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Stable carbon isotope discrimination and microbiology of methane formation in tropical anoxic lake sediments

R. Conrad[1], M. Noll[2], P. Claus[1], M. Klose[1], W. R. Bastos[3], and A. Enrich-Prast[4]

[1]Max-Planck-Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany
[2]BfR, Federal Institute for Risk Assessment, Division 74 "Hygiene and Microbiology", Thielallee 88–92, 14195 Berlin, Germany
[3]Laboratory of Environmental Biogeochemistry, University Federal of Rondonia, Rondonia, Brazil
[4]Laboratory of Biogeochemistry, Department of Ecology, Institute of Biology, University Federal of Rio de Janeiro, Brazil

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Correspondence to: R. Conrad (conrad@mpi-marburg.mpg.de)

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Abstract

Methane is an important end product of degradation of organic matter in anoxic lake sediments. Methane is mainly produced by either reduction of CO_2 or cleavage of acetate involving different methanogenic archaea. The contribution of the different methanogenic paths and of the diverse bacteria and archaea involved in CH_4 production exhibits a large variability that is not well understood. Lakes in tropical areas, e.g. in Brazil, are wetlands with high potential impact on the global CH_4 budget. However, they have hardly been studied with respect to methanogenesis. Therefore, we used samples from 16 different lake sediments in the Pantanal and Amazon region of Brazil to measure production of CH_4, CO_2, analyze the content of ^{13}C in the products and in intermediately formed acetate, determine the abundance of bacterial and archaeal microorgansisms and their community composition and diversity by targeting the genes of bacterial and archaeal ribosomal RNA and of methyl coenzyme M reductase, the key enzyme of methanogenic archaea. These experiments were done in the presence and absence of methyl fluoride, an inhibitor of acetoclastic methanogenesis. While production rates of CH_4 and CO_2 were correlated to the content of organic matter and the abundance of archaea in the sediment, values of ^{13}C in acetate and CH_4 were related to the ^{13}C content of organic matter and to the path of CH_4 production with its intrinsic carbon isotope fractionation. Isotope fractionation was small (average 10‰) for conversion of C_{org} to acetate-methyl, which was hardly further fractionated during CH_4 production. However, fractionation was strong for CO_2 conversion to CH_4 (average 75‰), which generally accounted for >50% of total CH_4 production. Canonical correspondence analysis did not reveal an effect of microbial community composition, despite the fact that it exhibited a pronounced variability among the different sediments.

BGD

7, 8619–8661, 2010

1 Introduction

Methane, an important greenhouse gas, is predominantly produced as an end product of the degradation of organic matter under anoxic conditions (Cicerone and Oremland, 1988). The actual CH_4 formation is catalyzed by methanogenic archaea that mainly use acetate or H_2+CO_2 as substrates, which in turn are the products of anaerobic organic matter degradation (Zinder, 1993). The relative contribution of acetotrophic and hydrogenotrophic methanogenesis to total CH_4 production significantly affects the $\delta^{13}C$ of the produced CH_4, since hydrogenotrophic methanogenesis expresses a larger kinetic isotope effect than acetotrophic methanogenesis (Whiticar et al., 1986). Since computation of global budget is constrained by the $\delta^{13}C$ of atmospheric CH_4, knowledge of the processes involved in CH_4 formation is important (Quay et al., 1991; Tyler, 1992). The processes involved in microbial CH_4 production during anaerobic degradation of organic matter constitute the most important sources in the global CH_4 cycle and occur in natural wetlands, in rice fields and other artificial wetlands, in the intestine of ruminants and termites, and in waste treatment systems (sewage, landfills). Natural wetlands are one of the largest individual sources accounting for 20–30% of the total atmospheric CH_4 budget, tropical wetlands being the most important ones (Lelieveld et al., 1998). Lakes within the floodplain of South American rivers (Amazon, Pantanal, Orinoco) have been found to be a significant source of atmospheric CH_4 (Bastviken et al., 2010; Devol et al., 1990; Marani and Alvala, 2007; Smith et al., 2000). However, the processes involved in CH_4 formation and their effects on the $\delta^{13}C$ of CH_4 have hardly been studied (Conrad et al., 2010b; Wassmann et al., 1992).

Methane production is achieved by a community of different microbial groups, which catalyze the stepwise degradation of organic matter (McInerney and Bryant, 1981; Zehnder, 1978). The process is initiated by the hydrolysis of polymers (e.g., polysaccharides) to monomers (e.g., sugars), which are then fermented by a variety of different bacteria to low-molecular-weight fatty acids, alcohols, CO_2, and H_2. Acetate is usually the most important primary fermentation product. Some bacteria can ferment sugars

BGD
7, 8619–8661, 2010

even to acetate as the sole fermentation product (homoacetate fermentation). However, acetate is also produced during secondary fermentation, during which primary fermentation products are further processed (Dolfing, 1988). Typical secondary fermentation reactions are for instance the conversion of fatty acids (e.g., propionate, butyrate, lactate) and alcohols (e.g., ethanol, propanol) to acetate, CO_2, and H_2. However, other degradation intermediates such as amino acids or aromatic compounds are also further degraded in secondary fermentation, always resulting in the production of acetate, CO_2, and H_2. Secondary fermentation is achieved by bacteria, which live in syntrophy with H_2-consuming and acetate-consuming methanogens, since secondary fermentation is only endergonic if the H_2 partial pressure, in particular, is kept at levels of a few pascal (Schink and Stams, 2006). The last step in organic matter degradation is then the production of CH_4 from the dismutation of acetate (acetoclastic methanogenesis: $CH_3COOH \rightarrow CH_4 + CO_2$) and from the reduction of CO_2 (hydrogenotrophic methanogenesis: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) (Zinder, 1993). Each step of organic matter degradation is crucial for the entire process and for CH_4 production. In systems with a dynamic input of organic matter, the degradation process may result in transient accumulation of intermediates, acetate in particular (Drake and Küsel, 2003). Thus, CH_4 production rates may be limited by each of the degradation steps involved. Only if the system has eventually achieved steady state, CH_4 production rates will be limited by the first initiating reaction step, the hydrolysis of complex organic matter (Billen, 1982).

The $\delta^{13}C$ of the produced CH_4 depends on several factors. One, mentioned above, is the different isotope fractionation during acetoclastic versus hydrogenotrophic CH_4 formation. This difference has been exploited for characterization of the predominant path of CH_4 formation (Conrad, 2005). This characterization can be done in a diagnostic way by comparing the $\delta^{13}C$ in both CH_4 and CO_2 (Hornibrook et al., 2000; Whiticar, 1999), or by quantification of the percent contribution of each path using mass balance equations based on the measurement of $\delta^{13}C$ in CH_4, CO_2 and acetate and on the knowledge of fractionation factors (Alperin et al., 1992). The latter approach, requiring a rather comprehensive analysis of $\delta^{13}C$ has not very often been applied (Avery

BGD
7, 8619–8661, 2010

et al., 1999; Conrad et al., 2009, 2010b; Sugimoto and Wada, 1993). Quantification of the hydrogenotrophic versus acetoclastic methanogenic paths by radiotracer technique also has not been used very often (reviewed by Conrad, 1999). However, these studies showed that the path of CH_4 production can vary to a large extent between different environments. It is presently unclear which environmental factors, including microbiological data, control the path of CH_4 production.

The $\delta^{13}C$ of the produced CH_4 is not only dependent on the fractionation during CH_4 production but also on the $\delta^{13}C$ of the substrate from which it is produced. A classical example of this interdependence is the use of $\delta^{13}C$ of CO_2 and CH_4 for calculation of an apparent fractionation factor $\alpha_{C\text{-app}}$ that is diagnostic for whether CH_4 is produced predominantly by hydrogenotrophic or acetoclastic methanogenesis. This diagnosis was pioneered by Whiticar et al. (1986) who used the synonym terms carbonate reduction and methyl fermentation, respectively. However, CO_2 is not only a substrate for methanogenesis, but is first of all a product of organic matter degradation. The same is the case with acetate, which is consumed by acetoclastic methanogenesis, while is produced by fermenting bacteria. The CO_2 and acetate may also have undergone stable isotope fractionation during the course of formation. Natural organic matter, the primary substrate, in turn is the product of photosynthesis, which fractionates stable carbon isotopes during assimilation of atmospheric CO_2 depending on the enzymatic mechanism (Zhang et al., 2002). For example, plants with C4 photosynthesis generally produce organic matter with relatively larger $\delta^{13}C$ than plants with C3 photosynthesis (Farquhar et al., 1989). Little is known how environmental conditions affect the isotopic composition of the substrates involved in methanogensis.

All the conversions and isotope fractionations during methanogenic degradation of organic matter are achieved by the microbial community in the respective environment. The microbial community is composed of a large diversity of bacteria and archaea which potentially may change from site to site. Most of these microorganisms have never been isolated so that their physiological characteristics are unknown (Pace, 1997; Rappe and Giovannoni, 2003). However, it is possible to gain information about

BGD
7, 8619–8661, 2010

diversity and structure of the microbial community by molecular fingerprinting and sequencing techniques (Amann et al., 1995). Thus it is possible to analyse the structure of bacterial and archaeal communities by targeting the gene coding for synthesis of ribosomal RNA, which is ubiquitous in all life (Woese, 2000). Furthermore, it is possible to analyze the structure of the community of methanogens by targeting the *mcrA* gene coding for a subunit of the methyl coenzyme M reductase, the key enzyme involved in CH_4 production (Lueders et al., 2001). However, microbial communities in tropical lake sediments have hardly been studied (Conrad et al., 2010b).

We studied 16 different lake sediments from the world largest tropical freshwater wetlands by measuring production rates of CH_4 and CO_2, analyzing many different variables and parameters involved in CH_4 production, analyzing the $\delta^{13}C$ of CH_4, CO_2, acetate (carboxyl and methyl group), and organic matter, determining isotopic fractionation factors, and analyzing the abundance and composition of microbial communities. The objective was to find out (1) which environmental variables control the rate of CH_4 production, (2) which environmental variables control the path of CH_4 production, and (3) which environmental variables control the $\delta^{13}C$ of CH_4, and its substrates.

2 Methods

2.1 Sampling

Sediment cores (6 cm diameter, 40 cm length) of floodplain lakes from the Amazon and Pantanal regions located in Brazil were taken using a corer sampler. The Amazonian lakes were located close to the city of Porto Velho and the Pantanal lakes were located close to the city of Ladario. The water temperature at the time of sampling was 25–35 °C. The upper 0–3 cm sediment were placed into plastic bottles and completely filled. The bottles were shipped by air freight to Marburg and there processed immediately. The sites, sampling time and main characteristics of the sediments are shown in Table 1 and are consecutively numbered (#1–#16). Some of the sites are identical to those

BGD
7, 8619–8661, 2010

described by Bastviken et al. (2010). The sediment of Pantanal Lake 1 was sampled in 2007 during the beginning of the wet season from the center (#8) and the margin (#9) of the lake, and sampled again in 2008 (#7) during the dry season from the center of the lake.

2.2 Incubation experiments

The incubation procedure was basically the same as described by Conrad et al. (2007, 2010b). About 9-mL aliquots of the sediment were transferred in triplicate into 27-mL sterile tubes, flushed with N_2, closed with butyl rubber stoppers and incubated over night 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 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, and $\delta^{13}C$ 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 $\delta^{13}C$ of acetate. The $\delta^{13}C$ of organic carbon ($\delta^{13}C_{org}$) was analyzed after air drying of the sediment at room temperature, removal of carbonate carbon by addition of HCl, followed again by air drying of the sediment slurry at room temperature.

2.3 Molecular analysis of the methanogenic archaeal community

The desoxyribonucleic 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 before (Conrad et al., 2008). Terminal restriction

BGD
7, 8619–8661, 2010

length polymorphism (T-RFLP) analysis of the 16S rRNA and *mcrA* genes were done as described previously in detail (Conrad et al., 2008; Noll et al., 2010). 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. Correspondence analysis and canonical correspondence analysis was done as described before (Conrad et al., 2008; Noll et al., 2010). Diversity indices richness [S], evenness [E] and Shannon index [H] of T-RFLP profiles were calculated. Richness was defined as the number of T-RFs with a relative abundance >1% within a given T-RFLP profile. To compare the dominance structure of the respective community structures as reflected by T-RFLP analysis, the evenness of the community fingerprint patterns was calculated as $E = (-\Sigma Ap_i \ln Ap_i)/\ln S$, where Ap is the fluorescence intensity of the i-th T-RF relative to the total fluorescence intensity, and S is the total number of T-RFs. The Shannon index was calculated as $H = -\Sigma Ap_i \log Ap_i$.

2.4 Chemical analyses

Methane, CO_2, and H_2 were analyzed by gas chromatography, acetate by high pressure liquid chromatography (HPLC) as described by Conrad et al. (2007). The C and N content of the sediments were quantified on a CHNS-elemental analyzer by the Analytical Chemical Laboratory of the University of Marburg. Sulfate was analyzed by ion chromatography and total iron by the ferrozin method (Conrad and Klose, 2006). The $\delta^{13}C$ (in units of permil) of CH_4 ($\delta^{13}C_{CH4}$) and CO_2 ($\delta^{13}C_{CO2}$) was analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS), the $\delta^{13}C$ of acetate ($\delta^{13}C_{ac}$) by HPLC-C-IRMS as described before (Conrad et al., 2007). The $\delta^{13}C$ of the methyl group of acetate ($\delta^{13}C_{ac\text{-}methyl}$) was determined after off-line pyrolysis (Conrad et al., 2007). Analysis of the $\delta^{13}C$ in organic matter was done at the Institute of Soil Science and Forest Nutrition (IBW) at the University of Göttingen (courtesy of Heinz Flessa) with an elemental analyzer (Fisons EA 1108) coupled to an IRMS.

BGD
7, 8619–8661, 2010

2.5 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 $\varepsilon \equiv 1 - \alpha$ (in units of permil). The $\delta^{13}C$ for a newly formed CH_4 ($\delta^{13}C_{new}$) was calculated from the $\delta^{13}C$ at two time points $t = 1$ ($\delta^{13}C_1$) and $t = 2$ ($\delta^{13}C_2$) by the following mass balance equation:

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

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

The fractionation factor for conversion of CO_2 to CH_4 is given by

$$\alpha_{CO2,CH4} = (\delta^{13}C_{CO2} + 1000)/(\delta^{13}C_{CH4\text{-}mc} + 1000) \quad (3)$$

where $\delta^{13}C_{CH4\text{-}mc}$ is the newly formed CH_4 (Eq. 2) derived from $H_2 + CO_2$, i.e., the CH_4 produced in the presence of 2% CH_3F, assuming that acetoclastic methanogenesis was then completely inhibited.

Relative contribution of $H_2 + CO_2$-derived CH_4 to total CH_4 was determined using the following mass balance equation (Conrad, 2005):

$$f_{H2} = (\delta^{13}C_{CH4} - \delta^{13}C_{CH4\text{-}ma})/(\delta^{13}C_{CH4\text{-}mc} - \delta^{13}C_{CH4\text{-}ma}) \quad (4)$$

where f_{H2} is the fraction of CH_4 formed from $H_2 + CO_2$, $\delta^{13}C_{CH4}$ the $\delta^{13}C$ of total produced methane, $\delta^{13}C_{CH4\text{-}mc}$ the $\delta^{13}C$ of CH_4 derived from $H_2 + CO_2$, and $\delta^{13}C_{CH4\text{-}ma}$ the $\delta^{13}C$ of CH_4 derived from acetate determined by:

$$\delta^{13}C_{CH4\text{-}ma} = \delta^{13}C_{ac-methyl} \quad (5)$$

where $\delta^{13}C_{ac\text{-}methyl}$ is the $\delta^{13}C$ of the methyl group of acetate accumulated. It is assumed, that the methyl group of acetate was converted to CH_4 without fractionation, i.e., $\varepsilon_{ac,CH4} = 0$.

Regression analysis was done using Origin 6.1 (Microcal, Northampton, MA, USA).

BGD
7, 8619–8661, 2010

3 Results

Sediments were sampled from 16 different lake sites in Brazilian Pantanal and Amazonia in 2006, 2007 and 2008. The geographical coordinates of the sites and some of the basic characteristics of the sediments are given in Table 1. The pH values of the lake sediments were in a range of pH 5.9 to 7.7 (average pH 6.75); the contents of organic carbon ranged between 0.8 and 12.5% (average 5.3%); $\delta^{13}C_{org}$ between −32.8 and −25.2‰; N_{tot} between <0.05% and 1.06%; and porewater sulfate concentrations between 0.3 and 95 µM. Hence, the chemical conditions of the lake sediments covered a rather broad range. The highest sulfate concentrations were found in samples taken in 2007 from Lake 1 and Lake 2.

The sediment samples were incubated in triplicate under anoxic conditions at 25 °C in the absence and presence of 2% methyl fluoride. Methyl fluoride served as inhibitor of acetoclastic methanogenesis (Janssen and Frenzel, 1997). A typical experiment is shown in Fig. 1 for Lake Jatoba sediment. The experimental results of all the lake sediments are shown in supplementary Fig. S1. In Lake Jatoba as in all the other lake sediments both CH_4 and CO_2 were produced during the course of the experiment. Production of CO_2 generally and production of CH_4 in most of the lake sediments started right from the beginning of the incubation, with rates gradually decreasing with time (Fig. 1a, b). Only the sediments sampled in Lake 1 and Lake 2 in 2007, which contained relatively much sulfate (Table 1), exhibited a lag phase before CH_4 production started (Fig. S1). Addition of CH_3F resulted in a lower CH_4 production due to inhibition of acetoclastic methanogenesis, but usually had a much smaller effect on production of CO_2. The rates are summarized in Table 2. The rates of CH_4 in the different lake sediments linearly increased with the content of C_{org} (Fig. 2a). Rates of CO_2 production were linearly related to those of CH_4 production in an almost proportional way (Fig. 2b).

The $\delta^{13}C$ values of the accumulating CH_4 and CO_2 were also measured during the course of incubation (Fig. 1c). The time period for which values of $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$ were averaged is indicated by arrows (Fig. 1c). The average values are

BGD
7, 8619–8661, 2010

summarized in Table 2 and in figures shown below. Values of $\delta^{13}C_{CH4}$ of newly formed CH_4 were calculated from those of accumulated CH_4 using mass balance (Eq. 2). The $\delta^{13}C_{CH4}$ values across all the different lake sediments (-71.8 ± 2.7‰) were generally much lower than values of $\delta^{13}C_{CO2}$ (-17.0 ± 2.0‰). The values of $\delta^{13}C_{CH4}$ decreased even further (-88.8 ± 2.6‰) when CH_3F was added to inhibit acetoclastic methanogenesis. However, CH_3F had only a relatively small effect on values (-20.8 ± 2.0‰) of $\delta^{13}C_{CO2}$ (Fig. 1c; Table 2). Values of $\delta^{13}C$ for CH_4 and for CO_2 were correlated to $\delta^{13}C_{org}$ (Fig. 3) and among themselves (not shown). However, they were not correlated to C_{org} or any other sediment characteristic that is not a $\delta^{13}C$ value or calculated thereof.

At the end of incubation, the concentration and $\delta^{13}C$ of acetate were analyzed (Table 3). Acetate concentrations increased during incubation in the presence of CH_3F, as acetoclastic methanogenesis was inhibited. In the uninhibited control, however, acetate concentrations were very low (generally <70 µM), often close or below to the detection limit (10 µM). The acetate accumulated in the presence of CH_3F was most probably due to acetate formation by fermenting bacteria, while that in the absence of CH_3F was the residual acetate after consumption by acetoclastic methanogens. Both sets of $\delta^{13}C$ values were correlated to each other in an almost proportional way (Fig. 4a) and were also positively correlated to $\delta^{13}C_{org}$, albeit with a slope larger than 2 (Fig. 4b). Furthermore, $\delta^{13}C$ of the methyl group of acetate was determined (Table 3). However, only values of $\delta^{13}C_{ac\text{-}methyl}$ in the presence of CH_3F were obtained from all the incubations, since acetate concentrations were sufficiently high under these conditions. These data were not significantly correlated with $\delta^{13}C_{org}$ albeit exhibiting a positive tendency (Fig. 4c). Accepting this tendency despite lacking significance, $\delta^{13}C_{ac\text{-}methyl}$ increased proportionally with $\delta^{13}C_{org}$ but with values that were about 10‰ lower. For the uninhibited incubations, however, the amounts of acetate retrieved were often not sufficient for determination of $\delta^{13}C$ of the methyl group, so that triplicate incubations were pooled. For many but not for all lake sediments, values of $\delta^{13}C$ of the acetate-methyl

BGD
7, 8619–8661, 2010

could thus be obtained.

In order to see a possible effect of acetate consumption on the $\delta^{13}C$ of the residual acetate, the difference was calculated between the $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}CH3F}$, i.e., acetate that was obtained in the absence and the presence of the inhibitor CH_3F. These differences were negatively correlated with $\delta^{13}C_{org}$ and changed from a positive to a negative value at a $\delta^{13}C_{org}$ of about −30‰ (Fig. 5a). A similar relationship using acetate-methyl instead of total acetate was not significant (Fig. 5b). The difference between $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}CH3F}$ was also negatively correlated with the intramolecular isotopic difference between carboxyl and methyl groups of acetate (see discussion), as shown by the difference of $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}methyl}$, both measured in the presence of CH_3F (Fig. 5c). However, the difference between $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}CH3F}$ exhibited no significant correlation with other parameters, such as CH_4 production rate, fraction of hydrogenotrophic methanogenesis, fractionation factors, microbial numbers, etc.

Assuming that the CH_4 in the presence of CH_3F was exclusively formed from reduction of CO_2 (CH_4-mc), the fractionation factor $\alpha_{CO2,CH4}$ (given as $\varepsilon_{CO2,CH4}$) for hydrogenotrophic methanogenesis was calculated from the $\delta^{13}C$ of CO_2 and of newly formed CH_4-mc (Table 2). Values of $\alpha_{CO2,CH4}$ ranged between 1.050 and 1.092 (average 1.075 ± 0.002). The values of the individual lake sediments did not reveal correlation with any other variable, e.g., C_{org} or $\delta^{13}C_{org}$. Furthermore, values of $\delta^{13}C$ of newly formed CH_4 were also used for calculation of the fraction ($f_{CO2,CH4}$) of CH_4 production by hydrogenotrophic methanogenesis using Eq. (4). The equation uses $\delta^{13}C$ of CH_4, of CH_4-mc and of CH_4-ma, the latter being the CH_4 formed from acetoclastic methanogenesis. The $\delta^{13}C_{CH4\text{-}ma}$ was assumed to be identical to the $\delta^{13}C$ of the acetate-methyl ($\delta^{13}C_{ac\text{-}methyl}$), which was measured in the acetate accumulating in the presence of CH_3F. We assumed that the acetate-methyl was quantitatively transformed into CH_4 so that fractionation did not occur. The values of $f_{CO2,CH4}$ are summarized in Fig. 6. Values of $f_{CO2,CH4}$ were generally higher than 50% demonstrating that hydrogenotrophic methanogenesis was more important than acetoclastic

BGD
7, 8619–8661, 2010

methanogenesis in all the lake sediments. The $f_{CO2,CH4}$ values showed a significant negative correlation with the $\delta^{13}C_{CH4\text{-}mc}$ (Fig. 6a), which was expected from Eq. (4), but no significant correlation with $\delta^{13}C_{ac\text{-}methyl}$ ($y = 74.5 + 0.26x$; $r^2 = 0.05$; $P = 0.41$). The $f_{CO2,CH4}$ values also showed no correlation with $\alpha_{CO2,CH4}$ ($r^2 = 0$) and CH_4 production rates ($r^2 = 0.03$), and the decreasing tendency with $\delta^{13}C_{org}$ ($r^2 = 0.11$) was not significant (Fig. 6b). It also correlated positively, but not proportionally, with the residual CH_4 production after inhibition of acetoclastic methanogenesis by CH_3F (Fig. 6c).

We also used the sediment samples to analyze the abundance, diversity and composition of the microbial community by using quantitative PCR and T-RFLP of the genes coding for the ribosomal rRNA of archaea and bacteria and of the *mcrA* gene coding for a subunit of the methylcoenzyme M reductase, the key enzyme of methanogenic archaea. The numbers of archaea (given as 16S rRNA gene copies) were generally one order of magnitude lower than those of the bacteria, but correlated with each other (Fig. 7a). Production rates of CH_4 increased linearly with the logarithm of numbers of archaea (Fig. 2c) and bacteria (not shown). In T-RFLP analysis a total of 17, 109, and 37 different terminal restriction fragments (T-RFs) of archaeal 16S rRNA genes, bacterial 16S rRNA genes and *mcrA* genes, respectively, were detected. On the average 9, 29, and 10, respectively, of these different T-RFs were found in each individual lake sediment. Thus, the richness of T-RFs in the combined lake sediments was larger than in the individual ones. The values of richness (S), Shannon diversity index (H), and evenness (E) for the different lake sediments and genes are summarized in Table 4. Neither of the diversity indices exhibited a significant correlation to the CH_4 production rates.

The T-RFLP patterns of the different genes were also analysed by canonical correspondence analysis (Fig. 8). The T-RFLP patterns of the lake sediments did not show a consistent clustering across the different genes indicating that bacterial and archaeal communities did not follow a geographical pattern. The T-RFLP patterns of the individual lakes were also not consistently correlated to one of the different vectors that indicate the chemical/physiological parameters measured, such as C_{org}, $\delta^{13}C_{org}$,

BGD
7, 8619–8661, 2010

CH_4 production, abundance of archaea and bacteria, $\delta^{13}C_{CH4}$, $\delta^{13}C_{CO2}$, $\delta^{13}C_{CH4\text{-}mc}$, $\delta^{13}C_{ac}$, and $\delta^{13}C_{ac\text{-}methyl}$.

Most of the T-RFs in the T-RFLP patterns of archaeal 16S rRNA gene could be assigned to groups of methanogenic archaea on the basis of published clone sequences: *Methanomicrobiales* (84 bp); *Methanobacteriales* (92 bp); *Methanosarcinaceae* (186 bp); *Methanosaetaceae* (284 bp); *Methanomicrobiales* and *Methanocellales* (393 bp); Rice cluster 2 (494 bp). These groups accounted on the average for <1%, 19%, 13%, 21%, 28%, and 5%, respectively, in total for 87% of all the different T-RFs. These methanogenic groups comprised both potentially acetoclastic methanogens (*Methanosarcinaceae* and *Methanosaetaceae*) and hydrogenotrophic methanogens (all the groups except *Methanosaetaceae*). Interestingly, $f_{CO2,CH4}$ showed a tendency to decrease with the relative abundance of T-RFs (284 bp length) representative for *Methanosaetaceae* (Fig. 7b). However, this tendency was statistically not significant.

4 Discussion

Incubation experiments with samples from 16 different lake sediments in tropical Brazil allowed estimating the activity and isotope fractionation involved in CH_4 production from organic matter. In particular, the data gave insight into the path of CH_4 production by hydrogenotrophic versus acetoclastic methanogenesis, the carbon isotope fractionation during acetate production, the control of isotopic composition of the produced CH_4, the control of CH_4 production rate, and the role of the microbial community.

4.1 Path of CH_4 production

The path of CH_4 production was determined by mass balance (Eq. 4) using measured values of $\delta^{13}C$ of acetate-methyl and of newly produced CH_4 in the presence and absence of CH_3F. The fraction of CH_4 produced by hydrogenotrophic methanogenesis

BGD
7, 8619–8661, 2010

($f_{CO2,CH4}$) was generally >50% (50–90%) and thus relatively large (Fig. 6). The determination assumed that the CH_4 produced in the presence of CH_3F was exclusively due to hydrogenotrophic methanogenesis, as acetoclastic methanogenesis was completely inhibited (Conrad, 2005). Previous studies with other lake sediments have shown that this assumption is robust (Conrad et al., 2007, 2009, 2010b). The measurement of $\delta^{13}C_{CH4\text{-}mc}$ also allowed the estimation of the fractionation factor ($\alpha_{CO2,CH4}$) involved in hydrogenotrophic methanogenesis resulting in a range that is consistent with literature data on aquatic sediments in which the energy provided by H_2 is limiting CH_4 production (Penning et al., 2005; Takai et al., 2008; Valentine et al., 2004). Thus, the $\alpha_{CO2,CH4}$ for the 16 different tropical lake sediments ranged between 1.050 and 1.092, also covering the $\alpha_{CO2,CH4}$ values of about 1.085 recently determined for two lake sediments in Amazonia (Conrad et al., 2010b).

The choice of $\delta^{13}C_{CH4\text{-}ma}$ assumed that acetate-methyl was not further fractionated during acetoclastic methanogenesis (Eq. 5), which is the case if acetate is consumed as rapidly as it is produced. This was most likely the case, since acetate concentrations were very low in all the lake sediments studied and only increased when acetoclastic methanogenesis was inhibited by CH_3F. The $\delta^{13}C$ of the background acetate in the different sediments increased almost proportionally and with a slope close to one with those of the acetate accumulated in the presence of CH_3F (Fig. 4a) also indicating that there was no significant fractionation during the degradation of acetate by methanogenesis. The error imposed by neglecting a putative fractionation during formation of $\delta^{13}C_{CH4\text{-}ma}$ would be relatively small since the theoretical fractionation factor for the conversion of acetate-methyl to CH_4 is at the maximum $\alpha_{ac,CH4} = 1.025$ when *Methanosarcina* species are the active methanogens (Gelwicks et al., 1994; Goevert and Conrad, 2009) or $\alpha_{ac,CH4} = 1.010$ when *Methanosaeta* species are the active methanogens (Penning et al., 2006; Valentine et al., 2004). Assuming that $\delta^{13}C_{CH4\text{-}ma}$ would be by 10‰ ($\alpha_{ac,CH4} = 1.010$) smaller than $\delta^{13}C_{ac\text{-}methyl}$ would result in values of $f_{CO2,CH4}$ ranging between 40% and 80%.

BGD
7, 8619–8661, 2010

Hence, the fraction of CH_4 produced by hydrogenotrophic methanogenesis was relatively large in the lake sediments (Conrad, 1999). This observation is consistent with determinations in other lake sediments (Conrad et al., 2009, 2010a, b), but cannot be explained by the complete degradation of organic matter according to

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4 \quad (6)$$

since

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (7)$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \quad (8)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (9)$$

where hydrogenotrophic methanogenesis can only achieve a maximum contribution of 33% (Conrad, 1999). Conceivable explanations are (1) that organic matter in the lake sediments is incompletely degraded with the preferential production of H_2 and the accumulation of residual organic substances having a higher oxidation state than the original C_{org}, or (2) that intermediate acetate is converted by syntrophic acetate oxidation instead of acetoclastic methanogenesis (Conrad et al., 2010a). Syntrophic acetate oxidation is the conversion of acetate to CO_2 plus H_2

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (10)$$

followed by hydrogenotrophic methanogenesis (Eq. 9).

The values of $f_{CO2,CH4}$ were positively correlated to the percentage of residual CH_4 production upon inhibition by CH_3F (Fig. 6c). Such correlation is expected, as the residual rates are the CH_4 production rates diminished by those of acetoclastic methanogenesis and should be equal to the rates of hydrogenotrophic methanogenesis. However, $f_{CO2,CH4}$ was not proportional to the residuals and increased with a relative slope of much smaller than one. Therefore, we have to assume that CH_3F inhibited not only

BGD
7, 8619–8661, 2010

acetoclastic methanogenesis but also to some degree hydrogenotrophic methanogenesis. Previous studies had shown that hydrogenotrophic methanogenesis is indeed sensitive to CH_3F if applied at a too high concentration (Conrad and Klose, 1999). It is hardly possible to optimize inhibition by CH_3F for each individual sediment, in particular, since quite small changes in CH_3F concentration can result in change of the inhibition pattern. In the present study we found that the unspecific inhibition of hydrogenotrophic methanogenesis became increasingly larger in those lake sediments that had a relatively low $f_{CO2,CH4}$.

4.2 Carbon isotope fractionation during acetate production

Acetate and CO_2, the substrates of methanogenesis, are formed from C_{org} and their $\delta^{13}C$ is due to isotope fractionation during production and consumption. As CO_2 was produced as well as consumed, even in the presence of CH_3F, its $\delta^{13}C$ was potentially affected by its formation during the degradation of C_{org} but also by its consumption during methanogenesis. The same is true for acetate, but only in the absence of CH_3F. In the presence of CH_3F, we may assume that acetate was only produced but no longer consumed. Therefore, the $\delta^{13}C$ of acetate should reflect the $\delta^{13}C$ of the substrate C_{org} and the effective fractionation factor during fermentation. Indeed, $\delta^{13}C_{acetate}$ increased with $\delta^{13}C_{org}$, but increased by a factor of 2.5 faster (Fig. 4b). Five out of 16 samples exhibited a $\delta^{13}C_{acetate}$ lower than $\delta^{13}C_{org}$, but most of the samples exhibited a higher $\delta^{13}C_{acetate}$. Possibly, the carboxyl group of acetate partially exchanged with the environmental CO_2 (DeGraaf et al., 1996), thus affecting the $\delta^{13}C$ of total acetate. Indeed, $\delta^{13}C$ of acetate-methyl was generally lower than $\delta^{13}C_{org}$. Furthermore, $\delta^{13}C_{ac\text{-}methyl}$ increased at the same rate (relative slope = 1.1) as $\delta^{13}C$ of C_{org} (Fig. 4c) as theoretically expected. However, it should be noted that the linear regression was not statistically significant for the number of data available. Nevertheless, the $\delta^{13}C$ of acetate-methyl was on the average by 10‰ lower than that of C_{org} indicating some fractionation during fermentative acetate production. Previous studies in lake sediments also found

BGD
7, 8619–8661, 2010

that acetate-methyl was more negative than $\delta^{13}C_{org}$, the difference ranging between 0 and 10‰ (Conrad et al., 2007, 2009, 2010b). Here, however, we observed a much larger range of 4–40‰. The smaller values can be explained from experiments with pure cultures of fermenting bacteria, in which $\delta^{13}C$ of acetate was only slightly different from $\delta^{13}C$ of the substrate (Blair et al., 1985; Penning and Conrad, 2006; Rinaldi et al., 1974). However, for $\delta^{13}C_{ac\text{-}methyl}$ being 40‰ more negative than $\delta^{13}C_{org}$ there is no immediate explanation, except that the effective substrate from which acetate was produced may have been more negative than the $\delta^{13}C_{org}$ measured.

Another interesting observation was that in some of the sediments the $\delta^{13}C$ of acetate accumulated in the presence of CH_3F was higher than that in the absence. The difference was only a few permil, and was larger for total acetate (Fig. 5a) than for acetate-methyl (Fig. 5b), but it was the same lake sediments where acetate carbon was fractionated this way. This way of fractionation is not expected, since the light carbon should be preferentially consumed in the absence of inhibitor and thus $\delta^{13}C$ in the residual acetate should be higher in the absence than in the presence of CH_3F. However, such a counterintuitive fractionation of acetate carbon was also observed in the sediment of a German lake (Conrad et al., 2009). In the present study we found a negative correlation of the difference between $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}CH3F}$ and the $\delta^{13}C_{org}$, and the unusual fractionation (i.e., difference between $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}CH3F} < 0$) was observed in those lake sediments, which had a $\delta^{13}C_{org}$ larger than about −30‰ (Fig. 5a). Interestingly, there was also a negative correlation of the difference between $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}CH3F}$ and the intramolecular isotopic difference between the carboxyl and the methyl group of acetate, and the unusual fractionation (i.e., difference between $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}CH3F} < 0$) was observed in those lake sediments, in which the intramolecular isotopic difference was relatively large (Fig. 5c). The intramolecular difference may be indicative for the acetate production process, being either fermentation of organic substrate or chemolithotrophic acetogenesis. In the latter pathway, acetate is synthesized from CO_2 (i.e., reversal of Eq. 10)

BGD
7, 8619–8661, 2010

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (11)$$

The acetate synthesized in this way exhibits only a small isotopic difference between methyl and carboxyl groups being on the order of <2‰ (Gelwicks et al., 1989). The acetate synthesized during fermentation, on the other hand, may have a much larger intramolecular isotopic difference being on the order of 24‰ (Blair et al., 1985). In the Brazilian lake sediments, the difference between $\delta^{13}C_{ac}$ and $\delta^{13}C_{ac\text{-}methyl}$ was between 5‰ and 17‰ (except Lake #15, Paca, Fig. 5) which corresponds to an intramolecular isotopic difference of 12–30‰. We hypothesize, similarly as before (Conrad et al., 2009), that acetate production during fermentation may be partially inhibited by CH_3F in such a way, that carbon isotope isotopic fractionation is affected. This could happen when different fermenting microorganisms exhibit different isotope fractionation and different susceptibility to inhibition by CH_3F. There are hardly studies of isotopic fractionation during fermentative acetate production (Blair et al., 1985; Penning and Conrad, 2006) although numerous different fermentation pathways exist in nature.

4.3 Control of the $\delta^{13}C$ of the produced CH_4

Our data show that the $\delta^{13}C$ of CH_4 and CO_2, the final products of the degradation of organic matter, but also the $\delta^{13}C$ of the intermediate acetate were controlled both by the fractionation during the degradation process and by the $\delta^{13}C$ of the original substrate. The effect of the fractionation during the degradation was most clearly seen by the decrease of the fraction ($f_{CO2,CH4}$) of hydrogenotrophic methanogenesis with increasing $\delta^{13}C_{CH4}$ (Fig. 6a). The fraction also decreased with increasing $\delta^{13}C_{CO2}$, since $\delta^{13}C_{CH4}$ was correlated with $\delta^{13}C_{CO2}$ (data not shown). Values of $f_{CO2,CH4}$ also showed a weak (and not significantly, $P = 0.21$) decreasing tendency with $\delta^{13}C_{org}$ (Fig. 6b), probably since C_{org} was the source for CO_2. However, $f_{CO2,CH4}$ did not show any correlation with the $\delta^{13}C$ of acetate-methyl ($y = 74.5 + 0.26x$; $r^2 = 0.05$; $P = 0.41$). The correlation of $f_{CO2,CH4}$ with $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$ but not with $\delta^{13}C_{ac\text{-}methyl}$ is

BGD
7, 8619–8661, 2010

likely due to the fact that generally more than 50% of the CH_4 was formed by hydrogenotrophic methanogenesis which exhibited a relatively strong fractionation (on the average $\alpha_{CO2,CH4} = 1.075$) in the lake sediments studied, whereas less than 50% of the CH_4 was produced from acetoclastic methanogenesis, which probably exhibited no or only low fractionation.

However, the $\delta^{13}C$ of the substrate, $\delta^{13}C_{org}$, also affected the $\delta^{13}C$ of CH_4, CO_2 and acetate (see discussion above), since these data obtained from the different lake sediments were all correlated to each other and to the $\delta^{13}C$ or organic matter. The relative increase of $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$ was larger than that of $\delta^{13}C_{org}$ with slopes of 2.9–3.5. On the average, the $\delta^{13}C$ of newly formed CH_4 was 43 ± 2‰ lower and that of CO_2 was 12 ± 2‰ larger than $\delta^{13}C_{org}$. These data show that the $\delta^{13}C$ of CH_4 and CO_2 were not only determined by the $\delta^{13}C_{org}$ but also by the fractionation during the degradation process. In the presence of CH_3F, the difference between $\delta^{13}C_{CH4-CH3F}$ and $\delta^{13}C_{org}$ was 60 ± 3‰, which is about 17‰ more than with $\delta^{13}C_{CH4}$ in the absence of CH_3F. This increase is due to the inhibition of acetoclastic methanogenesis by CH_3F and the then exclusive formation of CH_4 by hydrogenotrophic methanogenesis, which exhibits a larger fractionation factor than acetoclastic methanogenesis. The difference of 60‰ lower CH_4 and 12‰ larger CO_2 results in a difference of about 72‰ between $\delta^{13}C$ of CO_2 and of hydrogenotrophically formed CH_4, which corresponds to $\alpha_{CO2,CH4} = 1.075 \pm 0.002$ determined from the measured $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$ measured in the presence of CH_3F. In summary, fractionation had the larger effect on $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$ than the $\delta^{13}C_{org}$. However, $\delta^{13}C_{org}$ affected the $\delta^{13}C$ of acetate-methyl to a similar extent than fractionation during conversion of C_{org} to acetate (Fig. 4c).

BGD
7, 8619–8661, 2010

4.4 Control of CH_4 production rate

The data from 16 different lake sediments showed that the production rates of CH_4 and CO_2 were related to both the content of organic matter and the abundance of microorganisms in the sediment. The lake sediments with the highest organic carbon contents were from the Pantanal. Production rates of CH_4 almost doubled (relative slope = 0.83) with doubling of the organic matter content. These data show that organic matter content was a rate-limiting factor for CH_4 production. This is a reasonable conclusion, since the initial mobilization of organic substances is believed to be the rate-limiting step for further degradation and eventual CH_4 production (Billen, 1982). The dependency on organic matter content furthermore indicates that the CH_4 production process was under quasi steady state conditions. This is a reasonable conclusion, since acetate, or other organic intermediates apparently did not accumulate during the incubation but were below or close to the detection limit of the analysis. Acetate only accumulated, when its consumption by acetoclastic methanogens was inhibited with CH_3F. The observation that CH_4 production scales with the sediment organic matter content is in agreement with an analysis of lake characteristics determining CH_4 emission by Bastviken et al. (2004). These authors found that lake area, along with total phosphorous and dissolved organic carbon concentrations were the most useful variables for describing methane emissions on a global basis. Ebullition is the most likely fate (>50%) of the produced CH_4 especially in relatively shallow lakes. In lakes of the Pantanal, ebullition was found to account on the average for 91% of the total CH_4 flux (Bastviken et al., 2010).

Generally, only a small percentage of the total organic matter present in anoxic soils or sediments is used up for production of CH_4 and CO_2. In rice field soils, for example, 6–17% of the total soil organic matter was released as gas during the season (120 d) (Yao et al., 1999). Therefore, total sediment organic matter is only a proxy for the organic matter that is actually available for microorganisms and eventually degraded on the time scale used for our experiments. For rice field soil it was found that soil

BGD
7, 8619–8661, 2010

organic nitrogen was a good proxy for available organic materials predicting CH_4 production quite well, while total soil organic matter did not (Cheng et al., 2007; Yao et al., 1999). However, in the Brazilian lake sediments, sediment C_{org} was apparently a good predictor for potential CH_4 production.

Production rates of CO_2 also increased with organic matter content. The relative slope of CO_2 versus CH_4 production rates was 0.77. According to Eq. (6), complete degradation of organic matter should actually produce equimolar amounts of CO_2 and CH_4 (i.e., relative slope = 1.0). However, the measured CO_2 production rates covered only the gaseous portion of CO_2 in the incubations. Since most of the lake sediments were slightly acidic, bicarbonate content was negligible. The dissolved CO_2 can be calculated from Henry's law (Stumm and Morgan, 1981) and amounts to about 40% of the gaseous CO_2 for our experimental conditions. Hence, total CO_2 production rates were even slightly higher than CH_4 production rates. Such behaviour is frequently observed in acidic bogs (Galand et al., 2010; Heitmann et al., 2007; Keller et al., 2009; Yavitt and Seidmann-Zager, 2006), but also in lake sediments (Conrad et al., 2010b). It has been explained by the assumption that organic substances may act to some extent as electron acceptors for oxidation of organic matter, so that Eq. (6) is not rigorously valid. Nevertheless, this effect seemed to be rather small when considering the range of CH_4 and CO_2 production in the different lake sediments investigated.

Finally, production rates of CH_4 and CO_2 both correlated with the logarithm of archaeal and bacterial numbers in the sediment (Fig. 2c). This is plausible since microorganisms are catalyzing the biogeochemical processes, including degradation of organic matter to CO_2 and CH_4. Vice versa, the transformation of organic matter provides the energy for growth and maintenance of the microorganisms.

4.5 Role of the microbial community

Production rates of CH_4 more than quadrupled (relative slope = 4.4) when the abundance of archaea increased by an order of magnitude. The increase of microbial numbers with CH_4 production rates is reasonable since microorganisms can proliferate

BGD
7, 8619–8661, 2010

when energy supply increases, i.e. the rate of methanogenic organic matter degradation increases. The number of methanogenic archaea that can be maintained by a particular CH_4 production rate can be calculated from the microbial maintenance requirement which at 25 °C is constant at 4.5 kJ h^{-1} C-mol^{-1} biomass (Tijhuis et al., 1993). Using this constant parameter, theoretical calculations indicate that a CH_4 production rate of 100 nmol h^{-1} g^{-1} could maintain about 6×10^7 g^{-1} methanogenic archaea (Conrad, 2007). These calculations are based on the assumption that 1 C-mol microbial biomass is equivalent to 1.4×10^{14} microbial cells, a value which may be disputed because of the large range of possible cell sizes of archaea. In the Brazilian lakes we found between 1×10^7 and 2×10^9 copies of archaeal ribosomal genes per gram dry sediment (Figs. 2c, 7a). Since methanogenic archaea typically contain 3 ribosomal gene copies per cell (Klappenbach et al., 2001), the lake sediments correspondingly contained between 3×10^6 and 7×10^8 methanogenic archaea per gram sediment. However, the CH_4 production rates were only between 0.5 and 14 nmol h^{-1} g^{-1}, thus theoretically allowing the maintenance of about only 3×10^5 to 8×10^6 g^{-1} methanogens, which is 1–2 orders of magnitude lower than the actually measured values. This is a large discrepancy even when considering uncertainties in the theoretical calculation. We hypothesize that much of the microbial DNA, i.e., the ribosomal genes detected, may be preserved in dead cells and no longer take part in the metabolism and substrate turnover in the lake sediment.

If this is true, the analysis of DNA and of genes in the sediment would not reflect the active microbial community but mainly give an impression about the history of microbial activity. In sediments, in which metabolism is in a stable steady state, these two aspects would not be much different, but sediments which undergo seasonal changes might well result in strong differences. For the Brazilian lake sediments we have to expect the latter case, since these lakes are part of river floodplains which exhibit flooding pulses.

Such effect was probably the reason why sediment taken from the central of Lake 1 in 2008 (#8) behaved completely different from samples taken in 2007 (#10). Sample #8

BGD
7, 8619–8661, 2010

was taken at a time when the lake was just at the beginning of the wet season, while the samples #9–11 were taken during the time when water from the Paraguay River inundated the lakes. At this time, the sulfate content of the sediment (#8) was much larger than in #10, and CH_4 production exhibited a lag phase, probably because sulfate reducers outcompeted methanogens.

Analysis of the relative composition of the communities of bacteria, archaea and *mcrA*-containing methanogens showed quite a diversity in each of the different Brazilian lake sediments. The Shannon index of bacteria ranged between 2.5 and 3.9, whereas that for archaea and methanogens was a bit lower between 1.4 and 2.0. The evenness was generally higher than 0.67 and reached maximum values of 0.97. These diversity indices should not be taken as absolute numbers, since molecular fingerprinting techniques only reveal the relatively abundant taxa but cannot cover the less common microbes (Bent and Forney, 2008). However, the indices and the T-RFLP patterns can be compared across the different lakes. The comparison shows that the composition of the communities was different for each lake and exhibited no pattern with respect to geographical location, organic matter content, microbial abundance, activity or isotope fractionation. The composition patterns were also different with respect to bacteria, archaea and methanogens. The failure to detect a microbial community pattern that would be related to the microbial functionality of the lake sediments may have several reasons: (1) the number of lake sediments tested was still too limited to detect such a relationship; (2) such a relationship does not exist on the level of microbial genes detected but might exist on the level of microbial transcripts (expression of genes) or microbial proteins (transcription and translation of genes); (3) such a relationship is not visible on the level of global genes, such as ribosomal RNA or *mcrA* genes, but only when targeting genes that allow differentiating between different peculiar functions; (4) there is no such relationship at all, since diverse microbial species can replace each other to fulfil a particular functionality.

We found it an intriguing observation that $f_{CO2,CH4}$ in the different lake sediments exhibited a decreasing tendency when the relative percentage of acetoclastic

BGD
7, 8619–8661, 2010

Methanoseataceae increased (Fig. 7b). This is exactly what one would expect if *Methanoseataceae* but not other methanogens were indeed the populations responsible for CH_4 production from acetate. However, this relationship was statistically not significant. Nevertheless, this observation is encouraging for future research to eventually reveal a significant relation between microbial community patterns and microbial functionality if sufficient lake sediments are tested for relevant microbial genes and their transcription.

5 Conclusions

The study of various tropical lake sediments from Brazil demonstrated that the overall abundance of bacteria and archaea correlated well with the production rates of CH_4 and CO_2 showing that methanogenic degradation of organic matter is dependent on and may be limited by microbial activity. However, the study revealed no apparent correlation of the CH_4 production rates, the ^{13}C stable isotope discrimination, or the path of CH_4 production with the diversity or the composition of the resident methanogenic microbial communities. Although this result does not exclude that not-measured microbial parameters, e.g., the extent of gene expression or protein synthesis in the different groups of bacteria and archaea, do have a decisive effect on CH_4 production, it nevertheless indicates that the relative abundance of a particular microbial population is of little importance.

Production rates of CH_4 and CO_2 increased with the content organic matter showing that the availability of substrate may limit methanogenic degradation of organic matter.

However, neither organic matter content, microbial abundance, nor methanogenic transformation rates correlated with the $\delta^{13}C$ of CO_2, CH_4 or acetate. However, the isotopic data were a function of both the $\delta^{13}C$ of the primary substrate (organic matter) and the path of CH_4 production. In the tropical lake sediments the path of CH_4 production was dominated by hydrogenotrophic methanogenesis that exhibited relatively strong isotope fractionation with $\delta^{13}C_{CH4}$ being by about 43‰ lower than $\delta^{13}C_{org}$.

BGD
7, 8619–8661, 2010

Acetoclastic methanogenesis, on the other hand, which was comparatively less important, resulted only in relatively weak isotope fractionation. The $\delta^{13}C$ of the methyl group of acetate was on the average by only about 10‰ lower than $\delta^{13}C_{org}$. For the time being it remains unclear which environmental factors determine the relative importance of hydrogenotrophic versus acetoclastic methanogenesis. Therefore, it is presently not possible to theoretically predict the $\delta^{13}C$ of CH_4 and CO_2 from easily measurable lake variables. Further studies will focus the interaction between quality of degradable organic matter and microbial flora will eventually determine the path of CH_4 formation.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/7/8619/2010/bgd-7-8619-2010-supplement.pdf.

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BGD

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7, 8619–8661, 2010

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BGD
7, 8619–8661, 2010

Table 1. Sites of the lakes and main characteristics; mean ± SE, $n = 3$.

#	Lake	Region	Sampling time	Coordinates	pH	C_{org} (%)	$\delta^{13}C_{org}$	N_{tot} (%)	Sulfate (μM)
1	Arrozal	Pantanal	Jul 2007	19°00′50″ S 57°31′27″ W	6.30	6.28 ± 0.21	−25.18 ± 0.05	0.63 ± 0.03	5.6
2	Sinibu	Pantanal	Jul 2007	19°00′57″ S 57°24′17″ W	6.44	5.78 ± 0.50	−27.14 ± 0.05	0.49 ± 0.05	2.6
3	Jatoba	Pantanal	Jul 2007	19°02′22″ S 57°22′7″ W	6.53	0.80 ± 0.26	−29.26 ± 0.07	< 0.05	2.9
4	Anzol de Ouro	Pantanal	Jul 2007	19°00′58″ S 57°37′29″ W	6.89	12.5 ± 0.33	−27.95 ± 0.06	1.06 ± 0.04	15.3
5	Teresa	Pantanal	Nov 2008	18°57′38″ S 57°26′28″ W	6.83	1.69 ± 0.51	−27.85 ± 0.32	0.11 ± 0.02	0.3
6	Presa	Pantanal	Nov 2008	18°59′0″ S 57°25′0″ W	6.74	0.79 ± 0.12	−27.64 ± 0.46	0.07 ± 0.01	0.6
7	Lobo	Pantanal	Nov 2008	18°57′7″ S 57°36′54″ W	6.62	5.93 ± 0.13	−26.86 ± 0.10	0.55 ± 0.02	0.7
8	Lake 1, central	Pantanal	Nov 2008	19°1′45″ S 57°32′54″ W	7.67	8.52 ± 0.15	−29.16 ± 1.87	0.69 ± 0.01	0.3
9	Lake 1, margin	Pantanal	Jan 2007	19°1′45″ S 57°32′54″ W	6.52	11.7 ± 1.35	−30.07 ± 0.08	0.97 ± 0.08	60.4
10	Lake 1, central	Pantanal	Jan 2007	19°1′45″ S 57°32′54″ W	6.98	11.4 ± 0.19	−31.34 ± 0.16	0.92 ± 0.01	95.5
11	Lake 2	Pantanal	Jan 2007	19°1′50″ S 57°28′24″ W	6.93	3.06 ± 0.01	−29.51 ± 0.99	0.26 ± 0.01	36.4
12	Belmont	Amazonia	Aug 2007	08°39′3″ S 63°50′23″ W	5.88	5.70 ± 0.35	−28.55 ± 0.04	0.44 ± 0.00	4.1
13	Maravilha	Amazonia	Aug 2007	08°43′40″ S 63°55′52″ W	6.48	2.62 ± 0.01	−29.10 ± 0.03	0.18 ± 0.01	3.7
14	Laguinho	Amazonia	Aug 2007	08°26′47″ S 63°28′28″ W	6.80	1.59 ± 0.16	−29.50 ± 0.16	0.14 ± 0.00	4.5
15	Paca	Amazonia	Aug 2007	08°29′46″ S 63°27′56″ W	6.68	4.06 ± 0.01	−32.39 ± 0.06	0.35 ± 0.00	9.0
16	Puruzinho	Amazonia	Nov 2008	07°22′19″ S 63°23′22″ W	7.64	1.87 ± 0.04	−32.84 ± 2.53	0.24 ± 0.01	2.3

BGD
7, 8619–8661, 2010

Table 2. Production rates (nmol h^{-1} g-dw^{-1}) of CH_4 and CO_2, values of $\delta^{13}C$ (‰), and enrichment factor for CH_4 production from CO_2 in the incubations of different lake sediments with and without inhibitor (2% CH_3F); mean ± SE, $n = 3$.

	CH_4 production		CO_2 production		$\delta^{13}C$-CH_4		$\delta^{13}C$-CO_2		$\varepsilon_{CO2,CH4}$
#	control	+CH_3F	control	+CH_3F	Control	+CH_3F	Control	+CH_3F	mean
1	3.70±0.16	2.78±0.09	3.65±0.20	2.82±0.43	−59.03±0.47	−72.17±0.79	−7.19±0.32	−10.6±0.17	−66.31±2.82
2	6.00±0.57	4.02±0.07	4.69±0.39	4.91±0.68	−59.09±0.17	−75.44±0.33	−5.01±0.16	−8.32±0.12	−72.60±1.65
3	2.34±0.27	1.14±0.12	1.97±0.17	1.68±0.29	−65.29±0.59	−82.17±1.82	−11.0±0.25	−16.2±0.14	−71.88±4.68
4	14.3±0.80	7.26±0.18	10.2±0.13	7.86±0.66	−62.27±0.92	−80.00±0.93	−9.84±0.12	−11.6±0.17	−74.32±2.71
5	0.58±0.08	0.31±0.03	0.57±0.08	0.70±0.10	−83.65±1.67	−97.60±2.88	−16.3±0.29	−22.9±0.30	−82.80±7.54
6	1.48±0.10	0.33±0.03	1.04±0.01	0.74±0.04	−73.71±1.13	−97.41±0.84	−16.7±0.61	−21.0±0.12	−84.64±2.25
7	2.53±0.54	1.58±0.26	2.95±0.39	2.65±0.36	−64.54±1.75	−84.22±2.71	−8.23±0.28	−13.4±0.16	−77.28±5.47
8	2.86±0.25	1.99±0.28	3.59±0.15	3.52±0.45	−76.44±1.21	−91.11±1.37	−11.6±0.07	−16.6±0.26	−82.00±3.98
9	9.75±0.65	3.73±0.30	11.6±0.57	7.98±0.44	−66.18±0.46	−92.29±1.55	−22.6±0.97	−25.0±0.85	−74.13±10.28
10	8.04±1.18	5.34±0.50	5.39±0.99	6.84±2.96	−80.93±1.24	−100.9±1.60	−29.7±1.12	−32.2±1.33	−76.33±14.17
11	2.94±0.18	2.07±0.24	1.83±0.27	1.69±0.31	−82.83±0.74	−97.77±2.30	−26.8±1.41	−28.2±1.33	−77.13±15.24
12	4.52±0.43	1.92±0.05	6.98±0.09	3.86±0.13	−57.67±0.33	−69.85±0.90	−14.0±0.61	−17.3±0.37	−56.47±6.13
13	1.09±0.03	0.69±0.04	1.54±0.13	0.93±0.12	−78.01±1.31	−94.38±1.40	−21.1±0.74	−22.6±0.61	−79.26±7.35
14	0.92±0.11	0.22±0.03	1.84±0.06	1.24±0.09	−64.10±0.69	−85.99±0.46	−25.4±0.27	−30.6±0.27	−60.63±3.62
15	0.42±0.12	0.45±0.02	1.17±0.18	0.97±0.07	−94.50±3.68	−100.7±4.01	−30.9±0.10	−33.8±0.15	−74.39±13.44
16	1.98±0.21	1.16±0.14	2.04±0.07	1.90±0.17	−80.20±1.66	−98.71±2.47	−15.9±0.44	−22.1±0.08	−85.03±5.64
Mean ± SE	3.97±0.96	2.19±0.50	3.81±0.82	3.14±0.63	−71.78±2.74	−88.79±2.61	−17.0±2.05	−20.8±1.96	−74.70±2.01

BGD
7, 8619–8661, 2010

Table 3. Concentrations of acetate and values of $\delta^{13}C$ (‰) in total acetate and in acetate-methyl at the end of incubation of different lake sediments with and without inhibitor (2% CH_3F); mean ± SE, $n = 3$.

	Acetate (µM)		δ^{13}C-acetate (‰)		δ^{13}C-ac-methyl (‰)	
#	control	+CH_3F	control	+CH_3F	Control[b]	+CH_3F
1	24 ± 2	447 ± 13	−22.82 ± 0.49	−14.45 ± 0.22	−31.33	−29.72 ± 2.86
2	31 ± 7	925 ± 156	−19.82 ± 0.74	−15.96 ± 0.32	−26.35	−29.48 ± 0.96
3	63 ± 21	1330 ± 193	−22.09 ± 1.60	−24.98 ± 0.53	−30.57	−37.70 ± 0.50
4	21 ± 2	1217 ± 27	−24.13 ± 1.23	−21.63 ± 0.30	−29.83	−31.77 ± 0.09
5	0[a]	979 ± 107	−36.95[b]	−34.57 ± 0.40	−41.05	−46.58 ± 0.57
6	0[a]	2357 ± 104	−34.17[b]	−34.29 ± 0.18		−48.13 ± 0.60
7	0[a]	761 ± 67	−31.90[b]	−22.59 ± 0.13	−44.59	−39.77 ± 3.19
8	0[a]	205 ± 12	−36.49[b]	−28.49 ± 0.66	−45.44	−42.76 ± 0.67
9	70 ± 8	2922 ± 216	−19.75 ± 0.39	−20.78 ± 1.41	−33.10	−31.56 ± 1.77
10	62 ± 2	319 ± 146	-18.65 ± 0.33	−24.53 ± 2.06		−35.08 ± 1.14
11	52 ± 5	1191 ± 498	−19.22 ± 0.30	−23.67 ± 1.67	−32.09	−33.35 ± 1.22
12	39 ± 2	1297 ± 34	−23.09 ± 0.28	−19.08 ± 0.63	−38.47	−31.18 ± 0.60
13	23 ± 7	685 ± 23	−27.49 ± 0.50	−28.47 ± 1.01	−41.50	−39.80 ± 1.79
14	21 ± 1	1545 ± 95	−29.75 ± 0.21	−33.68 ± 0.29	−35.66	−40.13 ± 1.58
15	13 ± 1	152 ± 21	−33.77 ± 1.61	−43.83 ± 0.49	−35.66	−39.00 ± 1.43
16	0[a]	444 ± 63	−29.02[b]	−36.49 ± 0.49	−46.75	−47.75 ± 2.45
Mean ± SE	26 ± 6	1049 ± 190	−26.82 ± 1.61	−26.72 ± 2.02	−36.60 ± 1.73	−37.74 ± 1.58

[a] Below the detection limit of about 10 µM; [b] Single determination from pooled triplicates because of too low acetate concentration

BGD
7, 8619–8661, 2010

Table 4. Diversity indices (species richness *S*, evenness *E*, and Shannon index *H*) for T-RFLP patterns of the archaeal 16S rRNA, bacterial 16S rRNA and *mcrA* genes (mean ± SE).

	archaeal 16S rRNA			bacterial 16S rRNA			*mcrA*		
#	*S*	*E*	*H*	*S*	*E*	*H*	*S*	*E*	*H*
1	8.3±0.3	0.816±0.024	1.728±0.042	23.0±1.2	0.907±0.003	2.841±0.038	10.7±0.3	0.689±0.004	1.629±0.016
2	10.7±1.2	0.757±0.018	1.787±0.126	33.7±0.9	0.915±0.011	3.215±0.062	9.7±0.9	0.685±0.014	1.546±0.034
3	9.0±0.0	0.777±0.009	1.706±0.019	37.0±1.5	0.958±0.001	3.457±0.041	9.7±0.3	0.700±0.004	1.588±0.016
4	6.7±0.7	0.815±0.023	1.535±0.035	26.3±0.3	0.952±0.001	3.113±0.016	10.0±0.0	0.670±0.001	1.542±0.003
5	8.0±2.1	0.778±0.036	1.573±0.299	21.0±1.0	0.892±0.003	2.714±0.052	10.7±0.3	0.792±0.006	1.874±0.038
6	9.7±1.3	0.795±0.001	1.787±0.118	23.3±0.3	0.888±0.008	2.800±0.036	9.0±0.6	0.825±0.016	1.807±0.018
7	9.0±0.0	0.815±0.006	1.791±0.013	18.0±1.0	0.864±0.006	2.494±0.044	11.7±0.9	0.823±0.018	2.015±0.028
8	12.0±0.0	0.820±0.022	2.037±0.055	30.0±1.5	0.937±0.007	3.184±0.070	10.7±0.3	0.838±0.009	1.983±0.046
9	9.3±0.3	0.827±0.010	1.846±0.006	24.7±0.9	0.908±0.001	2.908±0.032	8.7±0.3	0.870±0.008	1.878±0.017
10	10.7±0.3	0.845±0.004	1.999±0.018	38.0±2.9	0.963±0.001	3.495±0.075	8.7±0.3	0.811±0.011	1.749±0.017
11	10.3±0.3	0.851±0.009	1.986±0.008	26.7±5.4	0.913±0.002	2.955±0.217	9.3±0.7	0.816±0.014	1.816±0.032
12	7.0±0.0	0.726±0.004	1.412±0.008	25.0±1.5	0.941±0.004	3.024±0.048	12.7±0.9	0.862±0.010	2.183±0.047
13	8.0±1.0	0.783±0.016	1.612±0.091	33.0±2.1	0.946±0.001	3.302±0.066	12.7±0.3	0.753±0.005	1.910±0.022
14	9.0±0.0	0.828±0.007	1.820±0.015	37.0±2.3	0.937±0.002	3.381±0.065	12.3±0.3	0.815±0.004	2.046±0.013
15	9.7±0.3	0.800±0.007	1.814±0.013	32.3±0.7	0.968±0.001	3.366±0.015	9.7±0.3	0.876±0.001	1.986±0.033
16	8.0±1.0	0.746±0.033	1.546±0.160	27.0±1.5	0.879±0.003	2.895±0.054	10.3±0.9	0.817±0.011	1.899±0.048
Mean ± SE	9.1±0.4	0.799±0.009	1.749±0.045	28.5±1.5	0.923±0.008	3.071±0.072	10.4±0.3	0.790±0.017	1.841±0.047

BGD
7, 8619–8661, 2010

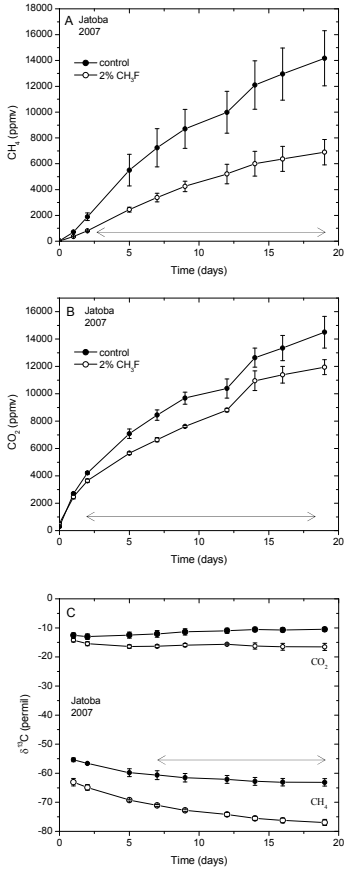

Fig. 1. Production of CH_4 and CO_2 in anaerobically incubated sediment of Lake Jatoba in the presence and absence of 2% methyl fluoride; **(A)** accumulation of CH_4 in the headspace; **(B)** accumulation of CO_2 in the headspace; **(C)** $\delta^{13}C$ of the accumulated CH_4 and CO_2; average ± SE; $n = 3$. The arrows indicate the period used for averaging data for determination of production rates or average $\delta^{13}C$.

BGD
7, 8619–8661, 2010

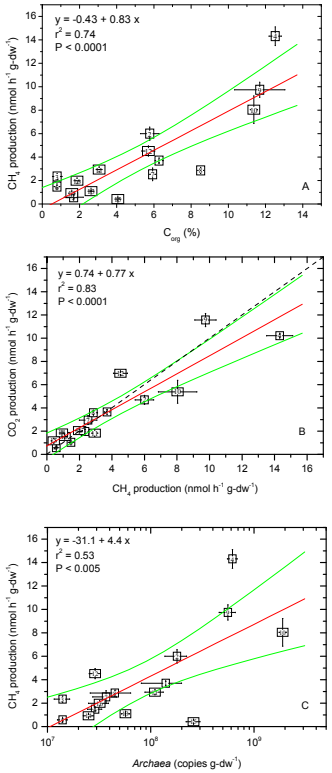

Fig. 2. Linear regression of production rates of CH_4 and CO_2 determined in 16 different lake sediments; **(A)** CH_4 production against the content of C_{org}; **(B)** CO_2 production against CH_4 production; **(C)** CH_4 production against numbers of archaea (as copies of the 16S rRNA gene); numbers of data points refer to the identity of the lake sediment as shown in Table 1; error bars give standard errors of n=3; the result of linear regression is given by a thin straight line with small-dotted curved lines as 95% confidence limit; the dashed straight line in (B) indicates the 1:1 proportionality line between the two production rates.

BGD
7, 8619–8661, 2010

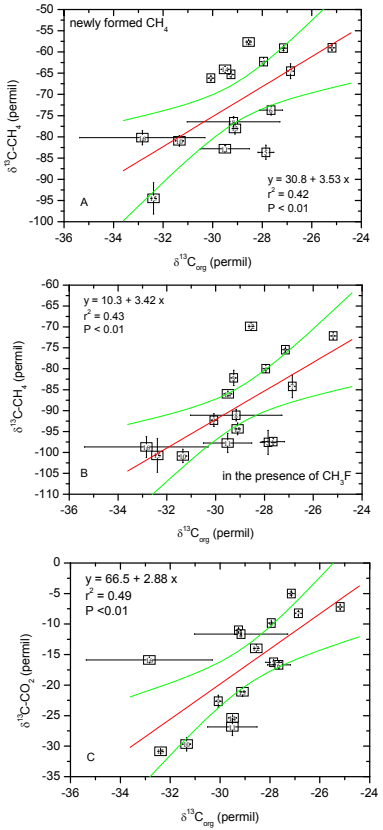

Fig. 3. Linear regression of the $\delta^{13}C$ of CH_4 and CO_2 against the $\delta^{13}C$ of sediment organic matter determined in 16 different lake sediments; **(A)** average $\delta^{13}C$ of newly formed CH_4 in the absence of CH_3F; **(B)** average $\delta^{13}C$ of newly formed CH_4 in the presence of CH_3F (= $\delta^{13}C_{CH4\text{-}mc}$); **(C)** average $\delta^{13}C$ of gaseous CO_2; other explanations see Fig. 2.

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7, 8619–8661, 2010

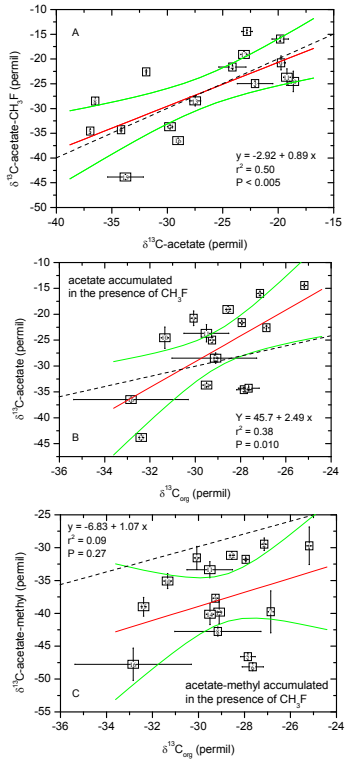

Fig. 4. Linear regression of the $\delta^{13}C$ of total acetate and acetate-methyl against the $\delta^{13}C$ of sediment organic matter determined in 16 different lake sediments; **(A)** average $\delta^{13}C$ of total acetate formed in the presence of CH_3F against that formed in the absence of CH_3F; **(B)** average $\delta^{13}C$ of total acetate formed in the presence of CH_3F against $\delta^{13}C$ of sediment organic carbon; **(C)** average $\delta^{13}C$ of acetate-methyl formed in the presence of CH_3F against $\delta^{13}C$ of sediment organic carbon; other explanations see Fig. 2.

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7, 8619–8661, 2010

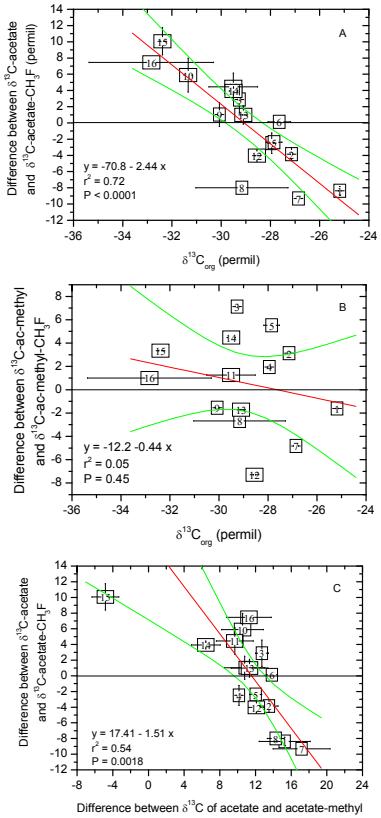

Fig. 5. Linear regression of the difference between the $\delta^{13}C$ of acetate or acetate-methyl measured in the absence and presence of CH_3F (Difference = $\delta^{13}C_{ac} - \delta^{13}C_{ac\text{-}CH3F}$) in 16 different lake sediments; **(A)** regression against the $\delta^{13}C$ of sediment organic matter using $\delta^{13}C$ of total acetate; **(B)** regression against the $\delta^{13}C$ of sediment organic matter using $\delta^{13}C$ of acetate-methyl; **(C)** regression against the acetate-intramolecular difference of $\delta^{13}C$ (Difference = $\delta^{13}C_{ac} - \delta^{13}C_{ac\text{-}methyl}$); other explanations see Fig. 2.

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7, 8619–8661, 2010

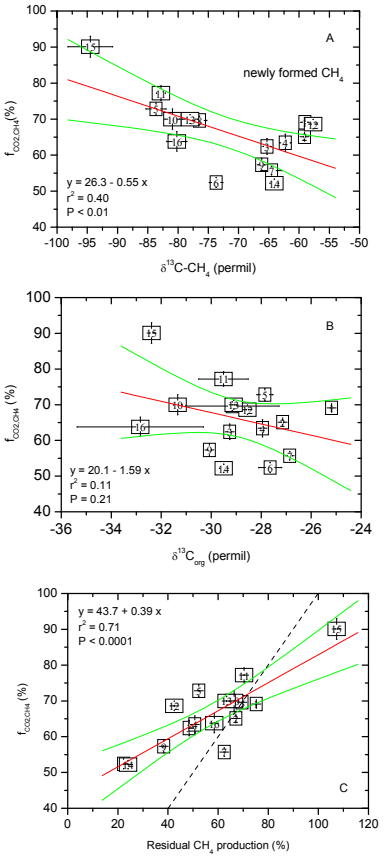

Fig. 6. Linear regression of the percent fraction ($f_{CO2,CH4}$) of hydrogenotrophic methanogenesis against different parameters determined in 16 different lake sediments; **(A)** against the average $\delta^{13}C$ of newly formed CH_4 in the absence of CH_3F; **(B)** against the $\delta^{13}C$ of sediment organic matter; **(C)** against the residual rate (percent of the uninhibited rate) of CH_4 production measured in the presence of CH_3F; other explanations see Fig. 2.

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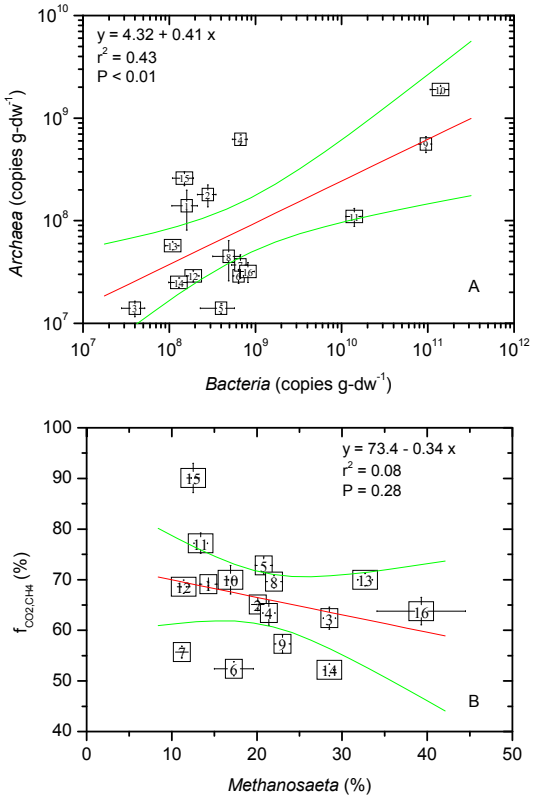

Fig. 7. Linear regression of **(A)** numbers of archaea against numbers of bacteria in the sediment (given as copies of the 16S rRNA gene); **(B)** the percent fraction ($f_{CO2,CH4}$) of hydrogenotrophic methanogenesis against the relative (percent) abundance of *Methanosaeta* spp. in the community of archaea determined in 16 different lake sediments; other explanations see Fig. 2.

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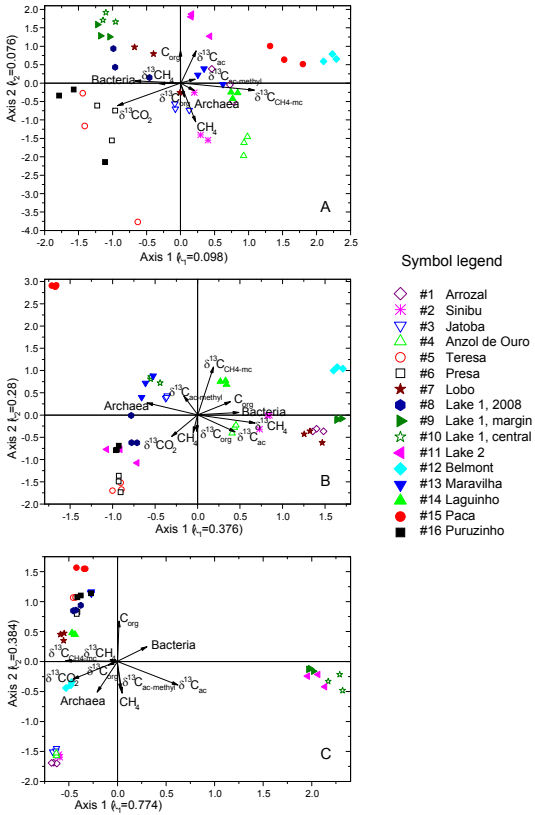

Fig. 8. Canonical correspondence analysis ordination plot for the effect of C_{org}, $\delta^{13}C_{org}$, CH_4 production, abundance of archaea and bacteria, $\delta^{13}C_{CH4}$, $\delta^{13}C_{CO2}$, $\delta^{13}C_{CH4\text{-}mc}$, $\delta^{13}C_{ac}$, and $\delta^{13}C_{ac\text{-}methyl}$ on the composition of the **(A)** archaeal 16S rRNA, **(B)** bacterial 16S rRNA, and **(C)** *mcrA* gene community in lake sediments, based on the relative abundances of respective T-RFs from the sediments of three replicate measurements. Symbol legend and eigenvalues of the first and second axes are included.

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