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Stable isotope fractionation during the methanogenic degradation of organic matter in the sediment of an acidic bog lake, Lake Grosse Fuchskuhle

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Abstract

Lake Grosse Fuchskuhle is an acidic bog lake, which develops an anoxic hypolimnion during the summer season, so that sediment organic matter is degraded anaerobically to CH_4 and CO_2. The $\delta^{13}C$ values of organic matter and its degradation products were used for determination of the mass balance, degradation path, and fractionation factors. Addition of methyl fluoride, an inhibitor of acetoclastic methanogenesis, increasingly inhibited CH_4 production and resulted in accumulation of acetate (and a little propionate), which then was an additional end product of organic matter degradation. The $\delta^{13}C$ of acetate-methyl group was slightly lower than that of organic matter, indicating a small fractionation (about 4‰) during the fermentative production of acetate. Chemolithotrophic acetogenesis was low (< 9%). The fraction of CH_4 produced from hydrogenotrophic methanogenesis was about 55–67%. After incubation for 20 h the average $\delta^{13}C$ of the degradation products was similar to that of sediment organic matter, both in the absence and the presence of CH_3F. The produced total inorganic carbon (TIC) was further converted to CH_4 with apparent enrichment factors of 56–67‰. The fraction of TIC that was actually converted to CH_4 was calculated to be about 33% and 23% in the absence and presence of CH_3F, respectively. These data are consistent with the assumption that about half of the organic matter was incompletely degraded, resulting in the production of only CO_2 and H_2, afterward converted to CH_4. The data further show that the reduction of CO_2 to CH_4 was partially inhibited in the presence of CH_3F.

In anoxic sediments of freshwater lakes organic matter is mainly degraded to methane and carbon dioxide (Rudd and Taylor 1980). The degradation is accomplished by a complex microbial community consisting of hydrolytic, fermenting, acetogenic, and methanogenic microorganisms (Zinder 1993). Methanogenic archaea use in the final degradation step either acetate or $H_2 + CO_2$, which in turn are produced by fermenting bacteria. The complete mineralization of organic matter such as polysaccharides to CH_4 and CO_2 theoretically proceeds by about two-thirds acetoclastic and one-third hydrogenotrophic methanogenesis. The initial hydrolysis of organic polymers is regarded as the rate-limiting step in the degradation process (Billen 1982). The availability of degradable organic material is one of the key factors controlling the activity of the microbial community so that production of CH_4 and CO_2 increases upon input of fresh algal biomass (Sander and Kalff 1993; Schwarz et al. 2008). However, organic matter is apparently not completely degraded, and residual substances remain. Recently we reported that the contribution of hydrogenotrophic methanogenesis increased with sediment depth from 35% to 60%, indicating that organic matter was incompletely and selectively oxidized in deeper sediment layers, thus favoring hydrogenotrophic over acetoclastic methanogenesis (Conrad et al. 2009).

Acetate and CO_2 are the two most important early products of fermentation of organic matter, which are subsequently further converted to CH_4 and CO_2. Whereas carbon isotope fractionation during the methanogenic process has frequently been studied, little is known about whether and to what extent fractionation occurs during the conversion of organic matter to acetate and CO_2. Acetate formation in bacterial cultures during fermentation of polysaccharides and sugars apparently exhibits only a small fractionation factor (Blair et al. 1985; Penning and Conrad 2006), while acetate formation during chemolithotrophic acetogenesis from H_2 plus CO_2 exhibits a large fractionation factor (Gelwicks et al. 1989). Production of CO_2 from organic matter may or may not exhibit fractionation. Thus, it was assumed that the CO_2 produced in anoxic marine sediments had the same $\delta^{13}C$ as organic matter (Blair 1998). On the other hand, the $\delta^{13}C$ of the CO_2 produced in farm soil was found to be about 5‰ smaller than that of soil organic matter (Werth and Kuzyakov 2008).

In order to better understand the degradation process of organic matter under anaerobic conditions, we decided to study the balance of ^{13}C in the degradation products compared to the sediment organic carbon and determine the path of CH_4 production from isotopic mass balance and isotopic fractionation. In contrast to CH_4, which is poorly soluble, CO_2 is not only found in the gas phase but also in aqueous form. However, the isotope fractionation for this equilibrium is only small. In addition, CO_2 equilibrates with bicarbonate exhibiting a relatively large fractionation factor. At neutral pH bicarbonate contributes significantly to the overall balance of $\delta^{13}C$, and even small changes in pH may have dramatic effects. Therefore, we decided to study the degradation process in an acidic sediment, where bicarbonate concentrations are low. We chose the sediment of Lake Grosse Fuchskuhle, an acidic bog lake in Northern Germany (Chan et al. 2002; Casper et al. 2003). A second objective was to quantify the path of CH_4 formation in an acidic environment from isotopic mass balance and isotopic fractionation.

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Methods

Sampling procedure—Dystrophic Lake Grosse Fuchskuhle is a 0.015-km^2 acidic bog lake located in Northern Brandenburg, Germany. The main characteristics of the lake and the microbial methanogenic community in the sediment have been described (Chan et al. 2002; Casper et al. 2003). The lake was artificially divided into four compartments in 1987 and finally in 1991, resulting in four nearly equal sized compartments, each with a different catchment area. In the present study we used the southwest basin, which is subject to allochthonic, mainly humic input. Several sediment cores (diameter 7.0 cm) were taken using a Jenkin sediment sampler (Ohnstad and Jones 1982) each in July 2006 and June 2009. At this time, the basin had developed an anoxic hypolimnion, and the in situ sediment temperatures were 9.1°C (July 2006) and 7.2°C (June 2009), respectively. The chemical and isotopic composition of organic matter in the sediment is most probably not homogenous in situ. For a representative sample, the upper 0–10 cm of the sediment cores were pooled, mixed, and stored at 4°C under a headspace of N_2. The samples were processed within 2 weeks. The pH of the sediment was measured with a glass electrode. Aliquots were used to analyze organic carbon content and its $\delta^{13}C$.

Incubation experiments—Incubation experiments were done as previously described (Conrad et al. 2009) using either sterile serum bottles (60 mL) or glass pressure tubes (27 mL). The vessels were filled with about 17 or 7 mL, respectively, of fresh sediment, flushed with N_2, and closed with butyl rubber stoppers and incubated overnight at 10°C. Then, the vessels were flushed again with N_2 and further incubated at 10°C. Although incubation temperature was close to in situ conditions, it cannot be excluded that the sampling and storage procedure had affected the microbial activity of the sediment samples. However, the effect of different treatments and temporal behavior should not be biased. The gas headspace of some of the vessels was supplemented with methyl fluoride (CH_3F) (Fluorochrome company) at concentrations between 0.5% and 3%. The incubations, which were kept without shaking in darkness, were either analyzed after an incubation time of 20 d (using the samples from July 2006 incubated in glass bottles) or were repeatedly analyzed over a total incubation period of 23 d (using the samples from June 2009 incubated in pressure tubes). The former was done by setting up triplicate incubations. The latter was done by setting up multiple tubes of which triplicates were sacrificed at each time point.

For analysis of gas samples, the vessels were heavily shaken by hand for about 30 s to equilibrate liquid and gas phase. Then gas samples were taken from the headspace and analyzed for CH_4 and CO_2 concentrations (gas chromatography [GC]) as well as $\delta^{13}C$ of CH_4 and CO_2 using gas chromatograph combustion isotope ratio mass spectrometry (GC-C-IRMS). Some gas samples were also analyzed for H_2 concentrations. Liquid samples were taken and analyzed for concentration using high-pressure liquid chromatography (HPLC) and $\delta^{13}C$ of acetate and propionate (HPLC-C-IRMS). Other fatty acids (e.g., butyrate) were not detected (< 20 μmol L^{-1}). The dry weight (dry wt) of the sediment was determined gravimetrically.

The analytical techniques (GC, HPLC) for measurement of CH_4, CO_2, H_2, and acetate as well as of the $\delta^{13}C$ (GC-C-IRMS, HPLC-C-IRMS) were the same as used before (Conrad et al. 2009). The determination of $\delta^{13}C$ in the methyl group of acetate by off-line pyrolysis followed by GC-C-IRMS has also been described (Conrad et al. 2009). The $\delta^{13}C$ in organic matter was analyzed at the Institute for Soil Science and Forest Nutrition (IBW) at the University of Göttingen, Germany, using an elemental analyzer coupled to mass spectrometer.

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

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

also expressed as isotopic enrichment factor $\varepsilon \equiv 1 - \alpha$ (in units of permil).

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

$$f_{CO_2CH_4} = \left(\delta^{13}C_{CH_4} - \delta^{13}C_{CH_4\text{-ac}}\right) / \left(\delta^{13}C_{CH_4\text{-}CO_2} - \delta^{13}C_{CH_4\text{-ac}}\right) \quad (2)$$

where f_{CO_2,CH_4} is the fraction of CH_4 formed from $H_2 + CO_2$, $\delta^{13}C_{CH_4}$ is the $\delta^{13}C$ of total produced methane, and $\delta^{13}C_{CH_4\text{-ac}}$ and $\delta^{13}C_{CH_4\text{-}CO_2}$ are the $\delta^{13}C$ of CH_4 derived either from acetate or $H_2 + CO_2$, which were determined by

$$\delta^{13}C_{CH_4\text{-ac}} = \delta^{13}C_{\text{ac-methyl}} \quad (3)$$

$$\delta^{13}C_{CH_4\text{-}CO_2} = \delta^{13}C_{CH_4\text{-}CH_3F} \quad (4)$$

where $\delta^{13}C_{\text{ac-methyl}}$ is the $\delta^{13}C$ of the methyl group of acetate accumulated, and $\delta^{13}C_{CH_4\text{-}CH_3F}$ is the $\delta^{13}C$ of CH_4 produced in the presence of CH_3F (i.e., with acetotrophic methanogenesis inhibited). For $\delta^{13}C_{CH_4}$ and $\delta^{13}C_{CH_4\text{-}CH_3F}$ the $\delta^{13}C$ for a newly formed CH_4 ($\delta^{13}C_{new}$) was used, which 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:

$$\delta^{13}C_2 = f_{new}\delta^{13}C_{new} + (1 - f_{new})\delta^{13}C_1 \quad (5)$$

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

Analogously, the relative contribution of $H_2 + CO_2$–derived acetate (chemolithotrophic acetate production) and organic matter–derived acetate (fermentative acetate production) to total acetate production was determined from the following mass balance:

$$f_{CO_2,ac} = \left(\delta^{13}C_{\text{ac-methyl}} - \delta^{13}C_{\text{ac-org}}\right) / \left(\delta^{13}C_{\text{ac-}CO_2} - \delta^{13}C_{\text{ac-org}}\right) \quad (6)$$

where $f_{CO_2,ac}$ is the fraction of acetate-methyl formed from $H_2 + CO_2$, $\delta^{13}C_{ac\text{-}methyl}$ is the $\delta^{13}C$ of total produced acetate-methyl, and $\delta^{13}C_{ac\text{-}org}$ and $\delta^{13}C_{ac\text{-}CO_2}$ are the $\delta^{13}C$ of acetate-methyl derived either from organic matter or $H_2 + CO_2$. For this calculation the $\delta^{13}C$ of accumulated acetate was used.

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 CH_4 dissolved in the liquid were less than 3% of the total and were neglected. The amounts of CO_2(aq) dissolved in the liquid were calculated from the solubility constant of CO_2 ($0.987 \times 10^{-1.27}$ mol L^{-1} bar^{-1}), those of bicarbonate (HCO_3^-) from the solubility constant of CO_2, the pH (6.0), and the dissociation constant ($10^{-6.464}$) of bicarbonate (Stumm and Morgan 1981). Total inorganic carbon (TIC) was defined as the sum of bicarbonate, gaseous, and dissolved CO_2. The $\delta^{13}C$ of dissolved CO_2 ($\alpha_{CO_2(aq)} = 0.9990$) and bicarbonate ($\alpha_{HCO_3} = 1.0092$) were calculated from the $\delta^{13}C$ of gaseous CO_2 and the corresponding fractionation factors α (Stumm and Morgan 1981), which are

$$\alpha_{CO_2(aq)} = \left(\delta^{13}C_{CO_2(aq)} + 10^3\right) / \left(\delta^{13}C_{CO_2(g)} + 10^3\right) \quad (7)$$

$$\alpha_{HCO_3} = \left(\delta^{13}C_{HCO_3} + 10^3\right) / \left(\delta^{13}C_{CO_2(g)} + 10^3\right) \quad (8)$$

The values of $\delta^{13}C_{CO_2(g)}$, $\delta^{13}C_{CO_2(aq)}$, and $\delta^{13}C_{HCO_3}$ were used to calculate $\delta^{13}C_{TIC}$ using the mole fractions of the different CO_2 species (Penning and Conrad 2006).

The average $\delta^{13}C$ ($\delta^{13}C_{av}$) of all products (x_i) was calculated by

$$\delta^{13}C_{av} = [1/(x_1 + x_2 + \ldots + x_n)] \left(x_1\delta^{13}C_{x1} + x_2\delta^{13}C_{x2} + \ldots + x_n\delta^{13}C_{xn}\right) \quad (9)$$

and used as computed equivalent to $\delta^{13}C_{org}$, from which the products eventually were formed. Values of the individual $\delta^{13}C_{xi}$ and x_i were all in terms of carbon atoms.

The fraction (f_{TIC,CH_4}) of TIC used for formation of CH_4 was calculated by assuming isotope fractionation in an open system, in which the input substrate is converted to a product plus residual substrate (Fry 2006) (Fig. 1)

$$f_{TIC,CH_4} = \left(\delta^{13}C_{TIC} - \delta^{13}C_{org}\right) / \varepsilon_{TIC,CH_4} \quad (10)$$

assuming that the $\delta^{13}C_{TIC}$ is that of the residual TIC that did not react to CH_4, while the TIC serving as input substrate has a priori the same $\delta^{13}C$ as organic matter. The value of $\delta^{13}C_{org}$ in Eq. 10 was assumed to be the measured value (−29.6‰) or to be equal to $\delta^{13}C_{av}$ calculated in Eq. 9. For $\delta^{13}C_{TIC}$ and $\delta^{13}C_{CH_4}$, the values measured at the end of incubation (Fig. 1) or between day 12 and day 23 of the incubation (Fig. 2) were used. Since CH_4 is exclusively formed from TIC when acetoclastic methanogenesis is inhibited by CH_3F, the isotopic enrichment factor can be

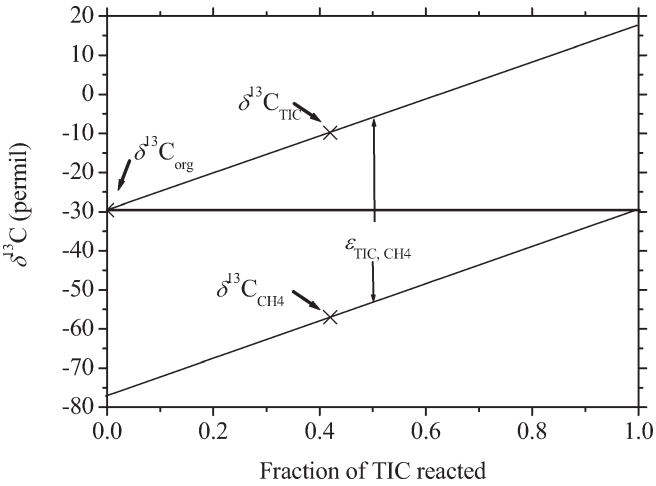

Fig. 1. Steady state fractionation during conversion of TIC to CH_4, showing the linear increase of $\delta^{13}C_{TIC}$ and $\delta^{13}C_{CH_4}$ as function of the fraction of the initial TIC converted to CH_4. It is assumed that the initial $\delta^{13}C_{TIC}$ is equal to $\delta^{13}C_{org}$.

calculated by

$$\varepsilon_{TIC/CH_4} = \delta^{13}C_{TIC} - \delta^{13}C_{CH_4\text{-}CH_3F} \quad (11)$$

Gibbs free energies (ΔG) of production of CH_4 and acetate were calculated from the actual concentrations of reactants and products and the standard Gibbs free energies using the following reaction equation:

$$4H_2(g) + CO_2(g) \rightarrow CH_4(g) + 2H_2O(l) \quad \Delta G^0 = -130.7 \text{ kJ} \quad (12)$$

$$4H_2(g) + 2CO_2(g) \rightarrow CH_3COO^-(aq) + H^+(aq) + 2H_2O(l) \Delta G^0 = -55.1 \text{ kJ} \quad (13)$$

Values of ΔG^0 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 at the incubation conditions were calculated from the ΔG^0 and the actual partial pressures of CH_4, CO_2, and H_2 and the actual concentrations of acetate and H^+ (pH 6.0) using the Nernst equation.

Results

Effect of different inhibitor concentrations—The sediment of Lake Grosse Fuchskuhle, SW basin had the following characteristics: water content, 95%; pH, 5.9 ± 0.1; organic carbon, 43–44% of dry matter; $\delta^{13}C$ of organic carbon ($\delta^{13}C_{org}$), −29.6‰ ± 0.1‰.

Sediment samples were anoxically incubated for 20 d at 10°C (in situ temperature) in the presence and absence of methyl fluoride (CH_3F), an inhibitor of acetoclastic methanogenesis. Incubation of sediment in the presence of methyl fluoride resulted in partial inhibition of CH_4 production and in accumulation of acetate. The accumulated gaseous and dissolved products of anaerobic degra-

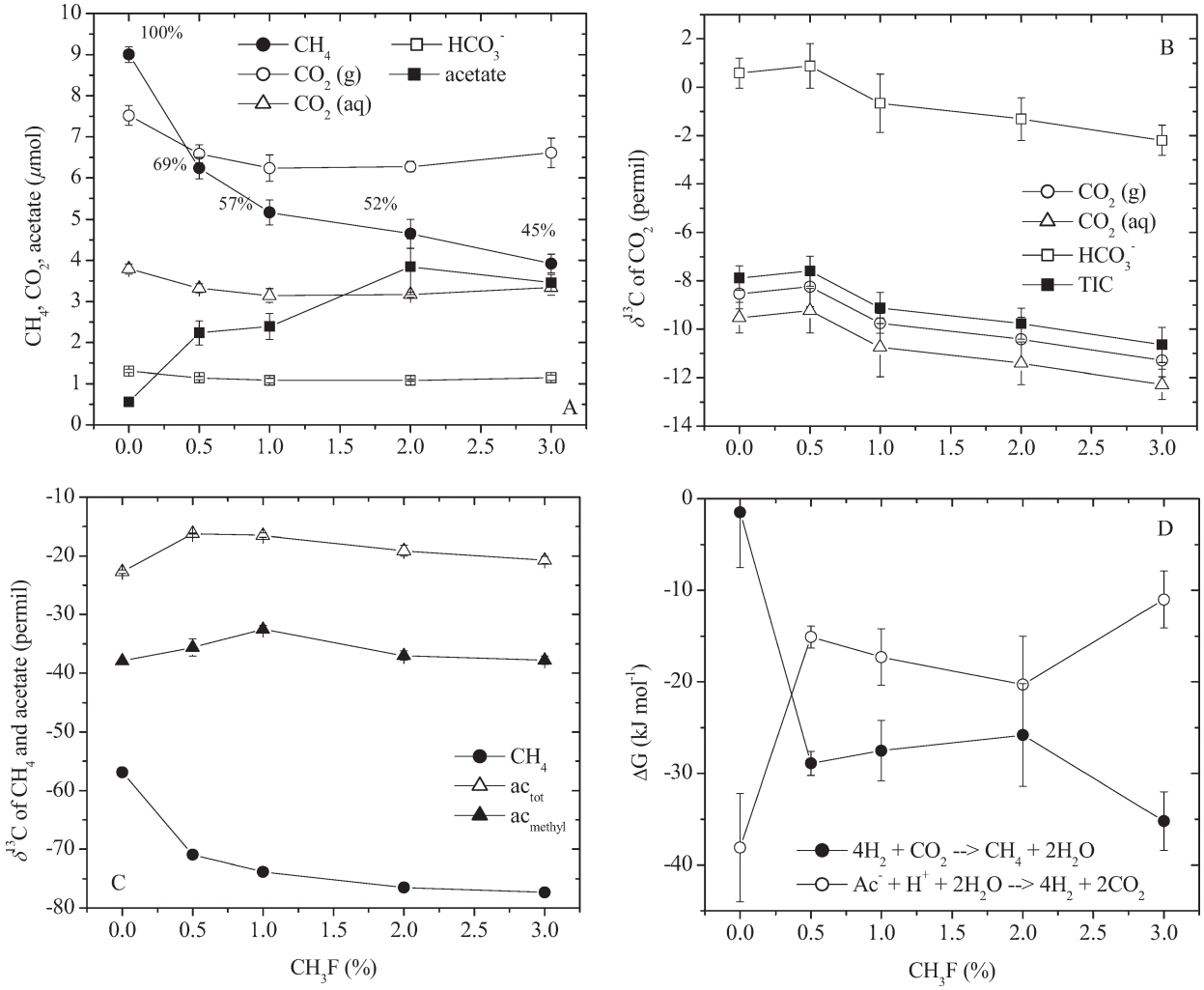

Fig. 2. Incubation of sediment sampled from Lake Grosse Fuchskuhle in July 2006 for 20 d under anaerobic conditions in the presence of different CH_3F concentrations (0–3%), showing (A) final amounts of CH_4, CO_2(g), CO_2(aq), bicarbonate, and acetate. The percentage values indicate the residual CH_4 production due to inhibition by increasing CH_3F concentrations; (B) $\delta^{13}C$ values of CO_2(g), CO_2(aq), bicarbonate, and TIC; (C) $\delta^{13}C$ values of CH_4, total acetate, and acetate-methyl; (D) Gibbs free energies of hydrogenotrophic methanogenesis and acetate oxidation (i.e., the reverse of chemolithotrophic acetogenesis); mean ± SE, $n = 3$.

dation of organic matter were analyzed. The extent of inhibition increased with increasing CH_3F concentration leveling off at about 2–3% CH_3F (Fig. 2A). The molar amount of accumulated acetate was less than that of the CH_4, which was not produced due to inhibition; e.g., 3.5 μmol acetate accumulated at 2% CH_3F, but 4.5 μmol CH_4 had not been produced (Fig. 2A). On the average, 60% ± 6% of the lacking amount of CH_4 accumulated as acetate. Small amounts of propionate also seemed to accumulate at 2% and 3% CH_3F, but the final concentrations were always lower than 30 μmol L^{-1}, which is only slightly above the detection limit (about 20 μmol L^{-1}).

Production of CO_2 was also inhibited but to a smaller extent than CH_4 production (Fig. 2A). Bicarbonate was less than 10% of TIC and thus contributed little to the $\delta^{13}C$ of TIC, although the $\delta^{13}C$ bicarbonate was much higher than that in CO_2(g) and CO_2(aq) (Fig. 2B). In general, $\delta^{13}C$ of the CO_2 species decreased slightly (ca. 2‰) with increasing CH_3F concentrations (Fig. 2B). However, $\delta^{13}C$ of the produced CH_4 decreased much more (up to 20‰) with increasing CH_3F concentrations (Fig. 2C). The $\delta^{13}C$ of the acetate, on the other hand, changed only little, but $\delta^{13}C$ in the methyl group of acetate was generally by about 15‰ lower than in total acetate (Fig. 2C). The $\delta^{13}C$ of propionate was between −27‰ and −25‰ (data not shown).

Addition of CH_3F also resulted in increased H_2 partial pressures, so that the ΔG of hydrogenotrophic methanogenesis decreased to about −30 kJ mol^{-1} CH_4 (Fig. 2D). The ΔG of acetate oxidation to H_2 + CO_2 was generally negative (Fig. 2D), while the reverse reaction, the hydrogenotrophic acetogenesis, decreased in the presence of CH_3F, but was generally in the endergonic range.

Temporal change—The incubation experiment was repeated using newly sampled sediment, applying a CH_3F concentration of 2%, and analyzing CH_4, CO_2, and acetate over a time period of 23 d. Methane was produced instantaneously with a constant rate (Fig. 3A,B). The

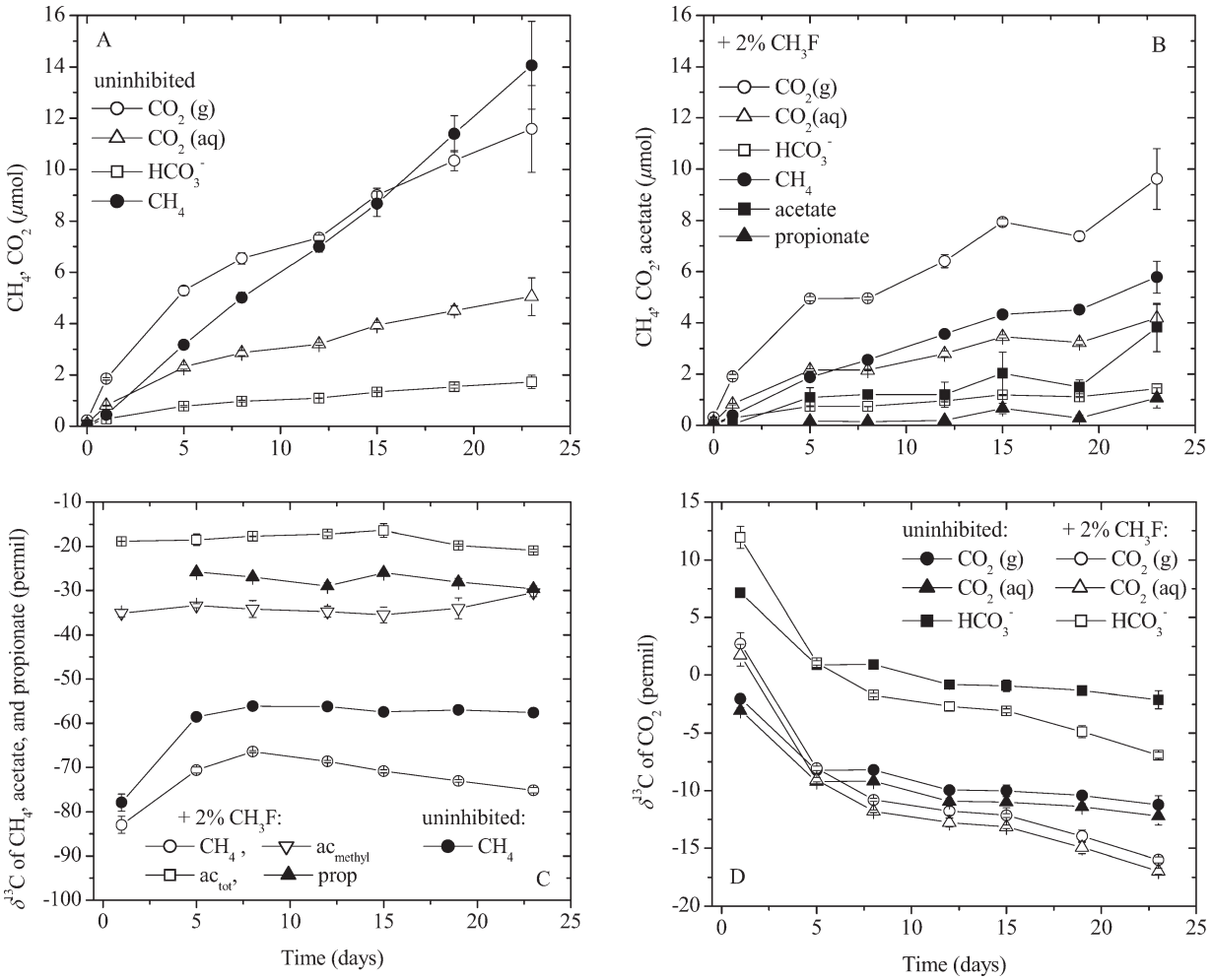

Fig. 3. Incubation of sediment sampled from Lake Grosse Fuchskuhle in June 2009 in the absence and presence of 2% CH_3F, showing the temporal change of (A) the amounts of CH_4, CO_2(g), CO_2(aq), and bicarbonate in the absence of CH_3F (uninhibited); (B) the amounts of CH_4, CO_2(g), CO_2(aq), bicarbonate, acetate, and propionate in the presence of CH_3F (inhibited); (C) $\delta^{13}C$ values of CH_4, total acetate, acetate-methyl, and total propionate in the presence and absence of CH_3F; (D) $\delta^{13}C$ values of CO_2(g), CO_2(aq), bicarbonate, and TIC in the presence and absence of CH_3F; mean ± SE, $n = 3$.

CH_4 production rates in the absence and presence of 2% CH_3F were 141 ± 27 and 40 ± 10 nmol h^{-1}g dry wt^{-1}, respectively. The residual activity in the inhibited sample was 28% ± 7%. The production rates of TIC, determined between day 12 and day 23, were almost the same as those of CH_4 production. Production rates of TIC were 130 ± 44 and 84 ± 30 nmol h^{-1}g dry wt^{-1} in the absence and presence of 2% CH_3F, respectively. The residual activity in the inhibited sample was 64% ± 23%. However, during the initial phase of incubation (0–8 d) production rates of TIC were higher, being about 1.9 times those of CH_4 production (Fig. 3A,B). Acetate accumulated in the presence of CH_3F, amounting to about 49% ± 8% of the CH_4 that was not produced. Propionate also accumulated, but only to a minor extent (Fig. 3B).

The $\delta^{13}C$ of the accumulated CH_4 ($\delta^{13}C_{CH_4}$) increased between day 1 and day 5 but then exhibited a constant value of −57.0‰ ± 0.3‰ (average data are generally given for the period 12–23 d) (Fig. 3C). The $\delta^{13}C_{CH_4}$ in the presence of CH_3F was more negative than without inhibitor. However, it decreased from −66.4‰ at day 8 to −75.1‰ at day 23 (Fig. 3C). The average value was −71.9‰ ± 1.4‰. The $\delta^{13}C$ of the acetate, which accumulated in the presence of 2% CH_3F, was constant at −18.5‰ ± 1.0‰. The $\delta^{13}C$ of the methyl group of acetate was by about 15‰ more negative, i.e., −33.7‰ ± 1.0‰ (Fig. 3C). The $\delta^{13}C$ of the carboxyl group of acetate is calculated to a value of −3.4‰ ± 3.1‰. The $\delta^{13}C$ of total propionate was −28.1‰ ± 0.8‰ (Fig. 3C).

The $\delta^{13}C$ of the accumulated CO_2(g) decreased from day 1 to day 5, but reached a relatively constant value of −10.4‰ ± 0.3‰ between days 12 and 23 (Fig. 3D). The $\delta^{13}C$ of the CO_2(aq) was only slightly more negative, but that of the HCO_3^- was by about 9‰ more positive (Fig. 3D). The pattern for the $\delta^{13}C$ of the different inorganic C species in the presence of CH_3F was similar to that in the absence, but did not reach a constant value, instead continually decreasing with incubation time (Fig. 3D).

Path of CH_4 production—The path of CH_4 production was calculated from the $\delta^{13}C$ of acetate-methyl and CH_4 formed in the presence of CH_3F (Eqs. 2–4). The calculation assumed that acetate-methyl was converted to CH_4 without further fractionation, since acetoclastic methanogenesis was limited by the availability of acetate, the concentration of which was close to the detection limit. It was further assumed that the CH_4 formed in the presence of CH_3F was exclusively due to hydrogenotrophic methanogenesis. The fractions (f_{CO_2,CH_4}) of CH_4 produced from hydrogenotrophic methanogenesis in the first experiment (Fig. 2) were 78% ± 3%, 71% ± 3%, 67% ± 3%, and 64% ± 3% at 0.5%, 1%, 2%, and 3% CH_3F, respectively. The value of f_{CO_2,CH_4} in the second experiment (Fig. 3) was 55% ± 1% when determined for day 12 to day 23. During the first 8 d, however, f_{CO_2,CH_4} was about 75%.

The fraction of chemolithotrophic acetogenesis, which was calculated using Eq. 6, was only very small ($f_{CO_2,ac} \approx$ 2–9%).

Balance of ^{13}C in products vs. organic C—The amounts of the different carbon species, which accumulated until the end of incubation, and their $\delta^{13}C$ were used for calculating the average $\delta^{13}C$ in all the measured degradation products of organic matter. These degradation products consisted of CH_4 and TIC in the absence of inhibitor and of CH_4, TIC, and acetate in the presence of inhibitor (the small amounts of accumulated propionate had no influence on the balance). In the first experiment, the average $\delta^{13}C$ of the degradation products was only by about 1.3‰ more positive than that of sediment organic matter (Fig. 4A). In the presence of CH_3F, this difference finally increased to 3.6‰ (Fig. 4A). In the second experiment, the average $\delta^{13}C$ of the degradation products finally also reached values that were similar to the $\delta^{13}C$ of the sediment organic matter, independent of the accounting of the products in the presence or the absence of 2% CH_3F (Fig. 4B). Again, the average $\delta^{13}C$ of the degradation products was by about 2‰ less negative in the presence than in the absence of CH_3F. In the beginning of the incubation the average $\delta^{13}C$ of the degradation products was less negative than that of sediment organic matter, but progressively became similar (Fig. 4B).

Residual total inorganic carbon—Assuming that production and consumption of TIC during the degradation of organic matter was in steady state (Fig. 1), the fraction of TIC (f_{TIC,CH_4}) that was converted into CH_4 can be calculated by knowing the $\delta^{13}C$ of the input-TIC, the $\delta^{13}C$ of the actual TIC measured, and the fractionation factor (ε_{TIC,CH_4}) for the conversion of TIC to CH_4. The value of ε_{TIC,CH_4} was calculated from the $\delta^{13}C_{TIC}$ and the $\delta^{13}C_{CH_4\text{-}CH_3F}$. In the first experiments the values at the end of incubation were between 63‰ and 67‰ at CH_3F concentrations ranging between 0.5% and 3.0% (Fig. 5A). In the absence of CH_3F, however, ε_{TIC,CH_4} was only 49‰ ± 0.5‰ (Fig. 5A). In the second experiment, ε_{TIC,CH_4} decreased with incubation time but finally reached relatively constant values between 56‰ and 60‰ in the presence of 2% CH_3F (Fig. 5B). The $\delta^{13}C$ of input-TIC was assumed to be the same as that of organic matter (Fig. 4). The values of f_{TIC,CH_4} in the absence of CH_3F varied within a range of 33–39% (Fig. 5B). In the presence of 2% CH_3F, f_{TIC,CH_4} decreased with incubation time from 37% to 23% (Fig. 5B) and also decreased with CH_3F concentration from 35% at 0.5% CH_3F to 27% at 3% CH_3F (Fig. 5A).

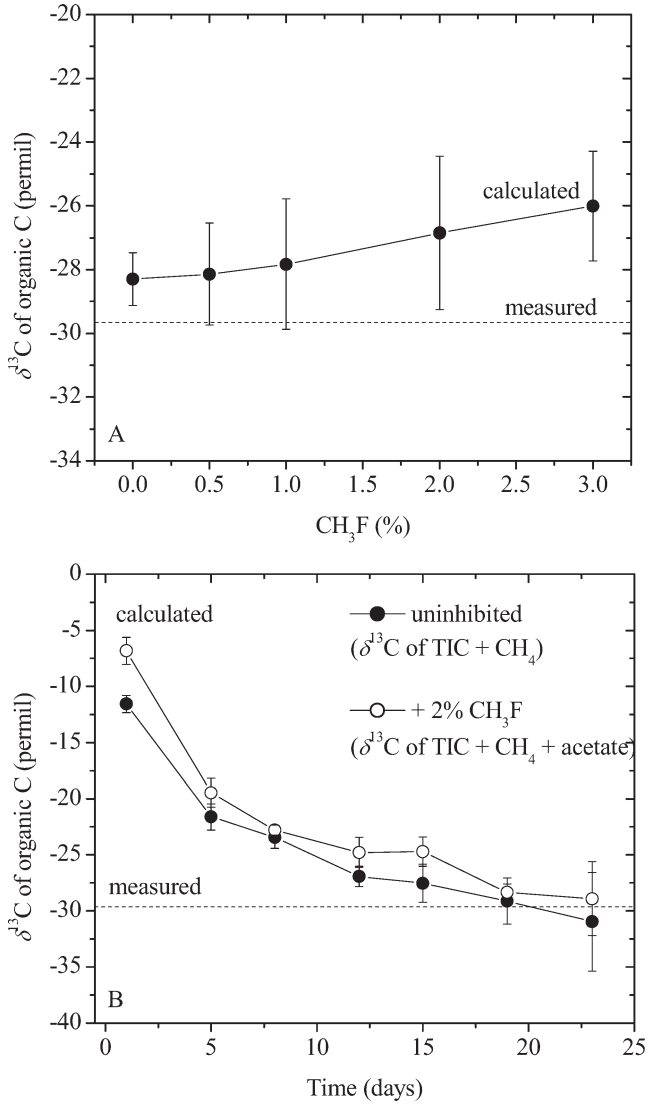

Fig. 4. Comparison of the average $\delta^{13}C$ of all degradation products of organic matter (calculated $\delta^{13}C_{org}$) with the actually measured $\delta^{13}C$ value of organic matter as function of (A) CH_3F concentration using sediment sampled in July 2006 and (B) incubation time in the absence and presence of 2% CH_3F using sediment sampled in June 2009; mean ± SE, $n = 3$.

Discussion

Anaerobic degradation and effect of methyl fluoride—Lake Grosse Fuchskuhle is a dystrophic bog lake and the sediment is rich in organic matter. Owing to the acidic pH, the sediment does not contain carbonates and the CO_2 is exclusively produced by the degradation of organic matter.

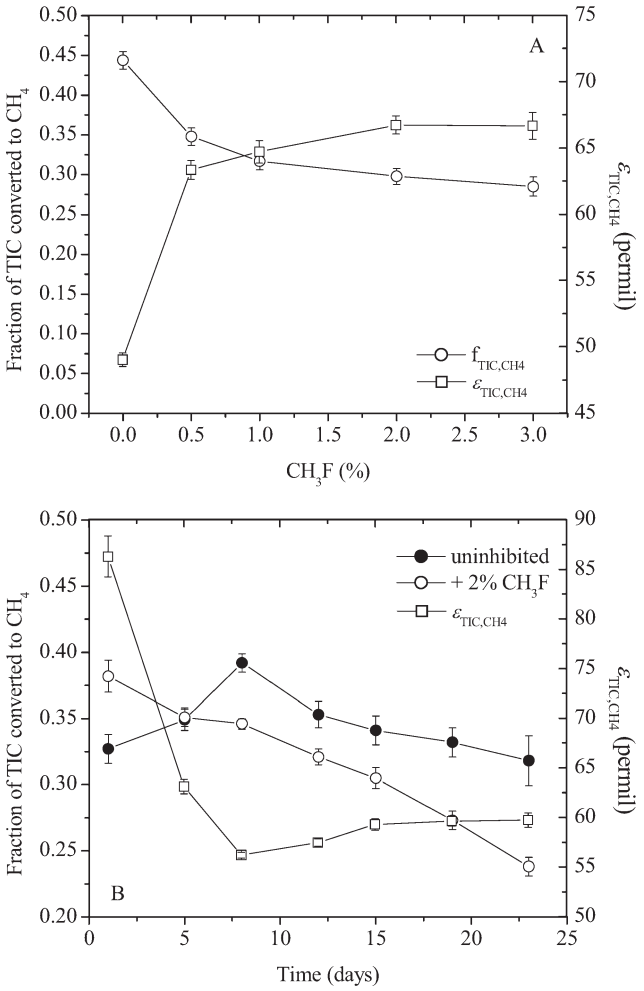

Fig. 5. Apparent isotopic enrichment factors (ε_{TIC,CH_4}) for the conversion of TIC to CH_4 in the absence of acetoclastic methanogenesis and fractions (f_{TIC,CH_4}) of TIC converted to CH_4. The values were determined (A) at the end of incubation of sediment in the presence of different CH_3F concentrations, and (B) during the incubation of sediment in the presence and absence of 2% CH_3F; mean ± SE, $n = 3$.

Incubation of sediment under anoxic conditions resulted in the immediate production of CH_4 and CO_2. The production rates of TIC first were almost two times larger than those of CH_4, but eventually both rates became equal. Equal rates of CH_4 and TIC production are consistent with the stepwise and complete degradation of organic compounds (e.g., polysaccharides) via fermentation to acetate, CO_2, and H_2 (Zinder 1993):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (14)$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \quad (15)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (16)$$

$$\text{Net}: \; C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4 \quad (17)$$

These equations show that 25% of the initially produced CO_2 is finally converted into CH_4.

The observation that production rates of TIC were larger than of CH_4 at the beginning of incubation indicates the presence of electron acceptors that allow the formation of CO_2 without concomitant production of a reduced compound, such as H_2 or CH_4. Interestingly, ratios of $CO_2 : CH_4$ larger than one have quite frequently been observed in anaerobic peat environments despite the fact that inorganic electron acceptors such as oxygen, nitrate, sulfate, or iron(III) were not available (Yavitt and Seidmann-Zager 2006). Such observations led to the speculation that humic substances themselves may act as electron acceptors (Heitmann et al. 2007).

Addition of CH_3F resulted in partial inhibition of CH_4 production and accumulation of acetate being consistent with inhibition of acetoclastic methanogenesis (Eq. 15). This behavior has been observed before in methanogenic lake sediments. Ideally, acetoclastic methanogenesis should be inhibited by CH_3F completely, whereas hydrogenotrophic methanogenesis should not be affected (Janssen and Frenzel 1997), i.e., only the reactions in Eqs. 14 and 16 operate, resulting in

$$\text{Net}: \; C_6H_{12}O_6 \rightarrow 2CH_3COOH + CH_4 + CO_2 \quad (18)$$

These equations show that 50% of the initially produced CO_2 is finally converted into CH_4. They further show that acetate would accumulate instead of CH_4 when acetoclastic methanogenesis is inhibited and that only one CH_4 and one CO_2 would be formed instead of three. However, the amount of acetate accumulated was smaller (about 50%) than the lacking amount of CH_4, and production of CO_2 and TIC was only about half as strongly inhibited by CH_3F than production of CH_4. These observations indicate that hydrogenotrophic methanogenesis was also partially inhibited by the addition of CH_3F. Such inhibition, which increases with concentration of CH_3F, has often been observed (Conrad and Klose 1999). In the present experiments, hydrogenotrophic methanogenesis was apparently already partially inhibited at the lowest CH_3F concentration (0.5%). Since comparison of the different basins of Lake Grosse Fuchskuhle has shown that those with high content of humic substances exhibit relatively low methanogenic activity (Casper et al. 2003), we assume that humic substances may have an inhibitory effect and thus have enhanced the effect of CH_3F. However, acetoclastic methanogenesis was more strongly inhibited by CH_3F than hydrogenotrophic methanogenesis. Inhibition of acetoclastic methanogenesis was probably complete at about 2% CH_3F, since accumulation of acetate then reached the maximum concentration (Fig. 2A) and the $\delta^{13}C$ of the accumulated CH_4 converged to the minimum value (Fig. 2C) that is typical for hydrogenotrophic methanogenesis being the sole CH_4-producing process.

Path of methane production—The path of CH_4 production was calculated by $\delta^{13}C$ mass balance of hydrogenotrophically ($\delta^{13}C_{CH_4\text{-}CO_2}$) and acetoclastically ($\delta^{13}C_{CH_4\text{-}ac}$) produced CH_4. The $\delta^{13}C_{CH_4\text{-}CO_2}$ was related to that

of methane produced in the presence of CH_3F when acetoclastic methanogenesis was inhibited. The $\delta^{13}C_{CH_4\text{-}CO_2}$ was typically much lighter than the $\delta^{13}C_{CH_4}$ formed in the absence of CH_3F. The isotopic difference between the hydrogenotrophically formed CH_4 and the CO_2(aq) is equivalent to the isotopic enrichment factor (ε_{CO_2,CH_4}) by hydrogenotrophic methanogenesis, since methanogens typically use CO_2 (rather than bicarbonate) for reduction to CH_4 (Vorholt and Thauer 1997). Values of ε_{CO_2,CH_4} were calculated to be about −77‰ to −71‰ (data not shown), which are similar to those (−78‰) in the sediment of oligotrophic Lake Stechlin, but lower than those found in eutrophic Lake Dagow (−90‰) or in Brazilian Lake Batata and Lake Mussura (−85‰) (Conrad et al. 2007, 2009, 2010). All these values are within the wide range of ε_{CO_2,CH_4} observed in aquatic environments (Whiticar et al. 1986; Conrad 2005). However, for the calculation of the path of CH_4 production, values of ε_{CO_2,CH_4} were not used. Instead, the $\delta^{13}C_{CH_4\text{-}CO_2}$ measured in the presence of 2% CH_3F was used in Eq. 2. The fraction (f_{CO_2,CH_4}) of H_2+CO_2–derived methane typically decreases with $\delta^{13}C_{CH_4\text{-}CO_2}$ becoming relatively more negative with respect to $\delta^{13}C_{CH_4}$. Values of f_{CO_2,CH_4} were relatively large in the sediment of Lake Grosse Fuchskuhle, since values of $\delta^{13}C_{CH_4\text{-}CO_2}$ were less negative. Increasing the CH_3F concentration from 2% to 3% did not result in a much lower $\delta^{13}C_{CH_4\text{-}CO_2}$ (Fig. 2C). However, increasing the incubation time tended to further decrease the $\delta^{13}C_{CH_4\text{-}CO_2}$ (Fig. 3C), so that values of f_{CO_2,CH_4} seemed to decrease with incubation time.

The δ^{13}C of acetoclastically produced CH_4 was assumed to be identical to the δ^{13}C of the acetate-methyl, from which CH_4 is formed by the methanogens. Values of f_{CO_2,CH_4} would become smaller when assuming fractionation during the conversion of acetate-methyl to CH_4. However, there was likely no isotopic fractionation during CH_4 formation from acetate, since acetate concentrations were so low that the acetate formed was probably consumed completely. In addition, δ^{13}C of acetate-methyl was not higher in the absence than in the presence of CH_3F, indicating absence of fractionation (Fig. 2C).

The values of f_{CO_2,CH_4} calculated from Eq. 2 were 55% to 67%, much higher than the theoretical value from Eqs. 14 and 17, which would have been 33%. In the beginning of incubation f_{CO_2,CH_4} was even higher. High values of f_{CO_2,CH_4} have been found before in sediments of lakes of different trophic levels (Conrad et al. 2009, 2010) and were interpreted by assuming that organic compounds in the sediment were only partially degraded (*see* discussion below). The analysis of methanogenic community in Lake Grosse Fuchskuhle revealed microbes using the hydrogenotrophic and also the acetotrophic path (Chan et al. 2002).

Degradation of organic matter and production of TIC—Since the sediment of Lake Grosse Fuchskuhle initially contained no inorganic carbon, all the CO_2 produced must originate from the decomposition of organic matter. The low pH of the sediment results in the chemical partitioning of TIC into only little bicarbonate (7.5% of TIC) and virtually no carbonate (< 0.01%), so that the δ^{13}C of TIC can be determined relatively precisely. In the absence of CH_3F, only TIC and CH_4 were the final net products of degradation of organic matter. After about 20 d of incubation, the average δ^{13}C of TIC and CH_4 was almost identical to that of sediment organic matter (Fig. 4). In the presence of CH_3F, acetate was an additional product, replacing the CH_4 that would have been formed by acetoclastic methanogenesis. Again, the average δ^{13}C of all products (i.e., TIC, acetate, and CH_4) was almost identical to that of sediment organic matter, except in the beginning of the incubation, when the average δ^{13}C of the products was higher than the $\delta^{13}C_{org}$ (Fig. 4B). Note, however, that the amount of accumulated products was still small when this imbalance happened, while the balance improved as more and more products accumulated.

The good balance of δ^{13}C between the products and the organic matter allows us to address the question of whether the conversion of organic matter to CO_2 resulted in carbon isotope fractionation. Note that the $\delta^{13}C_{TIC}$, which is observed, has already been affected by the partial conversion of CO_2 to CH_4 having a large isotopic enrichment factor. When CO_2 is converted to CH_4, the δ^{13}C of the residual TIC will linearly increase with the fraction of TIC converted to CH_4 due to carbon isotope fractionation (Fig. 1). If we a priori hypothesize that the originally produced CO_2 had a δ^{13}C identical to $\delta^{13}C_{org}$ (i.e., no fractionation), the fractions of TIC converted to CH_4 (f_{TIC,CH_4}) ranged between 33% and 39% (Fig. 5). These values are higher than the 25% expected from complete degradation of organic matter according to Eqs. 14–17. There are two conceivable explanations. In the first, we may assume that part of the organic matter was not completely degraded, e.g.,

$$C_6H_{12}O_6 + 2H_2O \rightarrow C_4H_8O_4 + 2CO_2 + 4H_2 \quad (19)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (20)$$

$$\text{Net}: \; C_6H_{12}O_6 \rightarrow C_4H_8O_4 + CH_4 + CO_2 \quad (21)$$

Note that 50% of the initially produced CO_2 is finally converted into CH_4 and that the residual organic matter, of which the actual chemical composition is unknown, has the same oxidation state as acetate. If we further assume that about 50% of the organic matter is degraded by these reactions (Eqs. 19–20) while the other 50% are completely degraded as shown in Eqs. 14–17, we would expect that about 37% ([50 + 25]/2) of the initially produced TIC is converted to CH_4. This value is similar to that actually determined (Fig. 5). Furthermore, about 66% ([33 + 100]/2) of the CH_4 would be formed from CO_2 reduction, which is also a similar percentage to that actually determined. Finally, the production rates of CH_4 and TIC would still be equal as expected from the results obtained (*see* Eqs. 17 and 21). Hence, our a priori assumption that the initially produced TIC had the same isotopic composition as that of organic matter is quite reasonable. This means that the conversion of organic matter to CO_2 exhibits only negligible fractionation.

In the second explanation, we may assume that acetate was converted to CO_2 and H_2 by syntrophic acetate-oxidizing bacteria instead of being consumed by acetoclastic methanogens, i.e.,

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (22)$$

$$2CH_3COOH + 4H_2O \rightarrow 8H_2 + 4CO_2 \quad (23)$$

$$12H_2 + 3CO_2 \rightarrow 3CH_4 + 6H_2O \quad (24)$$

$$\text{Net}: \; C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4 \quad (25)$$

Note that again 50% of the initially produced CO_2 is finally converted to CH_4 and that production rates of CO_2 and CH_4 are equal, so that the same conclusions are valid as described above for the incomplete degradation of organic matter. Syntrophic acetate oxidation is the reversal of hydrogenotrophic homoacetogenesis (Lee and Zinder 1988). Little is known about this process and the microorganisms involved, and nothing is known about isotope fractionation during this process (Nüsslein et al. 2003). Syntrophic acetate oxidation was thermodynamically feasible in sediment of Lake Grosse Fuchskuhle (Fig. 2D). Since acetoclastic methanogens have been demonstrated in this sediment (Chan et al. 2002), syntrophic acetate oxidizers would have to compete with them for acetate. Furthermore, we have to postulate that syntrophic acetate oxidation would have been completely inhibited by addition of CH_3F similarly as acetoclastic methanogenesis. We recently postulated that CH_3F indeed may inhibit syntrophic acetate oxidizers, based on the observation that in the sediments of Amazonian lakes $\delta^{13}C_{ac\text{-}methyl}$ changed with increasing CH_3F concentration (Conrad et al. 2010). However, such a change in $\delta^{13}C_{ac\text{-}methyl}$ was not observed in the present study. Although operation of syntrophic acetate oxidation in sediment of Lake Grosse Fuchskuhle cannot be excluded, we think that incomplete degradation of organic matter (explanation one) is the more parsimonious explanation for our observations.

In the incubations with 2% CH_3F, f_{TIC,CH_4} decreased with incubation time from 38% to 24% (Fig. 5B), i.e., much lower that the expected value of 50%, which is based on Eqs. 14 and 18. However, since the fraction of TIC converted to CH_4 decreased with the CH_3F concentration applied (Fig. 5A), we assume that the conversion of CO_2 to CH_4 was partially inhibited by CH_3F, which would also result in a decrease of f_{TIC,CH_4}. Partial inhibition is consistent with the observations that CH_4 production was not completely balanced by acetate accumulation and that production of CO_2 was not as much inhibited than that of CH_4 (Fig. 2A).

Acetate and propionate production—The $\delta^{13}C$ of acetate accumulated in the presence of CH_3F is no longer affected by isotope fractionation during further conversion (by either acetoclastic methanogens or syntrophic acetate oxidizers) and thus should have the $\delta^{13}C$ value that is characteristic for the acetate produced during fermentation of sediment organic carbon. The $\delta^{13}C$ of total acetate was about −16‰ to −23‰, that of acetate-methyl was about −30‰ to −38‰, and that of the carboxyl group was consequently about −11‰ to +3‰. The $\delta^{13}C$ of total acetate was 6–13‰ larger than the $\delta^{13}C$ of organic matter. Studies on other lake sediments found about 1–8‰ heavier $\delta^{13}C_{ac\text{-}tot}$ than $\delta^{13}C_{org}$ (Conrad et al. 2007, 2010; Heuer et al. 2010). However, we cannot exclude that the $\delta^{13}C$ of the acetate-carboxyl was partially affected by exchange with CO_2(aq) or bicarbonate, which both exhibited relatively large $\delta^{13}C$ in sediment of Lake Grosse Fuchskuhle. In sediment of carbonate-rich Lake Dagow, $\delta^{13}C_{ac\text{-}tot}$ was more than 10‰ heavier than $\delta^{13}C_{org}$ (Conrad et al. 2009). Therefore, the $\delta^{13}C$ of the carboxyl group may not represent the originally produced acetate carbon. However, the $\delta^{13}C$ of the methyl group most likely represents the originally produced acetate. Compared to organic carbon, the $\delta^{13}C$ of acetate-methyl was on the average by about 4‰ lower (maximum 9‰). Such a difference between organic C and acetate-methyl has also been observed in other lake sediments (Conrad et al. 2007, 2009, 2010) and may be indicative for the relatively moderate isotope fractionation during fermentation of organic matter to acetate. In other sediments, the large isotopic difference (about 30‰) between acetate-methyl and acetate-carboxyl (19–38‰) is also consistent with a formation process by fermentation, e.g., fermentation of polysaccharides (Blair et al. 1985; Penning and Conrad 2006), whereas formation by chemolithotrophic acetogenesis should result in similar isotopic composition (Gelwicks et al. 1989). Addition of different concentrations of CH_3F did not have a strong effect on the $\delta^{13}C$ of acetate or acetate-methyl (Fig. 2C), but the intramolecular difference between the carboxyl and methyl group decreased at the end of incubation from 32‰ to 19‰ (Fig. 3C), indicating that the acetate formation process might have been changing with increasing contribution of chemolithotrophic acetogenesis.

The measured H_2 partial pressures were too low for exergonic acetate production by hydrogenotrophic chemolithotrophic acetogenesis, but ΔG values were lower in the presence than in the absence of CH_3F (Fig. 2D). If we nevertheless assume that exergonic acetogenesis was perhaps possible within microniches, where H_2 partial pressures were higher than actually measured, we may roughly estimate the fraction of acetate-methyl produced from either fermentation of organic matter or chemolithotrophic acetogenesis. This estimation requires the knowledge of the $\delta^{13}C$ of acetate-methyl produced by either pathway. In case of fermentatively produced acetate-methyl we assume only a small isotopic enrichment (−4‰ to 0‰) during fermentation of C_{org} (−29‰) resulting in $\delta^{13}C_{ac\text{-}org}$ of −33‰ to −29‰. The CO_2 produced during fermentation would have the same $\delta^{13}C$ as C_{org}, but the acetate-methyl produced from this CO_2 by chemolithotrophic acetogenesis would be about −87‰ assuming an isotopic enrichment factor of −58‰ (Gelwicks et al. 1989). Using these values and a $\delta^{13}C_{ac\text{-}methyl} = -34$‰, only 2–9% of the acetate would be produced via chemolithotrophic

acetogenesis. Hence, chemolithotrophic acetogenesis was not of much importance.

Propionate was only produced in minor amounts when CH_3F was added. The amounts produced were too small to account in mass balance calculations concerning recovery of degraded organic carbon and, therefore, were neglected. The $\delta^{13}C$ of the produced propionate was only slightly (about 1‰) heavier than that of $\delta^{13}C_{org}$. We cannot exclude that this difference was caused by partial exchange of the carboxyl group of propionate with the CO_2 or bicarbonate of the sediment porewater, similarly as discussed above for acetate.

Isotope fractionation during methanogenic degradation of organic matter—The $\delta^{13}C$ of TIC, acetate, and CH_4 after incubation in the presence and absence of CH_3F balanced well with the $\delta^{13}C$ of the organic matter degraded in the sediment of Lake Grosse Fuchskuhle. The initial fermentation of organic matter to acetate and TIC exhibited only relatively small isotopic enrichment factors, resulting in $\delta^{13}C$ values of acetate-methyl only about 4‰ lower, and TIC also being similar to the $\delta^{13}C$ of organic matter. In the absence of CH_3F, about a third of the produced CO_2 was converted to CH_4. This percentage was consistent with the assumption that about half of the organic matter was completely degraded via acetate, CO_2, and H_2, while the other half was incompletely degraded producing only CO_2 and H_2. Addition of CH_3F completely inhibited acetoclastic methanogenesis but partially also inhibited hydrogenotrophic methanogenesis. The isotopic signature of the hydrogenotrophically produced CH_4 showed that more than 55% of the CH_4 production occurred by this path. The relatively high percentage of the hydrogenotrophic path might be explained with the operation of syntrophic acetate oxidation. However, a more parsimonious assumption is that many of the organic compounds in the sediment were only partially rather than fully degraded. Partial degradation means that the molecular weight of polymeric compounds decreased only little by release of CO_2 and reducing equivalents (Eq. 19), so that the residual compounds still had a high molecular weight. The assumption of partial degradation of organic matter is consistent with the presence of extraordinarily high amounts of organic matter in the sediment. The methanogenic paths in the sediment of the naturally acidic Lake Grosse Fuchskuhle are similar to the situations in neutral lake sediments, such as temperate lakes in Germany (Conrad et al. 2009) and tropical lakes in Brazil (Conrad et al. 2010). Most of acetate production occurred during fermentation of organic matter, and only less than 9% may have been produced by acetogenic reduction of CO_2.

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