that sulfate-reducing bacteria are directly involved in the oxidation of methane and other hydrocarbons.

## MATERIAL AND METHODS

Hydrate-bearing and hydrate-free samples were collected in the Green Canyon (GC) region of the Gulf of Mexico using a research submersible. Site description and detailed sampling procedures were reported in Sassen and MacDonald (1997). Hydrate-bearing samples were collected from site GC-234 (∼540 m depth) and site GC-286 (∼839 m depth). A hydrate-free sample (GC-cntrl) was collected ∼15 m from the hydrate-bearing sample at GC-286. Samples were frozen at −20 °C immediately upon recovery at the sea surface and kept frozen until analysis. Because of limited samples, results herein are preliminary and will be expanded later.

Extraction of fatty acids followed the procedures of White et al. (1979) and Zhang et al. (2002). Polar lipids were treated using mild alkaline methanolysis to produce fatty acid methyl esters (Fig. 1). The positions of double bonds in monounsaturated fatty acid methyl esters were determined by gas chromatography–mass spectrometry of the dimethyl disulfide adducts (Nichols et al., 1986).

Carbon isotopic properties of methylated fatty acids were determined on an HP 6890 gas chromatograph connected to a Finnigan MAT Delta$^{+}$-XL mass spectrometer via a GC-C III interface. Because of coelution of some fatty acid isomers, a composite $\delta^{13}C$ value was reported using the weighed average of isomers. Measurements were corrected for the methyl moiety (Abrajano et al., 1994; Zhang et al., 2002). Precision for an internal standard (19:0) was ±1.26‰ (n = 10). The $\delta^{13}C$ of bulk organic carbon (BOC) was determined on acidified samples by analyzing combusted $CO_2$ on a MAT 252 mass spectrometer. Precision for an organic standard (acetanilide) was 0.09‰ (n = 5). All carbon isotope ratios are reported relative to the Peedee belemnite standard.

## RESULTS

Phospholipid fatty acids are dominated by straight-chained $C_{14}$–$C_{18}$ fatty acids for all

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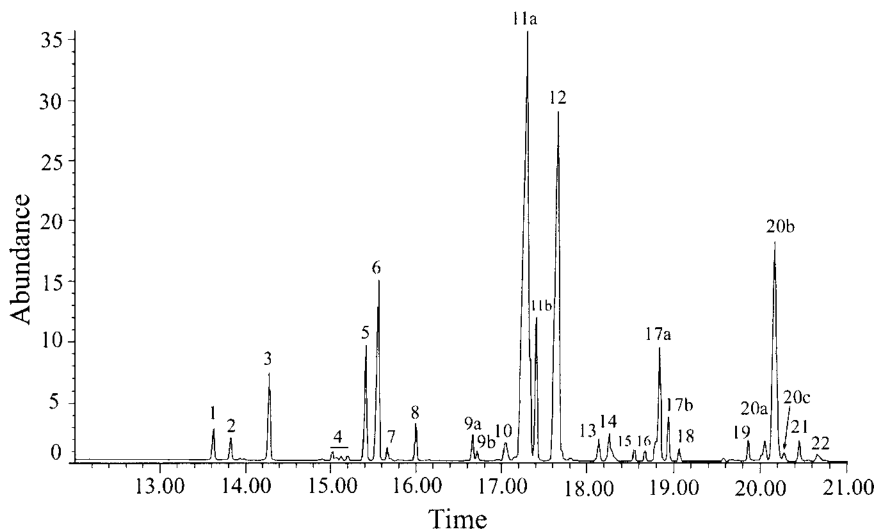


Figure 1. Capillary chromatograph of phospholipid fatty acids (as methyl esters) extracted from gas-hydrate sample GC-286 (1, i14:0; 2, 14:1ω5; 3, 14:0; 4, i15:1isomers; 5, i15:0; 6, a15:0; 7, 15:1isomers; 8, 15:0; 9a, i16:1a; 9b, i16:1b; 10, i16:0; 11a, 16:1ω9c; 11b, 16:1ω7c; 12, 16:0; 13, i17:1ω7c; 14, 10Me16:0; 15, i17:0; 16, a17:0; 17a, cy17:0a; 17b, cy17:0b; 18, 17:0; 19, 18:2ω6; 20a, 18:1ω9c; 20b, 18:1ω7c; 20c, 18:1ω7t; 21, 18:0; 22, dimethyl17:0). Fatty acid profiles of GC-234 and GC-cntrl are similar and thus not shown.

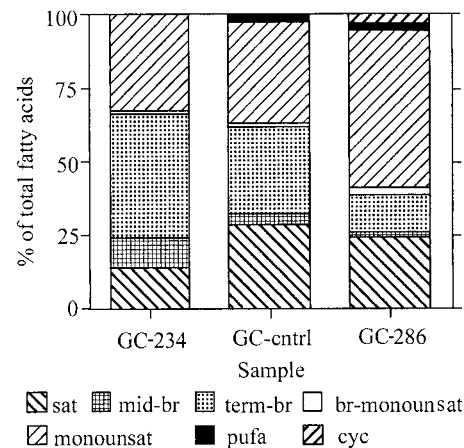


Figure 2. Distribution of saturated (sat), mid-chain branched saturated (mid-br), terminally branched saturated (term-br), branched-chain monounsaturated (br-monounsat), monounsaturated (monounsat), polyunsaturated (pufa), and cyclic-saturated (cyc) fatty acids.

samples. The fatty acids consist mainly of monosaturated, terminally branched saturated, and monounsaturated structures (Fig. 2). The mid-chain branched monosaturated, branched-chain monounsaturated, polyunsaturated fatty acids, and cyclic fatty acids in the $C_{14}$–$C_{18}$ range are minor components (Fig. 2). Fatty acids in carbon number greater than $C_{22}$ are below detection limits (data not shown).

In all samples, 16:0 isomer dominates the saturated fatty acids, i15:0 plus a15:0 isomers dominate the terminally branched saturated fatty acids, and 16:1 and 18:1 isomers dominate the monounsaturated fatty acids (Fig. 3). Although major fatty acids have similar distributions in all samples (Figs. 2 and 3), total phospholipid fatty acids were 11–30-fold higher in GC-234 (8.0 nmol/g) and GC-286 (22.1 nmol/g) than in GC-cntrl (0.7 nmol/g), which is consistent with enhanced microbial biomass at gas-hydrate deposits.

The $\delta^{13}C$ of individual fatty acids varies widely in GC-234 (−30.6‰ to −69.7‰) and GC-286 (−11.2‰ to −65.8‰). The variation is smaller in GC-cntrl (−27.6‰ to −39.6‰). Fatty acids most depleted in $^{13}C$ in GC-234 and GC-286 include a15:0, i17:0, and a17:0, whereas the most $^{13}C$-depleted fatty acid in GC-cntrl is a15:0 (Table 1). The depletion was as much as −37‰ relative to bulk organic carbon (BOC) in GC-234 (a17:0) and GC-286 (i17:0), but by only −11‰ in GC-cntrl (a15:0). Other fatty acids in GC-234 and GC-286, however, are enriched in $^{13}C$ relative to BOC. These include 15:0, cy17:0, and 18:0 in GC-234 and 14:1ω5, 15:0, i16:1, 18:1, and 18:0 in GC-286. In GC-cntrl, only 18:0 is enriched in $^{13}C$ relative to the BOC (Table 1).

## DISCUSSION

Lipids in marine sediments may be derived from terrestrial organic carbon, plankton, and in situ microorganisms or macroorganisms. Several lines of evidence indicate that phospholipid fatty acids analyzed here are mainly derived from in situ bacteria. First, white masses of bacteria (likely *Beggiatoa*) were visible to the eye at collection in GC-234 and GC-286. Second, long-chain (>$C_{25}$) *n*-alkyl fatty acids characteristic of leaf waxes from terrestrial higher plants (Huang et al., 1999) are below detection limits. Third, polyunsaturated fatty acids such as 18:4ω3, 18:5ω3, 20:5ω3, and 22:6ω3, which are enriched in planktonic organisms such as flagellate, diatoms, and dinoflagellates (Wakeham, 1995), are present in only trace abundance (<3%). Monosaturated or monounsaturated fatty acids such as 16:0, 16:1ω7c, and 18:1ω7c can be derived from cyanobacteria or diatoms (Wakeham, 1995). However, the contribution of these organisms to marine sediment is likely to be significantly diminished as their remains settle through the thick oxygenated water column (Wakeham et al., 1997). The distribution of phospholipid fatty acids closely fits the widely accepted characteristics of bacterial fatty acids (Perry et al., 1979). In particular, branched-chain fatty acids such as i15:0, a15:0, i17:0, a17:0, and

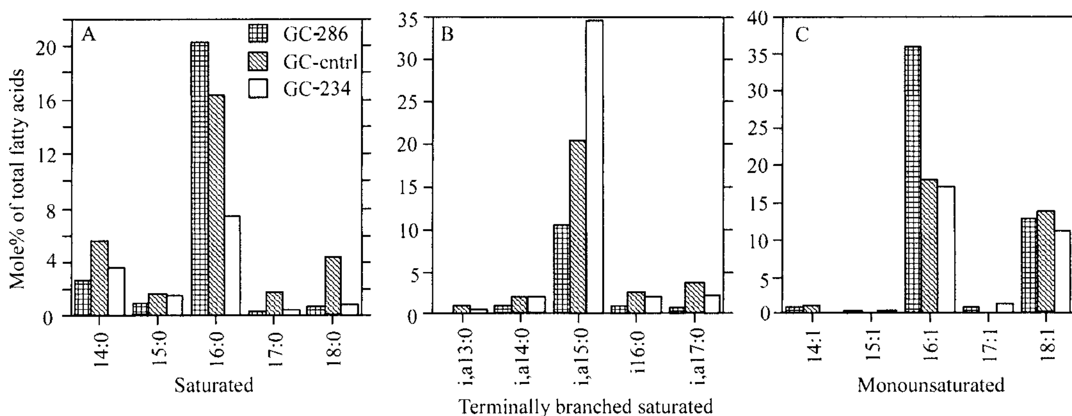


Figure 3. Relative abundance of individual fatty acids in gas-hydrate (GC-234, GC-286) and hydrate-free (GC-cntrl) samples. Terminally branched fatty acids included both iso (i) and anteiso (a) compounds. Monounsaturated fatty acids included all isomers.

TABLE 1. $\delta^{13}C$ AND $\epsilon$ OF FATTY ACIDS IN SEDIMENT SAMPLES FROM THE GULF OF MEXICO

| Fatty acids | Peak number | GC-234 | | GC-286 | | GC-cntrl | |
|---|---|---|---|---|---|---|---|
| | | $\delta^{13}C$ (‰) | $\epsilon$ (‰) | $\delta^{13}C$ (‰) | $\epsilon$ (‰) | $\delta^{13}C$ (‰) | $\epsilon$ (‰) |
| i14:0 | 1 | −41.5 | −12.8 | −35.9 ± 0.2 | −6.2 | −30.5 ± 0.7 | −1.5 |
| 14:1ω5 | 2 | N.D.† | N.D. | −11.2 ± 0.1 | 19.3 | N.D. | N.D. |
| 14:0 | 3 | −51.1 ± 1.1 | −22.7 | −36.6 ± 0.1 | −6.9 | −34.5 ± 0.5 | −5.6 |
| i15:0 | 5 | −47.6 ± 0.9 | −14.1 | −57.1 ± 0.9 | −28.0 | −36.0 ± 0.4 | −7.2 |
| a15:0 | 6 | −64.4 ± 0.8 | −36.4 | −63.5 ± 0.7 | −34.6 | −39.6 ± 0.2 | −10.8 |
| 15:0 | 8 | −33.2 ± 0.6 | 0.9 | −28.6 ± 0.4 | 1.3 | −32.5 ± 1.0 | −3.5 |
| i16:1 | 9 | N.D. | N.D. | −26.8 ± 0.5 | 3.2 | N.D. | N.D. |
| i16:0 | 10 | −35.5 ± 1.2 | −1.5 | −32.4 ± 0.6 | −2.6 | −34.0 ± 0.8 | −5.0 |
| 16:1 | 11 | −51.2 ± 0.9 | −22.8 | −33.1 ± 0.6 | −3.3 | −35.5 ± 0.5 | −6.6 |
| 16:0 | 12 | −47.4 ± 0.9 | −18.8 | −35.5 ± 0.5 | −5.8 | −35.5 ± 0.2 | −6.6 |
| i17:1ω7c | 13 | −54.8 ± 1.2 | −21.5 | −57.2 | −28.1 | −33.8 ± 0.6 | −4.8 |
| 10Me16:0 | 14 | N.D. | N.D. | −47.4 | −18.0 | N.D. | N.D. |
| i17:0 | 15 | −65.5 ± 2.2 | −32.6 | −65.8 ± 4.2 | −37.0 | −33.4 ± 2.1 | −4.4 |
| a17:0 | 16 | −69.7 ± 3.1 | −36.9 | −64.4 ± 2.5 | −35.6 | N.D. | N.D. |
| cy17:0 | 17 | −30.5 ± 0.7 | 3.6 | −35.4 ± 0.1 | −5.7 | −31.3 ± 1.7 | −2.2 |
| 18:1 | 20 | −49.5 ± 0.8 | −16.0 | −21.3 ± 0.9 | 8.9 | −32.5 ± 0.4 | −3.5 |
| 18:0 | 21 | −30.6 ± 0.9 | 3.6 | −29.8 | 0.1 | −27.6 ± 0.3 | 1.5 |
| dimethyl17:0 | 22 | −61.2 ± 1.4 | −28.2 | −59.5 ± 0.8 | −30.5 | N.D. | N.D. |
| BOC | N.A.‡ | −34.0 ± 0.2 | N.D. | −29.9 ± 0.3 | N.D. | −29.1 ± 3.7 | N.D. |

*Note*: Peak numbers refer to those in Figure 1. $\delta^{13}C$ is the mean ± one standard deviation (n = 1 to 4). $\epsilon = [(1000 + \delta^{13}C_{fatty\ acid})/(1000 + \delta^{13}C_{substrate}) - 1] \times 1000$. Isotopic compositions of i16:1, 16:1, cy17:0, and 18:1 represent the weighed-averages of coeluting isomers (Fig. 1). BOC is bulk organic carbon.

†N.D. = not determined.

‡N.A. = not applicable.

10Me16:0 are used as valid biomarkers for bacteria (most often sulfate-reducing bacteria) in marine sediment (Perry et al., 1979; Wakeham, 1995). In this study, the branched fatty acids compose 16%–52% of total fatty acids (Fig. 3). Given the predominance of sulfate reduction in hydrate-bearing sediment (Sassen et al., 1998), these branched fatty acids are most likely derived from sulfate-reducing bacteria.

Because gas hydrates are often associated with complex chemosynthetic communities (Fisher, 1990), the possibility exists that the degradation products of these large animals may contribute to the lipids in these samples. In sediment near GC-234, vestimentiferan tubeworms are found with *Beggiatoa* (Sassen et al., 1994). Only dead bivalves and living *Beggiatoa* are found at GC-286 and GC-cntrl (Sassen, personal observation). There is no reason to believe that living macrofauna are directly associated with samples. However, the widely occurring *Beggiatoa* is likely to contribute to the lipid pool.

The BOC is a mixture from several carbon sources with different $\delta^{13}C$. The $\delta^{13}C$ of sedimentary organic carbon in the Gulf of Mexico ranges from −19.7‰ to −21.7‰ (Gõni et al., 1998), that of crude oil is ∼−27‰ (Kennicutt et al., 1988), and that of methane averages ∼−43‰ for thermogenic origin and ∼−70‰ for biogenic origin (Brooks et al., 1986). The $\delta^{13}C$ of the BOC in this study ranges from −29.1‰ in the hydrate-free sample to −34.0‰ in the hydrate-bearing samples (Table 1). All values are consistent with the presence of various hydrocarbons. However, there is a strong contribution from methane-derived organic carbon in the hydrate-bearing samples.

The isotopic composition of fatty acids and fractionation with BOC suggest carbon utilization by different bacteria. Based on laboratory experiments using pure cultures, aerobic heterotrophs produce small fractionations (2.6‰ to −4.1‰) between fatty acids and the carbon substrate (Monson and Hayes, 1982; Blair et al., 1985; Abraham et al., 1998). Anaerobic heterotrophs produce greater negative fractionation (−9.4‰ to −21.0‰) (Teece et al., 1999; Zhang et al., 2002), and aerobic methanotrophs generate still greater negative fractionation (as much as −32.3‰) (Summons et al., 1994; Jahnke et al., 1999). However, some chemoautotrophic sulfur-oxidizing bacteria produce fatty acids enriched in $^{13}C$ relative to cell biomass (van der Meer et al., 1998).

The isotopic fractionations between fatty acids and the BOC in GC-cntrl indicate that aerobic organisms may be mainly responsible for the synthesis of 16:0, 16:1, and 18:1 ($\varepsilon$ = −3.5‰ to −6.6‰), but the sulfate-reducing bacteria may be responsible for the biosynthesis of a15:0 ($\varepsilon$ = −10.8‰) (Table 1). Both aerobes and the sulfate-reducing bacteria appear to live on petroleum hydrocarbons as a major carbon source. Results are consistent with molecular properties of long-chain alkanes of the crude oil, which show evidence of advanced bacterial oxidation (Sassen et al., 1994).

Isotopic fractionations between fatty acids and BOC from GC-234 range from 3.6‰ to −36.9‰ (Table 1), suggesting a more complex microbial community with different metabolic pathways. The extremely $^{13}C$-depleted a15:0, i17:0, and a17:0 fatty acids (Table 1) are likely from methane oxidation associated with sulfate-reducing bacteria. However, the $^{13}C$ of other fatty acids is consistent with multiple sources. *Beggiatoa* is certainly a component. Isotopic determination of *Beggiatoa* mats in the Gulf of Mexico yielded $\delta^{13}C$ of ∼−27‰ (Sassen et al., 1993). The lipid composition and isotope ratios of *Beggiatoa* are not reported. However, green and purple sulfur bacteria, which may use similar metabolic pathways as *Beggiatoa*, have fatty acids composed mainly of 16:0, 16:1, and 18:1 (van der Meer et al., 1998). If *Beggiatoa* also has these fatty acids and positive fractionation (2‰–16‰) versus cell biomass (van der Meer et al., 1998), the expected $\delta^{13}C$ values of fatty acids in *Beggiatoa* could range from −25‰ to −11‰, which would increase the $\delta^{13}C$ values of the overall fatty acid pool. Isotopic results from GC-286 are consistent with this conclusion. For GC-286, the large depletion in $^{13}C$ and large fractionation of i15:0, a15:0, i17:1ω7c, i17:0, a17:0, and dimethyl17:0 indicate methane oxidation by sulfate-reducing bacteria (Table 1). However, the enrichment in $^{13}C$ of i16:1, 18:0, and 18:1 are consistent with a contribution from *Beggiatoa*. Although 14:1ω5 in GC-286 showed the largest enrichment in $^{13}C$, its source is unknown.

Recent studies show that anaerobic methane oxidation may be mediated by consortia of methanogens and sulfate-reducing bacteria (e.g., Boetius et al., 2000). The mechanism is proposed to be (1) methanogens, running in reverse, oxidize methane to $CO_2$ and $H_2$ ($CH_4 + 2H_2O \rightarrow CO_2 + 4H_2$), or to acetate ($2CH_4 + 2H_2O \rightarrow CH_3COOH + 4H_2$), and (2) sulfate-reducing bacteria reduce sulfate by oxidizing $H_2$ or acetate from methane oxidation ($H^+ + 4H_2 + SO_4^{2-} \rightarrow HS^- + 4H_2O$; $CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$) (Hoehler et al., 1994; Valentine and Reeburgh, 2000). Methane carbon can be incorporated into biomass of sulfate-reducing bacteria via heterotrophic growth on $^{13}C$-depleted acetate or via chemoautotrophic growth on $^{13}C$-depleted $CO_2$ (Valentine and Reeburgh, 2000). In this study, the extremely $^{13}C$-depleted lipid biomarkers are a clear indication that sulfate-reducing bacteria are involved in the oxidation of methane in the Gulf of Mexico. We are currently working on a procedure to determine the $\delta^{13}C$ of archaeal lipids characteristic of methanogens, which are expected to be depleted in $^{13}C$ as well (Hinriches et al., 1999; Pancost et al., 2000).

Research on anaerobic methane oxidation highlights an important ecosystem in the gas-hydrate environment. For example, production of $H_2S$ by sulfate-reducing bacteria fuels chemoautotrophs in direct association with shallow gas-hydrate outcrops. This has implications for the global biogeochemical cycle of carbon, especially during the Late Proterozoic interglacials, when buried organic carbon had

abnormally low $\delta^{13}C$ values that were attributed to sulfide-oxidizing bacteria or other chemoautotrophs (Hayes et al., 1999). Furthermore, sulfate reduction coupled to methane oxidation affects alkalinity and favors carbonate precipitation. Without this coupling, precipitation of enormous volumes of carbonate rock at the seafloor is less likely. The coupled process of hydrocarbon oxidation and sulfate reduction may lead to sequestration of large amounts of carbon that otherwise would enter the water column and atmosphere, affecting global climate change.

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