

Methanogenic pathway, ^{13}C isotope fractionation, and archaeal community composition in the sediment of two clear-water lakes of Amazonia

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Abstract

We studied the methanogenic pathway and archaeal community composition in the sediment of two clear-water lakes, Lake Batata and Lake Mussura, in Amazonia. We measured CH_4 production and $\delta^{13}\text{C}$ of CO_2 , CH_4 , and acetate-methyl in the presence and absence of CH_3F , an inhibitor of acetotrophic methanogenesis. The fractionation factor of methanogenesis from CO_2 was rather high in both lake sediments, which was consistent with the low concentrations of H_2 and the small negative Gibbs free energy of hydrogenotrophic methanogenesis. The $\delta^{13}\text{C}$ of acetate-methyl was relatively low compared to that of organic matter and decreased further upon inhibition of acetate consumption by CH_3F . Collectively, the data possibly suggest involvement of syntrophic acetate oxidation besides acetotrophic methanogenesis. The isotopic data were used to calculate the percent contribution of CO_2 reduction to total methanogenesis, which was rather high (approximately 53–63%). Copy numbers of bacterial and archaeal 16S ribosomal ribonucleic acid (rRNA) genes were about 10-fold higher in Lake Mussura than in Lake Batata, indicating that microbial numbers were not a limiting factor for production rates of CH_4 , which were similar in both lake sediments. The composition of the archaeal community was analyzed by cloning and sequencing of the genes coding for 16S rRNA and methyl coenzyme M reductase (*mcrA*), demonstrating the presence of acetotrophic *Methanosaetaceae* and different hydrogenotrophic methanogenic orders (*Methanomicrobiales*, *Methanobacteriales*, *Methanocellales*) in both lake sediments. Although methanogenic communities and pathways were principally comparable to those found in lake sediments of the midlatitudes, there were several particularities, e.g., the possible involvement of syntrophic acetate oxidation.

Emission from wetlands is the single most important source (105–175 Tg yr⁻¹) in the global budget of greenhouse gas methane, and tropical wetlands contribute about 60% (Lelieveld et al. 1998). Process modeling of CH_4 emission arrives at even higher values, especially in the tropics (Walter et al. 2001), and these results are consistent with results from inverse modeling of atmospheric observations of CH_4 and its $\delta^{13}\text{C}$ values (Fletcher et al. 2004). Lakes within the floodplains of tropical rivers have been found to be an especially significant source of atmospheric CH_4 , e.g., the Amazon (Devol et al. 1990), Orinoco (Smith et al. 2000), and Pantanal (Marani and Alvala 2007). Much of the CH_4 flux seems to be based on ebullition, especially in the more shallow-water bodies (Marani and Alvala 2007), such that the ^{13}C isotopic signature of the emitted CH_4 is probably not affected by methanotrophic CH_4 oxidation in the water column. From measurements of $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of CH_4 and CO_2 in gas bubbles entrapped in sediments, Wassmann et al. (1992) tentatively concluded that CH_4 is mainly formed by “methyl fermentation,” i.e., acetotrophic methanogenesis. However, the pathway of microbial CH_4 formation and its effect on the ^{13}C isotopic signature of the formed CH_4 have to our knowledge not yet been studied in detail using sediments from tropical lakes. Also, the methanogenic microbial communities involved in CH_4 production have not yet been studied in tropical freshwater sediments. Conversely, such knowledge has been obtained from some lake sediments in midlatitudes.

However, even in midlatitude lake sediments, there are to our knowledge not many studies that have analyzed the methanogenic pathway and/or ^{13}C isotope fractionation of the methanogenic community, e.g. Lake Rothsee, Switzerland (Zepp-Falz et al. 1999), Lake Biwa, Japan (Koizumi et al. 2004), Lake Dagow, Germany (Chan et al. 2005), Lake Stechlin, Germany (Conrad et al. 2007), and Lake Kinneret, Israel (Nüsslein et al. 2001, 2003).

Methane in freshwater sediments is produced as terminal step in the degradation of organic matter under anoxic conditions. Organic matter is fermented to acetate, H_2 , and CO_2 , which are subsequently converted to CH_4 by methanogenic archaea. Acetotrophic methanogenesis ($\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2$) usually contributes more to total CH_4 production than hydrogenotrophic methanogenesis ($4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$), but the relative proportion can vary (Conrad 1999). The relative contribution of either pathway also affects the $\delta^{13}\text{C}$ of the produced CH_4 , since hydrogenotrophic methanogenesis expresses a larger kinetic isotope effect than acetotrophic methanogenesis (Whiticar 1999; Conrad 2005). Conversely, $\delta^{13}\text{C}$ values in CH_4 , CO_2 , and acetate can be used to delineate the relative contributions of the two methanogenic pathways if the relevant fractionation factors are known. Thus, $\delta^{13}\text{C}$ values measured in methanogenic environments have frequently been exploited to determine the relative importance of the acetotrophic vs. hydrogenotrophic path of CH_4 production (Conrad 2005). However, it has also been noticed that the fractionation factors involved in CH_4 production from either acetate or CO_2 can vary with the

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$\delta^{13}\text{C}$ of the substrate (i.e., organic carbon, acetate, and CO_2) (Conrad et al. 2009), the H_2 partial pressure and energetic conditions for hydrogenotrophic methanogenesis (Penning et al. 2005; Sugimoto and Fujita 2006), the acetate concentration (Govert and Conrad 2009), and the composition of the active methanogenic archaeal community (Penning et al. 2006; Govert and Conrad 2009). The importance of each of these controls can presently not be predicted for a particular environment, so that fractionation factors must be determined for each individual CH_4 -producing environment. The fractionation factor for hydrogenotrophic methanogenesis ($\alpha_{\text{CO}_2, \text{CH}_4}$ or $\epsilon_{\text{CO}_2, \text{CH}_4}$), which varies over a particularly large range, can conveniently be determined by specifically inhibiting acetotrophic methanogenesis using methyl fluoride (CH_3F) (Conrad et al. 2009).

In the present study, we investigated the pathway and methanogenic microbial community by which CH_4 was formed in anoxic sediments from tropical lakes, and the mechanism of stable carbon isotope fractionation during this process. We decided to study the sediments of two clear-water lakes, Lake Batata and Lake Mussura, within the floodplain of Trombetas River, a tributary of the Amazon River.

Methods

Sampling—The sampling sites have been described before (Melo et al. 2004). Lake Batata ($1^\circ 25' \text{S}$, $56^\circ 15' \text{W}$) and Lake Mussura ($1^\circ 35' \text{S}$, $56^\circ 26' \text{W}$) are located on the southern and northern side of Trombetas River, respectively. Trombetas River, a northern tributary of the Amazon River, originates from the Guyanas upland and is categorized as a clear-water river. The area and depth of the lakes change considerably due to seasonal flood pulses of the river that can amount to 7 m. At low water, Lake Batata is connected to Trombetas River only through a small channel, but at high water, the river floods over the lake banks. Lake Mussura, on the other hand, maintains only a single point of communication with the Trombetas River, even during the flooding period. The mixing regime of the two lakes is different. Whereas the mixing zone in Lake Batata extends from the surface to the bottom during the night, that in Lake Mussura is restricted to the upper 3-m layer during a 24-h cycle (Melo et al. 2004). Water characteristics in Lake Batata and Lake Mussura, respectively, were determined immediately before sampling: depth was 6.5 and 6.0 m; water temperature above sediment was 31°C and 30°C ; and the pH above sediment was 5.5 and 5.3. Sediment cores (6 cm diameter, 40 cm length) were taken during a high-water period in June 2005 using a corer sampler. The upper 0–3 cm of sediment were placed into plastic bottles and completely filled. The bottles were shipped by air freight to Marburg and processed there immediately. The main characteristics of the sediments are shown in Table 1.

Incubation experiments—The incubation procedure was basically the same as that described by Conrad et al. (2007). About 12-mL aliquots (about 2.2 g dry wt) of the sediment were transferred in triplicate into 60-mL sterile serum

Table 1. Main characteristics of the lake sediments.

Characteristic	Lake Batata	Lake Mussura
Organic carbon (%)	6.2 ± 0.03	6.0 ± 0.05
$\delta^{13}\text{C}_{\text{org}}$ (‰)	-31.99 ± 0.02	-31.91 ± 0.02
Total nitrogen (%)	0.61 ± 0.02	0.61 ± 0.01
Total iron ($\mu\text{mol g dry wt}^{-1}$)	196 ± 6	46 ± 1
Sulfate ($\text{nmol g dry wt}^{-1}$)	12	237
pH	6.9	7.2
CH_4 production ($\text{nmol h}^{-1} \text{ g dry wt}^{-1}$)	63.3 ± 1.9	45.5 ± 2.7
CO_2 production ^a ($\text{nmol h}^{-1} \text{ g dry wt}^{-1}$)	44.1 ± 0.7	59.0 ± 0.7
Bacteria (10^8 16S rRNA gene copies g dry wt ⁻¹)	5.7 ± 3.2	79.6 ± 10.7
Archaea (10^7 16S rRNA gene copies g dry wt ⁻¹)	2.8 ± 1.7	63.6 ± 7.4

^a CO_2 production in terms of TIC.

bottles, flushed with N_2 , closed with butyl rubber stoppers, and incubated overnight at 25°C . The exact amount of sediment was determined gravimetrically. Then, the bottles were flushed again with N_2 and further incubated at 25°C . The gas headspace of some of the bottles was supplemented with 0.5%, 1.0%, and 2.0% CH_3F (Fluorochrome Company) to specifically inhibit acetotrophic methanogenesis (Janssen and Frenzel 1997). Gas samples were taken repeatedly during the course of incubation and analyzed for CH_4 , CO_2 , H_2 , and $\delta^{13}\text{C}$ of CH_4 and CO_2 . At the end of incubation, the bottles were sacrificed for sampling of the liquid phase. Aliquots of the sediment slurry were centrifuged, and the supernatants were filtered through 0.2- μm polytetrafluoroethylene (PTFE) membrane filters and stored frozen (-20°C) for later analysis of concentration and $\delta^{13}\text{C}$ of acetate. The $\delta^{13}\text{C}$ of total carbon ($\delta^{13}\text{C}_{\text{tot}}$) was analyzed after air drying of the sediment at room temperature. The $\delta^{13}\text{C}$ of organic carbon ($\delta^{13}\text{C}_{\text{org}}$) was measured after removal of carbonate carbon by addition of HCl, followed by air drying of the sediment slurry at room temperature. There was no CO_2 bubble formation upon addition of HCl, and there was virtually no difference ($< 0.04\text{‰}$) between $\delta^{13}\text{C}_{\text{tot}}$ and $\delta^{13}\text{C}_{\text{org}}$.

Molecular analysis of the methanogenic archaeal community—The deoxyribonucleic acid (DNA) of the sediment samples was extracted with the Soil DNA Isolation Kit (MP) following the manufacturer's instructions as described in detail by Kolb et al. (2005). The abundance of archaeal and bacterial 16S ribosomal ribonucleic acid (rRNA) gene copies was determined by real-time polymerase chain reaction (PCR) as described previously (Conrad et al. 2008). Cloning and sequencing of the 16S rRNA and methyl coenzyme M reductase (*mcrA*) genes were done as described previously in detail (Conrad et al. 2008). The *mcrA* gene codes for a subunit of the methyl coenzyme M reductase, the key enzyme of CH_4 production that is unique to methanogenic archaea. Phylogenetic trees were calculated as described by Conrad et al. (2008). Sequences of 16S rRNA and *mcrA* genes were deposited in the GenBank

database under accession numbers FN432637-FN432723 and FN432549-FN432636, respectively.

Chemical analyses—Methane, CO₂, and H₂ were analyzed by gas chromatography, and acetate was analyzed by high-pressure liquid chromatography (HPLC) as described by Conrad et al. (2007). The C and N contents of the sediments were quantified on a CHN-elemental analyzer by the Analytical Chemical Laboratory of the University of Marburg. Sulfate was analyzed by ion chromatography, and total iron was analyzed by the ferrozine method (Conrad and Klose 2006). The δ¹³C (in units of per mil) values of CH₄ (δ¹³C_{CH₄}) and CO₂ (δ¹³C_{CO₂}) were analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS), and the δ¹³C of acetate (δ¹³C_{ac}) was analyzed by HPLC-C-IRMS as described before (Conrad et al. 2007). The δ¹³C of the methyl group of acetate (δ¹³C_{ac-methyl}) was determined after off-line pyrolysis (Conrad et al. 2007). Analysis of the δ¹³C in organic matter was done at the Institute of Soil Science and Forest Nutrition (IBW) at the University of Göttingen (courtesy of Heinz Flessa) using an elemental analyzer (Fisons EA 1108) coupled to an IRMS.

Calculations—Fractionation factors for a reaction A → B are defined after Hayes (1993):

$$\alpha_{A,B} = (\delta^{13}C_A + 1000) / (\delta^{13}C_B + 1000) \quad (1)$$

sometimes expressed as isotopic enrichment factor $\epsilon \equiv 1 - \alpha$ (in units of per mil). The δ¹³C for newly formed CH₄ (δ¹³C_{new}) was calculated from the δ¹³C at two time points, $t = 1$ (δ¹³C₁) and $t = 2$ (δ¹³C₂) by the following mass balance equation:

$$\delta^{13}C_2 = f_{\text{new}} \delta^{13}C_{\text{new}} + (1 - f_{\text{new}}) \delta^{13}C_1 \quad (2)$$

where f_{new} is the fraction of the newly formed C-compound relative to the total at $t = 2$.

The apparent fractionation factor for conversion of CO₂ to CH₄ is given by

$$\alpha_{\text{app}} = (\delta^{13}C_{\text{CO}_2} + 1000) / (\delta^{13}C_{\text{CH}_4} + 1000) \quad (3)$$

The relative contribution of H₂ + CO₂-derived CH₄ to total CH₄ was determined using the following mass balance equation (Conrad 2005):

$$f_{\text{H}_2} = (\delta^{13}C_{\text{CH}_4} - \delta^{13}C_{\text{CH}_4\text{-ma}}) / (\delta^{13}C_{\text{CH}_4\text{-mc}} - \delta^{13}C_{\text{CH}_4\text{-ma}}) \quad (4)$$

where f_{H_2} is the fraction of CH₄ formed from H₂ + CO₂, δ¹³C_{CH₄} is the δ¹³C of total produced methane, and δ¹³C_{CH₄-ma} and δ¹³C_{CH₄-mc} are the δ¹³C of CH₄ derived either from acetate or H₂ + CO₂, which were determined by:

$$\delta^{13}C_{\text{CH}_4\text{-ma}} = \delta^{13}C_{\text{ac-methyl}} + \epsilon_{\text{ac,CH}_4} \quad (5)$$

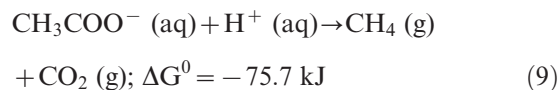
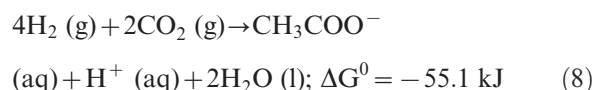
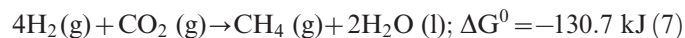
and

$$\delta^{13}C_{\text{CH}_4\text{-mc}} = \delta^{13}C_{\text{CO}_2} + \epsilon_{\text{CO}_2,\text{CH}_4} \quad (6)$$

where δ¹³C_{ac-methyl} is the δ¹³C of the methyl group of acetate accumulated, and $\epsilon_{\text{ac,CH}_4}$ and $\epsilon_{\text{CO}_2,\text{CH}_4}$ are the isotopic enrichment factors of acetotrophic and hydrogentrophic methanogenesis, respectively. The value of $\epsilon_{\text{CO}_2,\text{CH}_4}$, which is equivalent to $1 - \alpha_{\text{CO}_2,\text{CH}_4}$, is determined from Eq. 3 using δ¹³C_{CH₄-CH₃F} (δ¹³C of CH₄ produced in the presence of CH₃F, i.e., with acetotrophic methanogenesis inhibited) instead of δ¹³C_{CH₄}.

In general, calculations were done using the averaged data (± standard error) from triplicate incubations. Total amounts of gases in the headspace of the incubation vessels were calculated from the partial pressures using the volume of the gas space and the gas constant. The amounts of CH₄ dissolved in the liquid were less than 3% of the total and were neglected. The amounts of CO₂ dissolved in the liquid were calculated from the pH of the sediment, the solubility constant of CO₂, and the dissociation constant of bicarbonate (Stumm and Morgan 1981). Total inorganic carbon (TIC) was defined as the sum of bicarbonate, gaseous, and dissolved CO₂. The δ¹³C values of dissolved CO₂ (about 1‰ more negative) and bicarbonate (about 9‰ more positive) were calculated from the δ¹³C of gaseous CO₂ (Stumm and Morgan 1981). These values were used to calculate δ¹³C of TIC using the mole fractions of the different CO₂ species (Penning and Conrad 2006).

Gibbs free energies (ΔG) of production of CH₄ and acetate were calculated from the actual concentrations of reactants and products and the standard Gibbs free energies (Conrad et al. 2007) using the following reaction equations:



Values of ΔG⁰ of the reaction were calculated from tabulated values of the standard Gibbs free energies of formation at 298 K with the reactants and products at the gaseous (g), liquid (l), or aqueous (aq) state as indicated. The actual ΔG values at the incubation conditions were calculated from the ΔG⁰ and the actual partial pressures of CH₄, CO₂, and H₂ and the actual concentrations of acetate and H⁺ (pH 7) using the Nernst equation.

Results

The major characteristics of the sediments of the two lakes are summarized in Table 1. Both lake sediments exhibited similar contents of organic carbon and total nitrogen and had a neutral pH, but they differed in the other variables. In particular, Lake Mussura sediment showed lower iron and higher sulfate contents, and higher numbers of Bacteria and Archaea. The microbial numbers

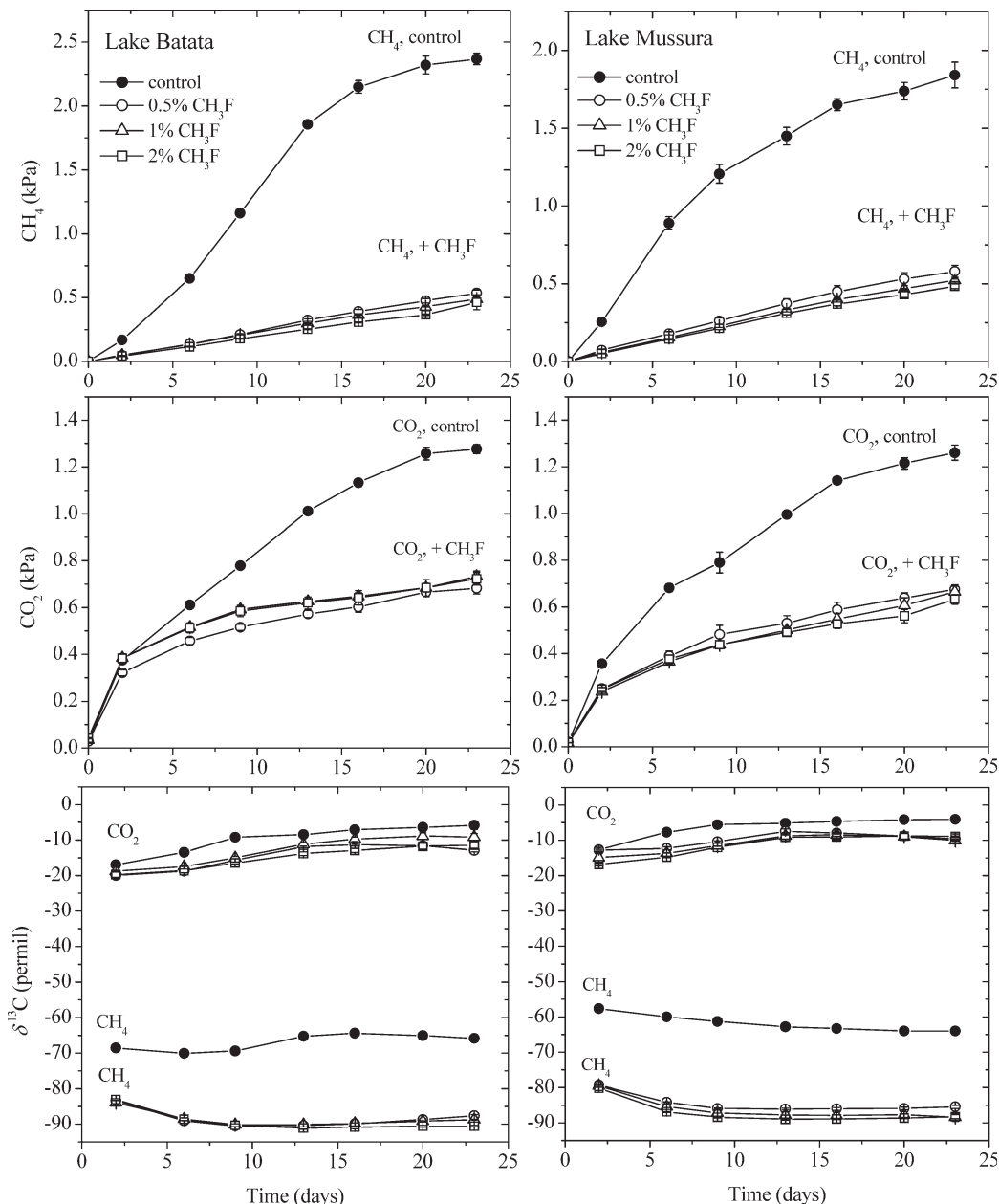


Fig. 1. Accumulation of gaseous CH_4 and CO_2 and their $\delta^{13}\text{C}$ values in sediment from Lake Batata and Lake Mussura incubated at 25°C in the absence and presence of methyl fluoride; CH_4 and CO_2 are given as partial pressures; 1 kPa is equivalent of about $20 \mu\text{mol}$ per total incubation; mean \pm SE, $n = 3$.

were determined by quantifying the copy numbers of the 16S rRNA genes, showing more than 10-fold higher bacterial than archaeal numbers. The values of $\delta^{13}\text{C}_{\text{org}}$ (-32‰) were virtually identical for both sediments and were insignificantly different from $\delta^{13}\text{C}_{\text{tot}}$, since the sediment contained virtually no carbonate.

Methane production in both lake sediments started immediately, accelerated slightly after about 2–5 d, and became slower after about 10–15 d of incubation (Fig. 1). Production rates of CH_4 and CO_2 , which were determined by linear regression between day 2 and 13, are shown in Table 1. Production rates of CH_4 and CO_2 were on the same order in both Lake Batata and Lake Mussura

sediments. In both sediments, addition of CH_3F resulted in partial inhibition of both CH_4 and CO_2 production (Fig. 1). The residual production rates of CH_4 and CO_2 in the presence of 0.5% CH_3F were in both lakes approximately 17% and decreased slightly to about 12% when 2% CH_3F was applied.

Hydrogen partial pressures under uninhibited conditions were very low, below 0.5 Pa in both sediments. However, they increased when CH_3F was applied (Fig. 2) and were then higher in Lake Batata than Lake Mussura sediment (Fig. 2). The Gibbs free energies (ΔG) of hydrogenotrophic methanogenesis (Eq. 7) were calculated for the actual incubation conditions and basically changed in parallel

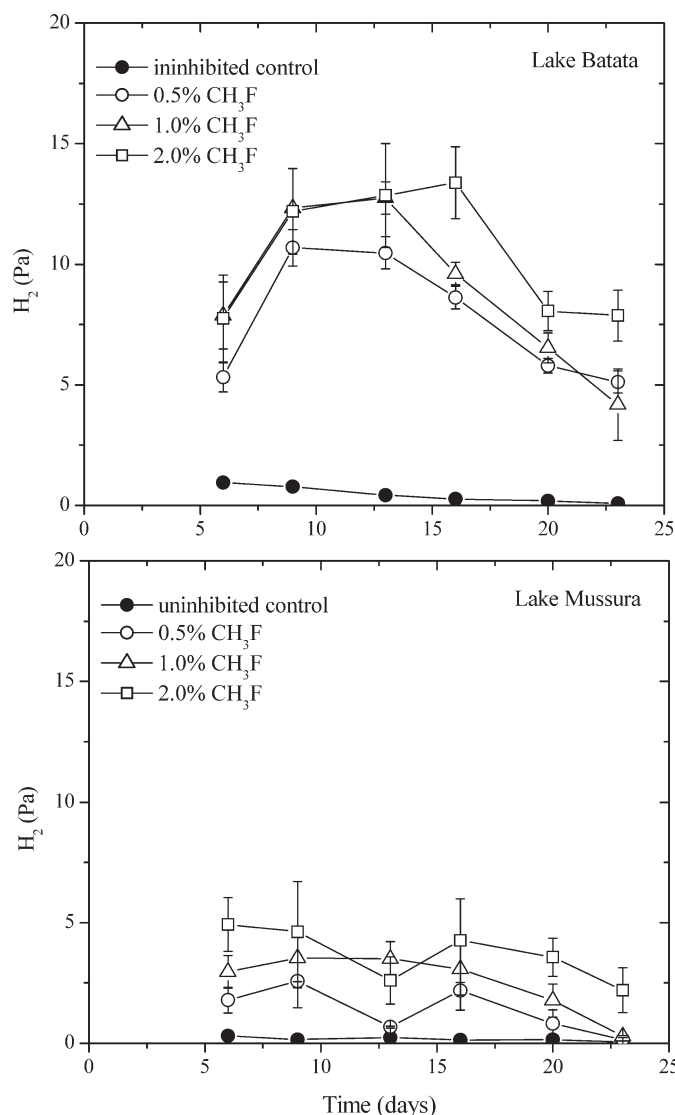


Fig. 2. Partial pressures of H_2 in sediment from Lake Batata and Lake Mussura incubated at $25^\circ C$ in the absence and presence of methyl fluoride; mean \pm SE, $n = 3$.

with the H_2 partial pressures. The values of ΔG averaged for days 9–20 are summarized in Table 2. The data show only slightly negative ΔG for Lake Batata sediment and even slightly positive ΔG for Lake Mussura sediment. However, ΔG values were more negative in the presence of CH_3F , reaching values of < -40 $kJ\ mol^{-1}$ and -30 $kJ\ mol^{-1}$ CH_4 in Lake Batata and Lake Mussura,

respectively. Since acetate concentrations were only measured at the end of incubation, ΔG of chemolithotrophic homoacetogenesis (Eq. 8) could only be calculated for this time point. The ΔG values were generally positive. In Lake Batata sediments, in the presence of 0 (control), 0.5%, 1.0%, and 2.0% CH_3F , ΔG values were 24, 8, 6, and 5 $kJ\ mol^{-1}$ acetate, respectively; in Lake Mussura sediment, ΔG values were 33, 24, 18, and 16 $kJ\ mol^{-1}$ acetate, respectively. Also, for acetotrophic methanogenesis (Eq. 9), the ΔG values could only be calculated at the end of incubation. The ΔG values were virtually the same in both sediments, i.e., -30 $kJ\ mol^{-1}$ CH_4 for the uninhibited control and -47 $kJ\ mol^{-1}$ CH_4 for all the CH_3F -treated incubations.

The values of $\delta^{13}C_{CO_2}$ in the incubated sediment increased from -16‰ to -6‰ in Lake Batata and from -13‰ to -4‰ in Lake Mussura (Fig. 1). The $\delta^{13}C$ values calculated for TIC were more positive by 3.2‰ and 4.7‰, respectively. In the presence of CH_3F , values of $\delta^{13}C_{CO_2}$ increased less (Fig. 1) and were on average a bit more negative (Table 3). However, $\delta^{13}C$ values of TIC were virtually the same in the control and in the CH_3F -treated sediment (data not shown). The values of $\delta^{13}C_{CH_4}$ (accumulated CH_4) were more negative by about 50‰ than $\delta^{13}C_{CO_2}$, i.e., approximately -70‰ to -65‰ in Lake Batata and approximately -64‰ to -58‰ in Lake Mussura (Fig. 1). Calculation of the $\delta^{13}C$ in the newly formed CH_4 (using Eq. 2) resulted in average $\delta^{13}C_{CH_4\text{-new}}$ (averaged from day 6 to 20) of -66‰ and -68‰ , respectively (Table 3). In the presence of 0.5–2.0% CH_3F , the values of $\delta^{13}C_{CH_4\text{-}CH_3F}$ (accumulated CH_4) were much more negative, reaching final values of -91‰ and -89‰ in Lakes Batata and Mussura, respectively (Fig. 1). Calculated values of the $\delta^{13}C$ in the newly formed CH_4 ($\delta^{13}C_{CH_4\text{-}CH_3F\text{-new}}$ using Eq. 2; averaged from day 6 to 20) were similar (Table 3). Using the values of $\delta^{13}C_{CH_4\text{-}CH_3F\text{-new}}$ together with $\delta^{13}C_{CO_2}$, it was possible to calculate the fractionation factor ϵ_{CO_2,CH_4} using Eq. 3 and the equivalent ϵ_{CO_2,CH_4} ; it was in the range of 82.9–86.5‰ (Table 3).

Acetate concentrations were only about 30 $\mu mol\ L^{-1}$ in both sediments, but values increased when incubated in the presence of CH_3F to millimolar levels (Table 4). The accumulated acetate accounted for 84–104% of the CH_4 that did not accumulate due to inhibition of acetotrophic CH_4 production. The $\delta^{13}C$ of total acetate ($\delta^{13}C_{ac}$) was more negative in sediment of Lake Batata (-29‰) than Lake Mussura (-26‰) and further decreased when accumulating due to inhibition of acetotrophic methanogenesis (Table 4). The decrease was increasingly larger at high than at low CH_3F concentrations, reaching values of

Table 2. Gibbs free energies of hydrogenotrophic methanogenesis (average of days 9–20).

Sample	Lake Batata		Lake Mussura	
	H_2 (Pa)	ΔG ($kJ\ mol^{-1}$)	H_2 (Pa)	ΔG ($kJ\ mol^{-1}$)
Control	0.42 ± 0.13	-5.0 ± 3.1	0.17 ± 0.02	$+2.4 \pm 1.1$
+0.5% CH_3F	8.89 ± 1.13	-39.3 ± 1.7	1.56 ± 0.48	-20.2 ± 3.5
+1.0% CH_3F	10.31 ± 1.44	-41.1 ± 1.8	2.98 ± 0.42	-28.0 ± 1.8
+2.0% CH_3F	11.62 ± 1.21	-42.8 ± 1.3	3.77 ± 0.44	-30.5 ± 1.4

Table 3. Values of $\delta^{13}\text{C}$ of CO_2 (taken from Fig. 1), of newly formed CH_4 (calculated from data of Fig. 1 using Eq. 2), and of the isotopic enrichment factor $\varepsilon_{\text{CO}_2, \text{CH}_4}$ ($a_{\text{CO}_2, \text{CH}_4}$ was calculated from $2 \delta^{13}\text{C}_{\text{CO}_2}$ and $\delta^{13}\text{C}_{\text{CH}_4\text{-new}}$ using Eq. 3 and then converted to $\varepsilon_{\text{CO}_2, \text{CH}_4}$). Mean values of $\delta^{13}\text{C}_{\text{CO}_2}$, $\delta^{13}\text{C}_{\text{CH}_4\text{-new}}$, and $a_{\text{CO}_2, \text{CH}_4}$ were calculated from the triplicate incubations for each date and then averaged for days 6–20.

Incubation	$\delta^{13}\text{C}_{\text{CO}_2}$	$\delta^{13}\text{C}_{\text{CH}_4\text{-new}}$	$\varepsilon_{\text{CO}_2, \text{CH}_4}$
Lake Batata			
Control	-8.94 ± 1.23	-66.07 ± 3.08	
0.5% CH_3F	-13.82 ± 1.46	-89.31 ± 1.74	82.9 ± 1.4
1.0% CH_3F	-12.45 ± 1.62	-89.68 ± 1.33	84.8 ± 1.0
2.0% CH_3F	-14.70 ± 1.26	-91.40 ± 0.87	84.4 ± 1.1
Lake Mussura			
Control	-5.44 ± 0.61	-68.04 ± 2.72	
0.5% CH_3F	-9.36 ± 0.88	-86.91 ± 0.81	84.9 ± 0.9
1.0% CH_3F	-10.28 ± 1.01	-88.84 ± 0.70	86.2 ± 1.0
2.0% CH_3F	-10.74 ± 1.15	-89.53 ± 0.87	86.5 ± 1.0

$\delta^{13}\text{C}_{\text{ac-CH}_3\text{F}}$ of -34‰ and -29‰ at 2% CH_3F in Lake Batata and Lake Mussura, respectively. Since the acetate concentrations in the uninhibited samples were very low, the triplicate samples had to be pooled for analysis of $\delta^{13}\text{C}$ of acetate-methyl. However, $\delta^{13}\text{C}_{\text{ac-methyl-CH}_3\text{F}}$ could be determined in each replicate of the inhibited samples, in which acetate had accumulated. Values of $\delta^{13}\text{C}_{\text{ac-methyl-CH}_3\text{F}}$ were always more negative by approximately 10–12‰ than $\delta^{13}\text{C}_{\text{ac-CH}_3\text{F}}$ (Table 4). The $\delta^{13}\text{C}$ values calculated for the acetate-carboxyl were correspondingly less negative. Hence, the intramolecular difference $\delta^{13}\text{C}_{\text{ac-carboxyl}} - \delta^{13}\text{C}_{\text{ac-methyl}}$ was approximately 20–24‰. At 0.5% CH_3F , $\delta^{13}\text{C}_{\text{ac-methyl-CH}_3\text{F}}$ was -40‰ and -36‰ for Lake Batata and Lake Mussura, respectively.

The $\delta^{13}\text{C}$ data averaged for days 6–20 were used to calculate the fraction (f_{H_2}) of total CH_4 production that was produced by hydrogenotrophic methanogenesis. The calculation used the data of newly formed CH_4 as signal for total CH_4 ($\delta_{\text{CH}_4\text{-new}}$) and that of newly formed CH_4 in the presence of CH_3F ($\delta^{13}\text{C}_{\text{CH}_4\text{-new-CH}_3\text{F}}$) as signal for CH_4 formed from hydrogenotrophic methanogenesis ($\delta^{13}\text{C}_{\text{CH}_4\text{-mc}}$). The $\delta^{13}\text{C}_{\text{CH}_4\text{-mc}}$ values were identical to those calculated from $\delta^{13}\text{C}_{\text{CO}_2}$ and $\varepsilon_{\text{CO}_2, \text{CH}_4}$ using Eq. 6. We made

two different assumptions concerning $\delta^{13}\text{C}_{\text{CH}_4\text{-ma}}$. We either assumed $\varepsilon_{\text{ac,CH}_4} = 0$, i.e., no further fractionation of the acetate carbon occurred during acetotrophic methanogenesis, so that $\delta^{13}\text{C}_{\text{CH}_4\text{-ma}}$ was identical to the $\delta^{13}\text{C}$ of acetate-methyl accumulated in the presence of CH_3F (Table 4), or we assumed that $\varepsilon_{\text{ac,CH}_4} = -10\text{‰}$, which is an isotope enrichment factor typical for *Methanosaetaceae* sp. (Penning et al. 2006). The f_{H_2} data were sensitive to the value chosen for $\varepsilon_{\text{ac,CH}_4}$, but they were also sensitive to the concentration of CH_3F because this concentration affected the $\delta^{13}\text{C}$ of acetate-methyl (Table 5).

The composition of the methanogenic archaeal community in the two sediments was determined by cloning of the archaeal 16S rRNA and the *mcrA* genes. The sequences of about 40 clones of each gene and sediment were used to calculate phylogenetic trees for the 16S rRNA (using DNA sequences) (Fig. 3) and *mcrA* genes (using the analogous amino acid sequences) (Fig. 4). This allowed affiliation of the individual sequences to particular taxa of Archaea or of methanogens. The results (Table 6) show that both lake sediments contained acetotrophic methanogens *Methanosaetaceae* but hardly any *Methanosarcinaceae*. Hydrogenotrophic methanogens were found in the orders *Methanomicrobiales*, *Methanobacteriales*, and *Methanocellales*. The *mcrA* gene sequences indicated that many clones of the *Methanomicrobiales* were affiliated with the Fen cluster, characterized by the acidophilic methanogenic isolates strain 6A and strain NTA (Bräuer et al. 2006). Furthermore, several groups of non-methanogenic archaea were present in the two sediments, *Crenarchaeota* in particular. Lake Mussura sediment contained a relatively large percentage of *Methanocellales* (Rice Cluster 1 or RC-1) and of RC-2 archaea, but otherwise, the differences between the two lake sediments were marginal.

Discussion

Methanogenic archaeal community—Lakes Batata and Mussura are clear-water lakes, i.e., they are in the floodplain of the clear-water Trombetas River. Clear-water rivers are the most common river types in Brazilian Amazonia, being more abundant than white-water or

Table 4. Acetate concentrations and $\delta^{13}\text{C}$ of total acetate, acetate-methyl, and acetate-carboxyl at the end of incubation of the sediments of Lake Batata and Lake Mussura. $\delta^{13}\text{C}_{\text{ac-carb}}$ was calculated using $\delta^{13}\text{C}_{\text{ac-carb}} = 2 \delta^{13}\text{C}_{\text{ac-tot}} - \delta^{13}\text{C}_{\text{ac-meth}}$.

Incubation	Acetate ($\mu\text{mol L}^{-1}$)	$\delta^{13}\text{C}_{\text{ac-tot}}$ (‰)	$\delta^{13}\text{C}_{\text{ac-meth}}$ (‰)	$\delta^{13}\text{C}_{\text{ac-carb}}$ (‰)
Lake Batata				
Control	32 ± 3	-28.91 ± 0.24	-40.52	-17.31
0.5% CH_3F	2930 ± 10	-30.44 ± 0.15	-40.44 ± 0.73	-20.43 ± 0.56
1.0% CH_3F	3175 ± 55	-32.94 ± 0.11	-41.84 ± 0.39	-24.11 ± 0.17
2.0% CH_3F	3045 ± 45	-34.52 ± 0.07	-44.27 ± 0.26	-24.76 ± 0.36
Lake Mussura				
Control	30 ± 5	-26.32 ± 0.90	-38.27	-14.38
0.5% CH_3F	2150 ± 20	-23.83 ± 0.31	-35.85 ± 0.39	-11.80 ± 0.86
1.0% CH_3F	2160 ± 120	-26.56 ± 0.13	-37.93 ± 0.56	-15.19 ± 0.32
2.0% CH_3F	2180 ± 40	-29.13 ± 0.07	-40.50 ± 0.62	-17.76 ± 0.65

Table 5. Fraction (f_{H_2} , given in %) of total CH_4 production due to hydrogenotrophic methanogenesis calculated for days 6–20 using $\delta^{13}C$ data and ϵ_{CO_2,CH_4} given in Tables 3 and 4.

Incubation	Lake Batata		Lake Mussura	
	$\epsilon_{ac,CH_4} = 0\text{‰}$	$\epsilon_{ac,CH_4} = -10\text{‰}$	$\epsilon_{ac,CH_4} = 0\text{‰}$	$\epsilon_{ac,CH_4} = -10\text{‰}$
0.5% CH_3F	53 ± 7	41 ± 9	63 ± 6	54 ± 7
1.0% CH_3F	51 ± 7	38 ± 9	59 ± 6	49 ± 7
2.0% CH_3F	46 ± 7	32 ± 9	56 ± 6	45 ± 8

black-water streams (Sioli 1984). Both lake sediments exhibited about 10 times higher numbers of Bacteria than Archaea, which has also been found in lake sediments from the midlatitudes (Zepp-Falz et al. 1999; Koizumi et al. 2004; Chan et al. 2005). The sediment of Lake Mussura, which has only a relatively small opening to the Trombetas River, showed about 10 times higher bacterial and archaeal numbers than the sediment of Lake Batata, which connects to the river over the lake banks during the high-water season. However, CH_4 production rates were similar or even a bit higher in Lake Batata than Lake Mussura sediment. Hence, microbial numbers were not a limiting factor for CH_4 production. Instead, recalcitrance of organic matter and substrate availability probably limited CH_4 production, and the population density of methanogens was probably also limited by the rate of CH_4 production. Using the procedure described by Conrad and Klose (2006), we roughly estimated the carrying capacity of the sediments for the population density of methanogens on the basis of available energy (ΔG , see following discussion), rates of CH_4 production (Table 1), maintenance energy of anaerobic microorganisms ($3.3 \text{ kJ h}^{-1} \text{ mol}^{-1} \text{ biomass-C}$), and biomass-C content of archaeal cells ($8 \times 10^{-15} \text{ mol C}$), and we arrived at $3\text{--}10 \times 10^7$ methanogens per g dry wt sediment. It seems that the methanogen population densities have already reached the carrying capacity in Lake Mussura sediment but not yet in Lake Batata sediment. Note, however, that the calculations are uncertain because of the assumed biomass content (i.e., size) of individual microbial cells, which may vary over more than an order of magnitude.

The archaeal community was also very similar in both lake sediments and showed only gradual differences in the percentage contribution of the different methanogenic orders and families. Hydrogenotrophic *Methanomicrobiales* and acetotrophic *Methanosaetaceae* are commonly found in lake sediments (Koizumi et al. 2004; Chan et al. 2005; Conrad et al. 2007). Interestingly, acetotrophic *Methanosarcinaceae* sp. have hardly been detected in lake sediments, even in a hypereutrophic lake (Priest Pot, England) (Earl et al. 2005). The existence of *Methanosaetaceae* instead of *Methanosarcinaceae* may be due to the fact that the former are much better adapted to low acetate concentrations than the latter (Jetten et al. 1992).

Hydrogenotrophic *Methanomicrobiales*, which is commonly found in methanogenic lake sediments, consisted in part of members of the so-called Fen cluster, which was first noticed as *mcrA* sequences in acidic fens and bogs (Galand et al. 2002). Isolation of acidophilic strains (strains 6A and NTA) of methanogens proved that the Fen cluster

belongs to *Methanomicrobiales* (Bräuer et al. 2006). Recently, members of the Fen cluster have also been detected on rice roots when grown in a riverbank soil (Conrad et al. 2008). The Fen cluster apparently also occurs in neutral lake sediments, as shown in the present study. Possibly, dispersion of Fen cluster methanogens is facilitated by the slightly acidic water of the two clear-water lakes. Hydrogenotrophic *Methanobacteriales* communities were also present in the two Amazonian lake sediments, mainly in Lake Batata. Members of this order are not frequently observed in lake sediments (Nüsslein et al. 2001; Earl et al. 2005). Most notably, hydrogenotrophic *Methanocellales* sp. were relatively abundant, in Lake Mussura sediment in particular. To our knowledge, *Methanocellales* sp. have rarely been found in lake sediment (only in Rothsee; Zepp-Falz et al. 1999), but they are common in rice (*Oryza sativa*) field soil (Ramakrishnan et al. 2001), and acidic peat (Sizova et al. 2003). Interestingly, large areas of the two lakes (Lake Mussura in particular) can be colonized by the aquatic macrophyte *Oryza glumaepatula*, which grows with the rising water level up to 6 m in length. It would be interesting to investigate whether it is the genus *Oryza* that allowed for the concomitant presence of *Methanocellales*. So far, only one isolate is available from the *Methanocellales*. This isolate exhibits an intriguing ability for syntrophic interaction with H_2 -producing bacteria (Sakai et al. 2008).

The other archaeal groups are *Crenarchaeota* and *Euryarchaeota*. Rice clusters RC-2 and RC-5, Thermoplasmatales and Group III and the Lake Dagow sediment (LDS) cluster are euryarchaeotal clusters that have been detected before in aquatic sediments (Glissmann et al. 2004; Conrad et al. 2007). RC-2, RC-5, and LDS are euryarchaeotal clusters with unknown phenotype and are probably not methanogenic. However, circumstantial evidence exists that RC-2 may have a methanogenic phenotype (Grosskopf et al. 1998). The *mcrA* tree, representing methanogenic archaea only, does not give a hint for a separate cluster that would be homologous to that of RC-2 on the 16S rRNA gene tree. Therefore, RC-2 either does not contain a *mcrA* gene, the *mcrA* gene is not amplified by the primers used, or the *mcrA* sequences of RC-2 are represented by one of the known *mcrA* phylogenetic groups (e.g., by *Methanocellales* or *Methanomicrobiales*) rather than by a separate cluster.

Isotope fractionation—In both sediments, organic carbon had a relatively low $\delta^{13}C$ value (-32‰). Such isotopically light organic carbon is often found in eutrophic lakes with anoxic hypolimnion, where it is probably caused by

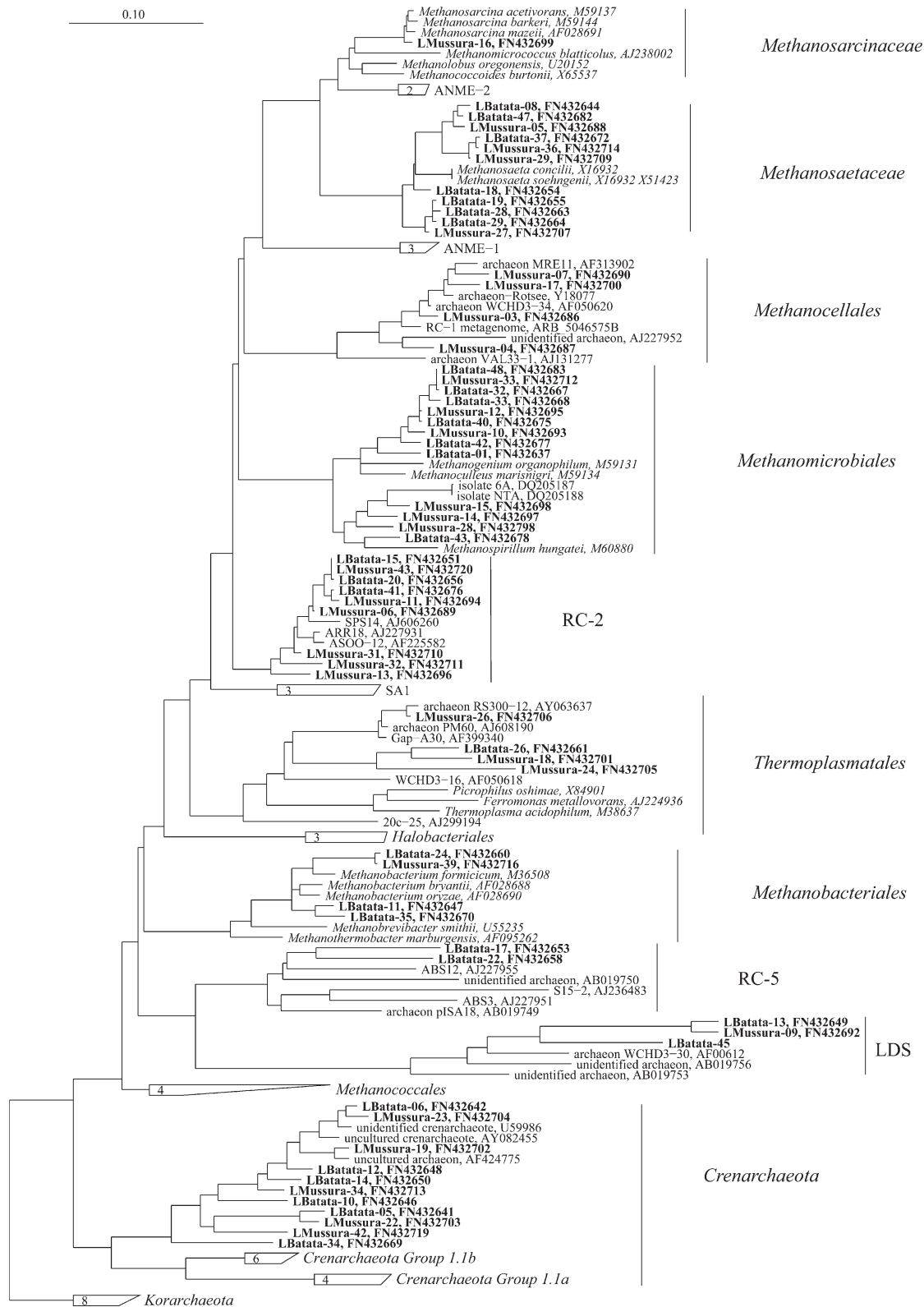


Fig. 3. Phylogenetic tree (neighbor joining) of a selection of characteristic partial 16S rRNA gene sequences retrieved from sediment from Lake Batata and Lake Mussura.



Fig. 4. Phylogenetic tree (neighbor joining) of a selection of characteristic partial *mcrA* amino acid sequences retrieved from sediment from Lake Batata and Lake Mussura.

Table 6. Percentage distribution of major archaeal phylogenetic groups among clone sequences of *mcrA* (44 clones each) and 16S rRNA genes (44 and 39 clones each, respectively) retrieved from sediment of Lake Batata and Lake Mussura.

Archaea	Lake Batata		Lake Mussura	
	<i>mcrA</i>	16S rRNA	<i>mcrA</i>	16S rRNA
<i>Methanosaetaceae</i>	34	18	23	18
<i>Methanosarcinaceae</i>	0	0	0	2
<i>Methanocellales</i> (RC-1)	5	0	16	10
<i>Methanomicrobiales</i>	52	27	48	15
<i>Methanobacteriales</i>	9	9	13	3
RC-2		7		26
<i>Thermoplasmatales</i> and Group III		2		8
RC-5		5		0
LDS		5		3
<i>Crenarchaeota</i>		27		15

sedimentation of anaerobic microbial biomass (Teranes and Bernasconi 2005). In our own studies, we found such a low $\delta^{13}\text{C}_{\text{org}}$ (-31%) in sediments of eutrophic Lake Dagow (Conrad et al. 2009) but a higher $\delta^{13}\text{C}_{\text{org}}$ (-26%) in oligotrophic Lake Stechlin (Conrad et al. 2007). The reason for the low $\delta^{13}\text{C}_{\text{org}}$ value in the two Amazonian clear-water lakes, which are nutrient poor, is presently unknown.

Since carbonate is not of importance in sediment of Lake Batata and Lake Mussura, the $\delta^{13}\text{C}_{\text{CO}_2}$ is eventually derived from $\delta^{13}\text{C}_{\text{org}}$. Values of $\delta^{13}\text{C}_{\text{CO}_2}$ were generally higher (by 16–19%) than $\delta^{13}\text{C}_{\text{org}}$ and further increased during incubation. The isotopic difference between $\delta^{13}\text{C}_{\text{org}}$ and $\delta^{13}\text{C}_{\text{CO}_2}$ probably reflects isotope fractionation during heterotrophic processes in the sediment, including methanogenesis. The values of $\delta^{13}\text{C}_{\text{CH}_4}$, on the other hand, were much lower (by 34–38%) than $\delta^{13}\text{C}_{\text{org}}$. The values of $\delta^{13}\text{C}_{\text{CH}_4}$ and $\delta^{13}\text{C}_{\text{CO}_2}$ were comparable to those observed by Wassmann et al. (1992) in gas bubbles that were stirred out of sediments from the Amazonian floodplain at the Ilha de Marchantaria close to Manaus.

Production of CH_4 is basically the anaerobic disproportionation of organic substances to CO_2 and CH_4 (e.g., $\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 3\text{CO}_2 + 3\text{CH}_4$). Using this equation, we calculated the expected $\delta^{13}\text{C}$ value of organic matter using the $\delta^{13}\text{C}$ values of TIC and CH_4 as input. For Lake Mussura sediment, we obtained $\delta^{13}\text{C}_{\text{org}}$ values of -32.8% to -31.1% , which were almost identical to the $\delta^{13}\text{C}_{\text{org}}$ actually measured. For Lake Batata sediment, however, we obtained values of -41.2% to -34.2% , which were significantly more negative than the $\delta^{13}\text{C}_{\text{org}}$ actually measured. The most likely explanation is that only a tiny fraction of the organic carbon is decomposed during the relatively short incubation period and that some substances making up the organic matter may have different initial $\delta^{13}\text{C}$ and/or may be decomposed with different isotope fractionation factors. Therefore, an unbalanced distribution of $\delta^{13}\text{C}$ to CH_4 and CO_2 during the degradation process, such as observed for Lake Batata sediment, is quite likely. Studies on vegetated tropical peat have shown that the difference in $\delta^{13}\text{C}$ between the substrate and the produced CH_4 is generally more negative for more recalcitrant substrates, indicating that H_2 compared to acetate becomes the more important methanogenic precur-

sor (Miyajima et al. 1997). In sediment of eutrophic Lake Dagow, the importance of hydrogenotrophic methanogenesis increased with sediment depth (Chan et al. 2005; Conrad et al. 2009), where organic matter presumably became more recalcitrant. Similar observations have been made in peat soil (Hornibrook et al. 1997; Popp et al. 1999). Therefore, we assume that the organic matter in the two lake sediments was relatively recalcitrant, and that the fermentation in case of Lake Batata resulted in production of a higher proportion of CH_4 than CO_2 as expected from the disproportionation of organic matter. This is reflected in the relative production rates of CH_4 and CO_2 (Table 1). This assumption is also consistent with a model of biogenic CH_4 formation in the marine environment that is under the control of labile organic carbon flux and availability of oxidants (Blair 1998). According to this model, the labile organic carbon flux should be higher in Lake Batata than in Lake Mussura, which is not unlikely given their different relationship to the annual flood pulse (see Methods).

The $\delta^{13}\text{C}$ of acetate carbon exhibited a strong intramolecular difference of $> 20\%$, which is consistent with the pattern observed in glucose-fermenting *Escherichia coli* (Blair et al. 1985) and in anoxic paddy soil decomposing fresh leaves (Sugimoto and Wada 1993). Studies of isotope fractionation in saccharide-fermenting *Clostridium papyrosolvens* have shown that $\delta^{13}\text{C}_{\text{ac}}$ of total acetate is only slightly ($< 3\%$) lower than $\delta^{13}\text{C}_{\text{org}}$ (Penning and Conrad 2006). However, since CH_4 is exclusively formed from the methyl group of acetate, it is the $\delta^{13}\text{C}$ of acetate-methyl that is relevant for the further flow of carbon to CH_4 . The $\delta^{13}\text{C}_{\text{ac-methyl}}$ value was more negative by 8‰ and 6‰ than the $\delta^{13}\text{C}_{\text{org}}$ in the sediment of Lake Batata and Lake Mussura, respectively. The $\delta^{13}\text{C}_{\text{ac-carboxyl}}$, however, was much more positive than $\delta^{13}\text{C}_{\text{org}}$. The relatively high $\delta^{13}\text{C}_{\text{ac-carboxyl}}$ may be caused by isotopic exchange of the carboxyl group of acetate with the pool of CO_2 (DeGraaf et al. 1996). This explanation is not unlikely since δ_{CO_2} was isotopically heavier than $\delta^{13}\text{C}_{\text{ac-carboxyl}}$ in the two Amazonian sediments. Although the mechanistic reasons for the relatively large difference between $\delta^{13}\text{C}_{\text{ac-methyl}}$ and $\delta^{13}\text{C}_{\text{org}}$ are not completely clear, we have also observed such a difference in the sediments of Lake Stechlin (Conrad et al.

2007) and Lake Dagow (Conrad et al. 2009). The difference between $\delta^{13}\text{C}_{\text{ac-methyl}}$ and $\delta^{13}\text{C}_{\text{org}}$ was larger in Lake Batata than Lake Mussura sediment, indicating differences in fermentation of organic matter (*see* previous discussion).

Effect of methyl fluoride—Methyl fluoride is a relatively specific inhibitor of acetotrophic methanogenesis. Hydrogenotrophic methanogenic archaea, homoacetogenic, sulfate-reducing and syntrophic fermentative bacteria seem not to be inhibited, albeit only a limited number of species have been tested (Janssen and Frenzel 1997). In methanogenic rice-field soil, increasing concentrations of CH_3F were found to progressively inhibit also hydrogenotrophic methanogenesis (Conrad and Klose 1999). Recently, we hypothesized that acetate production by some bacterial groups that ferment organic substances may also be affected (Conrad et al. 2009). Hence, interpretation of data generated by the use of inhibitors must be done cautiously. Nevertheless, addition of CH_3F largely caused the effects that were theoretically expected from specific inhibition of acetotrophic methanogenesis: (1) CH_4 production was partially inhibited, and instead acetate accumulated stoichiometrically, consistent with acetate being exclusively consumed by acetotrophic methanogenesis; (2) the CH_4 produced in the presence of CH_3F was isotopically much lighter than in the absence, consistent with hydrogenotrophic methanogenesis; and (3) the methyl group of the acetate accumulating in the presence of CH_3F was isotopically lighter than without inhibition, as expected when it is no longer consumed and thus no longer experiences a kinetic isotope effect. Since an increase of CH_3F concentration did not increase inhibition and did not decrease $\delta^{13}\text{C}_{\text{CH}_4}$ much further, it is likely that acetotrophic methanogenesis was completely inhibited. We assume, however, that some part of the hydrogenotrophic methanogenesis was inhibited by even the lowest CH_3F concentration applied. Since partial inhibition of hydrogenotrophic methanogenesis resulted in increase of H_2 partial pressure (Fig. 2), and consequently in more negative ΔG (Table 2) values, isotope fractionation ($a_{\text{CO}_2,\text{CH}_4}$) and $\delta^{13}\text{C}_{\text{CH}_4\text{-mc}}$ may possibly be affected (*see* following discussion). However, $a_{\text{CO}_2,\text{CH}_4}$ should decrease and $\delta^{13}\text{C}_{\text{CH}_4\text{-mc}}$ should increase with decreasing ΔG (Penning et al. 2005), but this was not observed (Fig. 1; Table 3). Therefore, the $\delta^{13}\text{C}_{\text{CH}_4}$ that was exclusively produced from hydrogenotrophic methanogenesis in the presence of CH_3F was most probably not significantly biased by partial inhibition of this reaction.

Consequently, the isotopic enrichment factor $\varepsilon_{\text{CO}_2,\text{CH}_4}$ can be calculated from $\delta^{13}\text{C}_{\text{CH}_4\text{-mc}}$ and $\delta^{13}\text{C}_{\text{CO}_2}$. These values were on the order of about $\varepsilon_{\text{CO}_2,\text{CH}_4} = -85\%$ ($a_{\text{CO}_2,\text{CH}_4} = 1.085$), which is more negative than that found in oligotrophic Lake Stechlin sediment (-78%) (Conrad et al. 2007) but less negative than in eutrophic Lake Dagow sediment (-90%) (Conrad et al. 2009). Values of $\varepsilon_{\text{CO}_2/\text{CH}_4}$ in other aquatic environments approximately range from -90% to -40% (Conrad 2005; Itoh et al. 2008), so fractionation in Lake Batata and Lake Mussura sediment was relatively strong. The calculation of $\varepsilon_{\text{CO}_2,\text{CH}_4}$ used the $\delta^{13}\text{C}_{\text{CO}_2}$ in the gas phase. If the $\delta^{13}\text{C}$ of the dissolved CO_2

were used instead, there would have been only a small effect, i.e., resulting in 1% smaller $\varepsilon_{\text{CO}_2,\text{CH}_4}$ values. Methanogenic archaea (also homoacetogenic bacteria) are using CO_2 rather than bicarbonate as active species when reducing it to CH_4 (Vorholt and Thauer 1997). Therefore, it is not necessary to consider the $\delta^{13}\text{C}$ of bicarbonate. The strong fractionation ($\varepsilon_{\text{CO}_2/\text{CH}_4}$) indicates that H_2 supply, and thus availability of energy for the hydrogenotrophic methanogens, was low (Penning et al. 2005). Using the empirical relationship between $\Delta\text{G}_{\text{CO}_2,\text{CH}_4}$ and $a_{\text{CO}_2,\text{CH}_4}$ published by Penning et al. (2005), we calculated $\Delta\text{G}_{\text{CO}_2/\text{CH}_4}$ values of -23 to -19 kJ mol^{-1} CH_4 and -19 to -17 kJ mol^{-1} CH_4 for sediment of Lake Batata and Lake Mussura, respectively. This Gibbs free energy is just sufficient for the molar production of 1/4 adenosine triphosphate per CH_4 produced from H_2 and CO_2 , which is the smallest useful energy quantum (Thauer and Morris 1984).

The acetate, which accumulates in the presence of CH_3F , should be equivalent to that being produced during fermentation of organic matter and now being no longer consumed. The $\delta^{13}\text{C}$ values of the acetate (both total and acetate-methyl) that accumulated in the presence of CH_3F were slightly more negative than those that accumulated in its absence. In principle, this can be expected, since acetate consumption in the absence of CH_3F would prefer the isotopically lighter acetate, so that the residual acetate would become enriched in ^{13}C . However, the $\delta^{13}\text{C}$ of the accumulated acetate became progressively more negative as the concentration of CH_3F was increased, despite the fact that acetotrophic methanogenesis was already fully inhibited at the lowest CH_3F concentration (Table 4). Therefore, the lower $\delta^{13}\text{C}$ can hardly have been a result of a change in the acetate consumption pathway, but must instead have been due to a change in the acetate production pathway. We recently hypothesized that CH_3F may have a small effect on some fermenting bacteria producing acetate by fermentation of organic matter (Conrad et al. 2009). The present data set is consistent with this hypothesis. However, the effect was only small, about 4%, $\delta^{13}\text{C}_{\text{ac-methyl}}$ across the different CH_3F concentrations (0–2%).

Methanogenic pathway—The relative contribution of hydrogenotrophic and acetotrophic methanogenesis to total CH_4 production can be calculated by using Eq. 4. The value of f_{H_2} is calculated using the $\delta^{13}\text{C}$ of CH_4 measured in the presence ($\delta^{13}\text{C}_{\text{CH}_4\text{-mc}}$) and absence ($\delta^{13}\text{C}_{\text{CH}_4}$) of CH_3F and the $\delta^{13}\text{C}_{\text{ac-methyl}}$. We used only the $\delta^{13}\text{C}$ of newly produced CH_4 , but use of accumulated CH_4 would make only a marginal difference. However, since $\delta^{13}\text{C}_{\text{CH}_4\text{-mc}}$ decreased slightly with increasing CH_3F concentration, values of f_{H_2} decreased accordingly (Table 5). The simultaneous decrease of $\delta^{13}\text{C}_{\text{ac-methyl}}$ and $\delta^{13}\text{C}_{\text{CH}_4\text{-ma}}$ also resulted in decrease of f_{H_2} , thus enhancing the effect of increasing CH_3F concentration on decreasing f_{H_2} . We calculated f_{H_2} by assuming $\varepsilon_{\text{ac},\text{CH}_4}$ equal to 0 or -10% . Since acetate concentrations were very low, it is more likely that the produced acetate was immediately consumed and thus was not isotopically fractionated (i.e., $\varepsilon_{\text{ac},\text{CH}_4} = 0$). Furthermore, we assume that the $\delta^{13}\text{C}_{\text{ac-methyl}}$ at the lowest CH_3F concentration is the most likely value

for the newly produced acetate. Note that this is not much different from the $\delta^{13}\text{C}_{\text{ac-methyl}}$ in the absence of inhibition. Therefore, the most likely values of f_{H_2} were 53% and 63% for Lake Batata and Lake Mussura, respectively. Hence, CH_4 production in the two Amazonian lake sediments was dominated by hydrogenotrophic methanogenesis. Lake sediments often exhibit a dominance of acetotrophic methanogenesis, as theoretically expected when polysaccharides are the main carbon substrates and are mineralized completely (Conrad 1999). However, dominance of hydrogenotrophic methanogenesis has also been observed (Conrad 1999) and has been attributed to incomplete degradation of organic matter (Conrad et al. 2009). Note that the f_{H_2} values observed are considerably larger than those derived from CH_4 production rates in the presence and absence of CH_3F , which indicated only about < 17% contribution of hydrogenotrophic methanogenesis. This difference indicates that CH_3F not only inhibited acetotrophic methanogenesis, but also contributed a significant part of hydrogenotrophic methanogenesis (see previous discussion).

Energetic conditions—While the Gibbs free energy of acetotrophic methanogenesis was generally negative, that of hydrogenotrophic methanogenesis was only negative in the presence of CH_3F . In uninhibited sediment, however, ΔG of hydrogenotrophic methanogenesis was close to zero or even slightly positive. The reason was that the H_2 partial pressures in the uninhibited sediment were very low and only increased upon CH_3F treatment. We assume that CH_3F treatment resulted in partial inhibition of hydrogenotrophic methanogenesis and thus in accumulation of H_2 that was no longer utilized. In uninhibited sediment, on the other hand, H_2 was efficiently utilized, probably by cell-to-cell contact, so that H_2 partial pressures were kept low and resulted in calculation of a ΔG that was unrealistically high for the obviously ongoing hydrogenotrophic methanogenesis. We have observed similarly high (not so negative) $\Delta\text{G}_{\text{CO}_2, \text{CH}_4}$ in the deeper sediment layers of oligotrophic Lake Stechlin (Conrad et al. 2007), and also in sediment of eutrophic Lake Dagow (Glissmann et al. 2004); whereas sediments of mesotrophic Lake Constance (Schulz and Conrad 1996) and Lake Kinneret (Nüsslein et al. 2001) had lower (more negative) $\Delta\text{G}_{\text{CO}_2, \text{CH}_4}$ values (i.e., -40 to -20 kJ mol^{-1} CH_4). We presently do not know how these differences in measured $\Delta\text{G}_{\text{CO}_2, \text{CH}_4}$ come about, but we assume that it is caused by the spatial structuring of the methanogenic community that degrades organic matter to CH_4 . We hypothesize that relatively low H_2 partial pressures and consequently relatively high $\Delta\text{G}_{\text{CO}_2, \text{CH}_4}$ values indicate operation of hydrogenotrophic methanogenesis in microbial consortia with juxtaposed syntrophic partners facilitating interspecies H_2 transfer (Conrad et al. 1985). We furthermore hypothesize, that high $a_{\text{CO}_2, \text{CH}_4}$ (negative $\varepsilon_{\text{CO}_2, \text{CH}_4}$) values, which indicate a more negative $\Delta\text{G}_{\text{CO}_2, \text{CH}_4}$ than calculated from the sediment H_2 concentrations (see previous discussion), are likewise an indicator for juxtaposed H_2 transfer (Penning et al. 2005; Sugimoto and Fujita 2006). It is intriguing that the sediments of Lake Batata and Lake Mussura contained hydrogenotrophic

Methanocellales (see previous), which have the peculiar characteristic for growth in syntrophic partnership (Sakai et al. 2008).

The low H_2 partial pressures in the two Amazonian sediments also resulted in positive $\Delta\text{G}_{\text{ac}, \text{CO}_2}$ for hydrogenotrophic homoacetogenesis. Hence, acetate formation should only marginally be affected by this process, unless it also occurs in microbial consortia with juxtaposed interspecies H_2 transfer like hydrogenotrophic methanogenesis. The large difference between $\delta^{13}\text{C}_{\text{ac-methyl}}$ and $\delta^{13}\text{C}_{\text{ac-carboxyl}}$ is consistent with the absence of acetate production via homoacetogenesis, which should exhibit only a marginal intramolecular difference (Gelwicks et al. 1989). By contrast, the low H_2 partial pressures would make the operation of syntrophic acetate oxidation thermodynamically feasible, since syntrophic acetate oxidation is the reversal of hydrogenotrophic homoacetogenesis (Eq. 8) (Lee and Zinder 1988). We know very little about this process and the microorganisms involved, and we know virtually nothing about isotope fractionation (Nüsslein et al. 2003). We hypothesize that syntrophic acetate oxidizers may be sensitive to CH_3F , thus resulting in the progressive increase of isotopically light acetate that was no longer consumed, such as observed when increasing concentrations of CH_3F were added to the Amazonian sediments. This interpretation would reconcile the relatively large differences in f_{H_2} that were calculated either from ^{13}C data or from the inhibition of CH_4 production by CH_3F , and it might also offer an explanation for the generation of progressively more negative $\delta^{13}\text{C}_{\text{ac-methyl}}$ in the presence of CH_3F when assuming kinetic isotope fractionation by the syntrophic acetate oxidizers.

The microbial communities in the sediments of two clear-water lakes in Amazonia produced CH_4 with similar rates and to a large extent by the hydrogenotrophic pathway. Production rates of CH_4 were probably limited by the recalcitrance of organic matter rather than by the numbers of bacteria and archaea, which were higher in Lake Mussura than in Lake Batata. The methanogenic community consisted in both lake sediments of hydrogenotrophic methanogens, mainly from the orders of *Methanomicrobiales*, but also from *Methanobacteriales* and *Methanocellales*. Acetotrophic methanogens were represented by the family of *Methanosetaeaceae*. Isotopic fractionation during hydrogenotrophic methanogenesis was large, being consistent with the limited supply of H_2 that resulted in only a small negative Gibbs free energy for hydrogenotrophic methanogenesis or acetogenesis. The methyl group of acetate also exhibited a pronounced isotope fractionation with respect to the $\delta^{13}\text{C}$ of organic matter. This effect was enhanced by increasing concentrations of CH_3F used for inhibition of acetate consumption, possibly suggesting that acetate might not only be consumed by acetotrophic methanogens but also by acetotrophic fermenting bacteria (syntrophic acetate oxidation). Thus, this study of the two tropical lake sediments revealed many particularities, but it also showed that the methanogenic archaeal communities and processes were in many ways similar to those in lake sediments from midlatitudes.

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