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Comparison of Acetate Turnover in Methanogenic and Sulfate-Reducing Sediments by Radiolabeling and Stable Isotope Labeling and by Use of Specific Inhibitors: Evidence for Isotopic Exchange†

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Acetate turnover in the methanogenic freshwater anoxic sediments of Lake Vechten, The Netherlands, and in anoxic sediments from the Tamar Estuary, United Kingdom, and the Grosser Jasmunder Bodden, Germany, the latter two dominated by sulfate reduction, was determined. Stable isotopes and radioisotopes, inhibitors (chloroform and fluoroacetate), and methane flux were used to provide independent estimates of acetate turnover. Pore water acetate pool sizes were determined by gas chromatography with a flame ionization detector, and stable isotope-labeled acetate was determined by gas chromatography-mass spectrometry. The appearance of acetates with a different isotope labeling pattern from that initially added demonstrated that isotopic exchange occurred during methanogenic acetate metabolism. The predominant exchange processes were (i) D-H exchange in the methyl group and (ii) ^{13}C-^{12}C exchange at the carboxyl carbon. These exchanges are most probably caused by the activity of the enzyme complex carbon monoxide dehydrogenase and subsequent methyl group dehydrogenation by tetrahydromethanopterine or a related enzyme. The methyl carbon was not subject to exchange during transformation to methane, and hence acetate with the methyl carbon labeled will provide the most reliable estimate of acetate turnover to methane. Acetate turnover rate estimates with these labels were consistent with independent estimates of acetate turnover (acetate accumulation after inhibition and methane flux). Turnover rates from either radioisotope- or stable isotope-labeled methyl carbon isotopes are, however, dependent on accurate determination of the acetate pool size. The additions of large amounts of stable isotope-labeled acetate elevate the acetate pool size, stimulating acetate consumption and causing deviation from steady-state kinetics. This can, however, be overcome by the application of a non-steady-state model. Isotopic exchange in sediments dominated by sulfate reduction was minimal.

Low-molecular-weight organic acids are important intermediates in the anaerobic degradation of organic matter as the end products of fermentation and as substrates for terminal carbon mineralization (3, 4, 11). Acetic acid is particularly important with respect to the carbon flow through the indigenous microbial community and its final mineralization to carbon dioxide and methane; it is the principal substrate for methanogenesis in freshwater sediments (5, 10) and for sulfate reduction in marine sediments (12, 16). Accurate determination of the turnover of pore water acetate would give a direct estimate of the rate of anaerobic organic matter degradation. However, previous approaches with radiotracer techniques in marine environments have yielded overestimates of the acetate turnover compared with independent measurements of anaerobic carbon mineralization (e.g., sulfate reduction [2, 14] and ammonia release [6]). Those overestimates have mainly been ascribed to overestimates of the free pore water acetate concentration (15). However, carboxyl exchange of [U-^{14}C]acetate and deviation from pseudo-first-order kinetics may also be sources of errors in the determination of acetate turnover.

A large variety of stable isotope-labeled forms of acetate have become available; combined with mass-spectral detection techniques, this provides the potential to develop a method for measuring acetate turnover rates while avoiding the hazards, legislation, and expense of radiochemical approaches. Recently, however, an attempt to measure acetate turnover in a freshwater environment through the use of deuterated acetate also resulted in overestimation compared with methanogenesis (9). In the present study, radiolabeling, stable isotope labeling, and inhibition methods are compared in freshwater sediments from Lake Vechten, The Netherlands. For comparison, some key experiments were also performed with brackish-water sediments from the Grosser Jasmunder Bodden, Rügen, Germany, and in marine sediment from the Tamar Estuary, Plymouth, United Kingdom.

MATERIALS AND METHODS

Sampling and sites. Freshwater sediment samples were taken with a modified Jenkin mud sampler on 25 October 1994, at a depth of 10.5 m, from the eastern depression of Lake Vechten. Lake Vechten is a freshwater mesotrophic monomictic lake located in the center of the Netherlands and has been the subject of around 30 years of ongoing research (7, 9). Brackish-water sediment samples were taken from the Grosser Jasmunder Bodden with a shipboard multicorer on 10 October 1994. Estuarine sediment samples were obtained by hand from an intertidal mudflat at Southdown, Tamar Estuary, with Plexiglas cores (7-cm inner diameter [i.d.]) on 12 April 1994. In all cases, the cores were transported back to the laboratory and processed within 3 h of sampling.

Preparation of sediment slurries. The 0- to 10-cm depth horizon of a core from Lake Vechten was transferred with a polythene bag to a 1-liter bottle, and the headspace was flushed with nitrogen for 5 min before the bottle was sealed.

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TABLE 1. Combined results of measurement of acetate cycling rates with different stable isotope-labeled and radiolabeled acetate in replicate slurries taken from bottom sediment of Lake Vechten[a]

Isotope	Turnover time (h^{-1})		Pool size (μM)	Turnover rate (μM/h)	Uptake rate (R_c) (μM/h)	Production rate (R_p) (μM/h)	Accumulation (μM/h) after inhibition by:	
	Initial label	Σ Labeled acetates					Chloroform	Fluoroacetate
$^{13}CH_3COO^-$	6	6	5	30	23.3	17	10	ND[b]
	3.8	3.8	7.2	27.3	24.4	16.6	15.2	ND
	5.1	5.1	6.7	34.2	14.4	3.7	ND	18.5
$^{13}CH_3{}^{13}COO^-$	5	3.6	5.3	22	13	6	12	ND
	4.2	3	6.5	19	15.3	8.5	12.7	ND
	4.2	3	6	18	20.2	9	ND	18.5
$^{13}CD_3COO^-$	7.4	3	6.5	17.6	18.4	8.1	9.7	ND
	5.1	2.2	6.3	13.8	11.2	5.4	13.8	ND
	7.4	3.1	5.2	16.4	18.7	6	ND	17.3
Mean (SD)	5.6 (1.3)	3.6 (1.1)	6.1 (0.7)	22 (6.5)	17.7 (4.3)	8.9 (4.5)	10.5 (4.6)	18.1 (0.7)

[a] For $^{14}CH_3COO^-$, turnover times from $^{14}CH_4$ plus $^{14}CO_2$ and $^{14}CH_4$ production were 6.7 and 7.1 h^{-1}, respectively; the pool was 1 to 2 μM; and the turnover rate was 7 to 14 μM/h. For $^{(14)}CH_3{}^{(14)}COO^-$, turnover times from $^{14}CH_4$ plus $^{14}CO_2$ and $^{14}CH_4$ production were 5.1 and 6.2 h^{-1}, respectively; the pool was 1 to 2 μM; and the turnover rate was 6 to 12 μM/h.

[b] ND, not done.

The contents of the bottle were shaken thoroughly to homogenize the sediment. Replicate subsamples (10 to 25 ml) of the sediment slurry were removed with an nitrogen-flushed syringe and needle (2 mm [i.d.]) and transferred to nitrogen-filled serum vials capped with butyl rubber septa. The headspaces were then flushed with nitrogen before the vials were incubated overnight at the in situ temperature (8°C), prior to addition of labeled acetate. Similarly, the 2- to 7-cm depth horizon from Grosser Jasmunder Bodden sediment was mixed and dispensed under helium into serum vials and incubated overnight at the in situ temperature (10°C), prior to addition of labeled acetate. Unlike the Lake Vechten and Grosser Jasmunder Bodden sediments, which were mixed without dilution, the Tamar sediment was slurried to facilitate sample manipulation. The 1- to 4-cm depth horizon was diluted (1:1, wt/wt) with filter-sterilized degassed seawater (taken at the time of sampling) under nitrogen, dispensed anaerobically, and incubated overnight (10°C) as above.

Stable isotope time courses. Immediately before the introduction of labeled acetate, a time zero sample (1.5 ml) was removed with a syringe and transferred to an Eppendorf centrifugation vial. This was centrifuged with minimal delay (5 to 10 min at 4,000 to 5,000 $\times$ *g* at 7°C), and the Eppendorf vials were stored on ice before and after centrifugation. Pore water was collected and stored frozen at −20°C until used for analysis. Stable isotope-labeled acetate was introduced as 5 mM stock solution of the sodium salt (Aldrich, Beerse, Belgium) to a final concentration of ca. 10 μM. The slurry was mixed thoroughly, and incubation was started. Subsamples (1.5 ml) were removed with a syringe at selected time intervals, and the pore water was collected as described above. The incubation period was taken as the time between introduction of label and start of centrifugation. After 2 to 3 h of incubation, chloroform (0.1%, vol/vol) or a solution of sodium fluoroacetate (final concentration, 0.1%, wt/vol) was added as an inhibitor. The slurries were shaken thoroughly upon addition of inhibitors, and a new spike of label (ca. 10 μM) was added. The actual free concentration was calculated from the amount of wet sediment, converted to volume by using the porosity and density of the slurry.

Radioisotope time courses. Replicate slurries were injected with 5 μl of stock isotope solutions of either [1-(2)-^{14}C]acetate, termed dual label (9.25 kBq, 2.18 GBq $mmol^{-1}$; Amersham, Little Chalfont, United Kingdom), or $^{14}CH_3COONa$, termed mono label (37 kBq, 2.18 GBq $mmol^{-1}$; Amersham), or $H^{14}CO_3Na$ (37 kBq, 240 MBq $mmol^{-1}$; Sigma Radiochemicals, Poole, United Kingdom). The slurries were thoroughly mixed immediately upon addition of radiolabel and incubated as for stable isotope slurries before fixation by the addition of 4 M NaOH solution.

Core incubations with chloroform. Four subcores (stainless steel, 2 cm [i.d.] by 20 cm long) were taken from two replicate cores (7 cm [i.d.]) from Lake Vechten. The uppermost 10 cm of the sediment was given an injection of chloroform in a vertical line through the center of the subcore, leaving ca. 5 μl of chloroform per 3.14 ml of wet sediment. After approximately 1, 2, and 4 h, the sediment was extruded with a plunger and sliced at 1-cm intervals, and the selected slices (0 to 1, 1 to 2, 3 to 4, and 7 to 8 cm) were transferred to Eppendorf vials for extraction of pore water as described above.

GC-flame ionization detection and GC–mass-selective detection. Determination of volatile fatty acids (VFA) was carried out by gas chromatography (GC) (Hewlett-Packard 5890A apparatus) with HP-FFAP capillary columns (10 m long by 0.53 mm [i.d.] by 1 μm [film thickness] and 15 m long by 0.32 mm [i.d.] by 0.53 μm [film thickness]) with helium as the carrier gas at flow rates of 16 and 4 ml min^{-1}, respectively (8). Samples were acidified with formic acid (Suprapur; Merck) to 0.3% (vol/vol) before being injected into the apparatus. For the wide-bore (0.53-mm) column, 5 μl of sample was introduced via a split/splitless injection port (170°C) with a 90- by 4-mm (i.d.) glass liner, and detection was performed with a flame ionization detector. Samples were injected with the oven temperature at 50°C, rising at 10°C min^{-1} to 120°C, which was held for 1 min. Concentrations were calculated from the peak areas (Interscience integrator) by relating them to those of gravimetric standards: sodium acetate trihydrate (pro analyse; Merck), acidified with formic acid to 0.3% (vol/vol), and corrected for blanks.

The narrow-bore (0.32 mm [i.d.]) column was used in combination with a cooled injection system (CIS 3; Gerstell Analytical Systems, Middelburg, The Netherlands) and an HP 5970 mass-selective detector. Samples (1 μl) were introduced into the cooled injector at 35°C, and the liner was heated at a rate of 10°C s^{-1} to 100°C and then at 5°C min^{-1} to 200°C. The GC oven program was the same as used for the wide-bore column. The end of the narrow-bore column was linked through a transfer line (150°C) to the ionization chamber of the mass-selective detector (ionization energy, 70 eV). Spectral data were processed with an HP-300 computer. Labeled VFA were analyzed in single-ion monitoring mode. Acetic acid and its stable isotopes were monitored at *m/z* 60 to 64. Concentrations were calculated from the peak areas of *m/z* 61 (corrected for 2.2% contribution from the natural peak at *m/z* 60), 62, and 64 by comparison with gravimetric standards ($^{13}CH_3COONa$, $^{13}CH_3{}^{13}COONa$, and $^{13}CD_3COONa$, all acidified with formic acid to 0.3% [vol/vol]). Good linear calibration was achieved (r^2 = 0.9997), and there was no significant difference in the sensitivity of the molar response factors for deuterium and ^{13}C-labeled acetates. Quantitation of acetic acid at *m/z* 60 was not possible because of high and irreproducible blanks. In the absence of added acetate, high and irreproducible signals are found at *m/z* 60. For further details on manual tuning and external standardization, see reference 9.

^{14}C determinations. The amount of $^{14}CH_4$ produced was determined by a modified method described by Cragg et al. (6a). The headspace of each vial was flushed with oxygen-free nitrogen (flow rate, 80 ml min^{-1}) for 40 min, passing the gas through a CuO furnace at 850°C (Carbolite, Sheffield, United Kingdom) to oxidize labeled methane to CO_2. Labeled carbon dioxide was trapped in a system of three scintillation vials in series, each containing 10 ml of CO_2-absorbing scintillant [800 ml of toluene, 100 ml of phenylethylamine, 5 g of 2,5-diphenyloxazole (PPO), 0.1 g of 1,4-bis(5-phenyloxazolyl)benzene (POPOP)]. In addition, a cold trap containing methanol at −25°C was included to prevent carryover of aerosols. Mean recovery of $^{14}CH_4$ was typically 95 to 99% with this system. Next, $^{14}CO_2$ was removed from the samples by acidification with 1.5 ml of 2 M H_2SO_4. The headspace of the acidified vials was flushed with oxygen-free nitrogen, and the gas flow passed through two empty 50-ml traps at room temperature (to prevent physical carryover of label) before collection of labeled CO_2 as described above. The trapping efficiency of this stage was typically >95%, and the mean recovery was 98%. Between samples, each system was flushed for 10 min with oxygen-free nitrogen at 100 ml min^{-1} to minimize cross-contamination. Residual [^{14}C]acetate was measured by adding 3 ml of the acidified pore water to 10 ml of Instagel (Packard Instruments) for liquid scintillation counting. All radioisotope samples were counted with a Rackbeta liquid scintillation counter (Pharmacia-LKB, Milton Keynes, United Kingdom) with external standard and quench correction.

Calculation of rates. Turnover rates and separated rates of consumption (R_c) and production (R_p) were calculated from the changes in measured concentrations of labeled acetate and corresponding cold pool sizes (Table 1). Rate constants were calculated directly by regression of the logarithm of the initial added labeled acetate and the sum of all labeled acetates (see Results). Acetate pool sizes were averaged over the same time intervals as rate constants were determined. Turnover rates were calculated from the average pool size multiplied by the rate constants based on the sum of all labeled acetates. This is appropriate for steady-state conditions, at which rates of acetate consumption (R_c) and production (R_p) are in balance. Introduction of a small amount of labeled acetate [Ac*] does not significantly change the acetate pool size, as is assumed for ^{14}C-labeled acetate:

$$d[\mathrm{Ac}^*]/dt = R_c \times [\mathrm{Ac}^*]/[\mathrm{Ac}]_{(t=0)}$$

Depletion of labeled acetate shows pseudo-first-order kinetics such that

$$(k) = d \ln \{[\mathrm{Ac}^*]/[\mathrm{Ac}^*]_{(t=0)}\}/dt = \{R_c/[\mathrm{Ac}]_{(t=0)}\}$$

Thus, acetate turnover rate = acetate rate constant (k) × acetate pool size. With addition of stable isotopes, however, there is a significant increase in the initial acetate pool size, which subsequently is reduced throughout the experiment (see Fig. 1 and 2):

$$[\mathrm{Ac}]_{\mathrm{pool}} = [\mathrm{Ac}]_{\mathrm{spiked}} + (R_p - R_c) \times t$$

where $[\mathrm{Ac}]_{\mathrm{spiked}}$ = initial pool size + added labeled acetate. Thus, the differential equation for the consumption of labeled acetate becomes

$$d[\mathrm{Ac}^*]/dt = R_c \times [\mathrm{Ac}^*]/\{[\mathrm{Ac}]_{\mathrm{spiked}} + (R_p - R_c) \times t\}$$

The solution of this equation is

$$d \ln[\mathrm{Ac}^*]/d \ln \{[\mathrm{Ac}]_{\mathrm{spiked}} + (R_p - R_c) \times t\} = R_c/(R_p - R_c)$$

Therefore, regression of ln (label) against ln (pool) gives a value for $R_c/(R_p - R_c)$ and regression of the decrease of the pool size with time gives $(R_p - R_c)$, so that R_c and R_p may be calculated separately.

RESULTS

Characteristic anaerobic terminal oxidation processes at the sites. At the time of sampling, Lake Vechten was stratified and oxygen was depleted in the hypolimnion. Nitrate was absent from the pore water samples, and the sulfate concentration was at a threshold value of ca. 5 to 10 μM. The dominant terminal oxidation process at this freshwater site was methanogenesis. In the Tamar Estuary, depth-integrated sulfate reduction accounted for up to 60% of whole-core oxygen uptake whereas methanogenesis was responsible for ≤0.5%. In addition, 92% of sulfate reduction was due to acetate metabolism, on the basis of molybdate inhibition experiments (17). In the Grosser Jasmunder Bodden, pore water sulfate concentrations decreased with depth from a maximum of 6.5 mM at the sediment surface; however, 3 mM sulfate was still present at a depth of 20 cm. Minimal levels of methane were measured in the Grosser Jasmunder Bodden sediment, suggesting that anaerobic terminal oxidation was also dominated by sulfate reduction at this brackish water site.

Uptake of $^{13}CD_3COO^-$. The uptake of added $^{13}CD_3COONa$, injected into a mixed section (1 to 10 cm) of Lake Vechten sediment to a calculated initial concentration of ca. 10 μM, was monitored over time by GC–mass-selective detection (Fig. 1). The added labeled acetate was rapidly removed, as reflected by the rapid decrease of the *m/z* 64 peak with time. At the same time, additional labeled acetates appeared, demonstrating the formation of intermediates during the uptake of $^{13}CD_3COO(H)$ (Fig. 1). The relative importance of the *m/z* 61 peak indicates that $^{13}CH_3COO(H)$ is formed by exchange of methyl deuteriums with hydrogen. The total acetate pool decreased during the experiment because of the initial elevation of the acetate pool by the addition of label (natural pool size, 1 to 2 μM [see below]).

Similar experiments performed with both brackish water sediment from the Grosser Jasmunder Bodden and a slurry from the Tamar Estuary revealed that this D-H exchange is virtually absent under sulfate-reducing conditions.

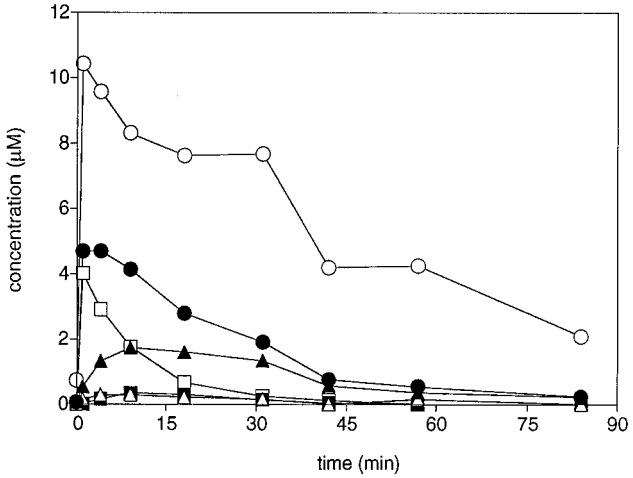

FIG. 1. Uptake of $^{13}CD_3COO(H)$ (*m/z* 64) and the release of intermediates in a mixed section (0 to 10 cm) of Lake Vechten sediment. ○, total pool; □, *m/z* 64; ■, *m/z* 63; △, *m/z* 62; ▲, *m/z* 61; ●, *m/z* 61 to 64.

Uptake of $^{13}CH_3{}^{13}COO^-$. The uptake of added $^{13}CH_3{}^{13}COONa$ (calculated initial concentration, ca. 10 μM) into Lake Vechten sediment showed a similar appearance of the *m/z* 61 peak concomitant with the depletion of the added label, *m/z* 62, with time (Fig. 2). In addition, the *m/z* 46 peak ($[^{13}COOH]^{+\cdot}$) was monitored in this experiment to establish the position of the ^{13}C label in the *m/z* 61 moiety. Since the depletion with time of the *m/z* 46 peak matched that of the *m/z* 62 peak, it must be concluded that $^{13}COOH$ has come only from $^{13}CH_3{}^{13}COOH$ and thus that the *m/z* 61 peak corresponds to $[^{13}CH_3COOH]^{+\cdot}$ and exchange of the carboxyl group has occurred. Similar experiments with the sulfate-reducing sediments of the Grosser Jasmunder Bodden and the Tamar Estuary showed that some carboxyl exchange took place (data not shown), although to a much smaller extent than in Lake Vechten sediments.

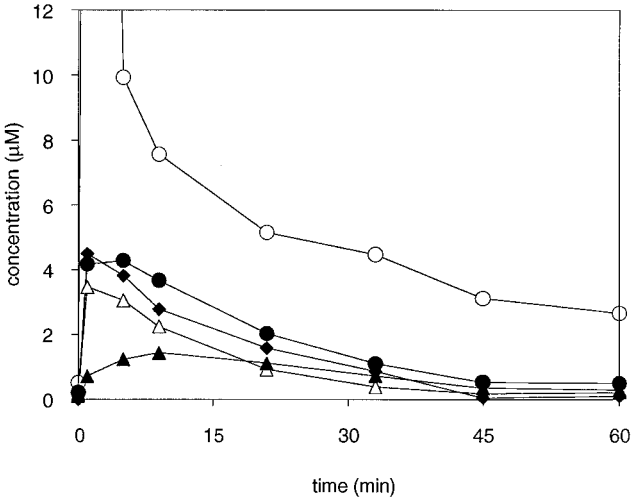

FIG. 2. Uptake of $^{13}CH_3{}^{13}COO(H)$ (*m/z* 62) and the release of intermediates in a mixed section (0 to 10 cm) of Lake Vechten sediment. ○, total pool; △, *m/z* 62; ▲, *m/z* 61; ●, *m/z* 61 to 62; ◆, *m/z* 46.

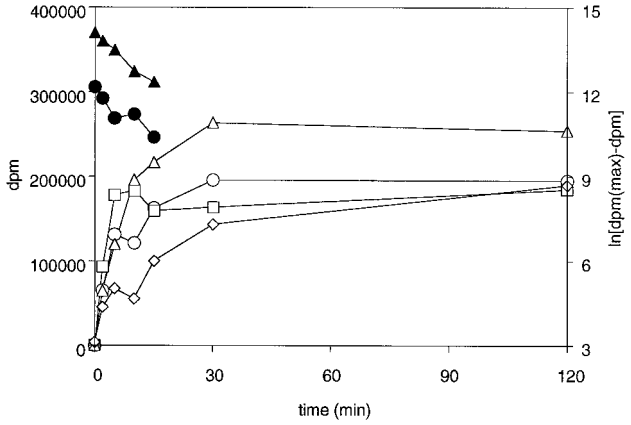

FIG. 3. Release of $^{14}CH_4$ and $^{14}CO_2$ from injected $^{14}CH_3COO(H)$ (monolabeled) and [1-(2)^{14}C]$^{(14)}CH_3{}^{(14)}COO(H)$ (dual labeled) in a mixed section (0 to 10 cm) of Lake Vechten sediment. △, CH_4 from monolabeled acetate (values shown are 20% of real data); ▲, ln CH_4 from monolabeled acetate; ◇, CO_2 from monolabeled acetate; ○, CH_4 from dual-labeled acetate; ●, ln CH_4 from dual-labeled acetate; □, CO_2 from dual-labeled acetate.

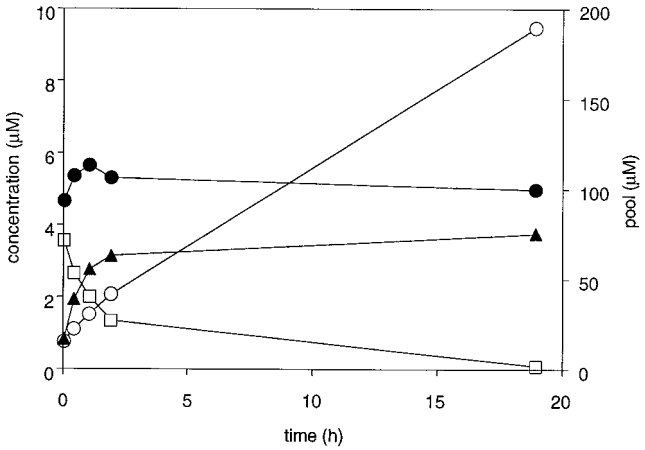

FIG. 4. Chloroform inhibition of the uptake of $^{13}CD_3COO(H)$ (*m/z* 64), release of intermediates, and accumulation of acetate in a mixed section (0 to 10 cm) of Lake Vechten sediment. ○, total pool; □, *m/z* 64; ▲, *m/z* 61; ●, *m/z* 61 to 64.

Uptake of $^{14}CH_3COO^-$ and [1-(2)-^{14}C]acetate. ^{14}C-labeled acetate turnover data from Lake Vechten showed that the methyl group of $^{14}CH_3COO(H)$ was transformed predominantly to methane, with a respiratory index [$^{14}CO_2/(^{14}CO_2 + {}^{14}CH_4)$] of 0.10, which is consistent with previous results (0.24 in reference 4 and 0.20 in reference 1). [1-(2)-^{14}C]acetate was ultimately transformed into an equal mixture of methane and carbon dioxide, although the latter was notably more rapidly formed than methane (Fig. 3). Whereas [^{14}C]methane production required about 15 min, the formation of $^{14}CO_2$ was apparently complete after only 5 min, indicating that the carboxyl group is more rapidly transformed into CO_2 than the methyl group is transformed to CH_4. The turnover rate constants of the formation of methane from acetate were 7.1 ± 0.5 and 6.2 ± 1.5 h^{-1} from methyl-labeled and dual-labeled acetate, respectively. The free pool of acetate in the pore waters of three parallel subslurries was, however, at or below the detection limit of 2 μM of the GC method and hence was assumed to be in the 1 to 2 μM range. Therefore, only a range for the acetate turnover rate can be provided, amounting to approximately 6 to 14 μM h^{-1}.

Methane production. Two replicate slurries were used to measure the methane flux, and a third was used to estimate methanogenesis from $H_2/H^{14}CO_3^-$. The increase of methane in the headspace was linear with time (r^2 = 0.9997) and amounted to 10.2 μmol kg of wet sediment^{-1} h^{-1}. Methane production from carbonate was 1.3 μmol kg of wet sediment^{-1} h^{-1}, accounting for 13% of the total methane flux, with the remainder being 8.9 μmol kg of wet sediment^{-1} h^{-1}. Corrected for a density of 1.16 kg liter^{-1} and dry weight of 25%, methane production from acetate amounts to 10.2 μM liter^{-1}.

Chloroform and fluoroacetate inhibition. Approximately 3 h after each turnover experiment with stable isotope-labeled acetate, chloroform (0.1% [vol/vol]) or fluoroacetate (0.1% [wt/vol]), followed by more label, was added. The results demonstrated, using $^{13}CD_3COONa$, that acetate uptake in Lake Vechten sediment was blocked (Fig. 4). Although the D-H exchange continued very slowly (since the *m/z* 64 peak decreased), the consumption of methyl carbon was effectively zero during the first 20 h of incubation, since the sum of all labeled acetate is virtually constant. The acetate pool increased linearly for the first 4 h, and this was used to estimate the acetate production rate. It should be noted that the rate of acetate production decreased over a longer incubation period.

Generally, in chloroform inhibition experiments, measured the labeled acetate concentration was lower than the calculated addition (ca. 10 μM). This, however, was not the case when fluoroacetate was used as the inhibitor. Acetate accumulation rates were also higher than those when chloroform was used. As a result of inhibition, there was an accumulation of propionate and higher VFA up to hexanoate. It is noteworthy that chloroform also inhibited acetate metabolism in a mixed section of sulfate-reducing Grosser Jasmunder Bodden sediment (Fig. 5). [*methyl*-^{13}C]acetate, injected 1 h later than the chloroform addition, was not metabolized. The only difference between Lake Vechten and Grosser Jasmunder Bodden sediments was that the former produced mainly branched higher VFA whereas the latter produced mainly straight-chain VFA. At both sites, the relative percent VFA accumulation during inhibition was similar: approximately 80% acetate, 15% propionate, and 5% higher VFA.

Chloroform inhibition in intact subcores of Lake Vechten sediment was also studied for direct comparison with the

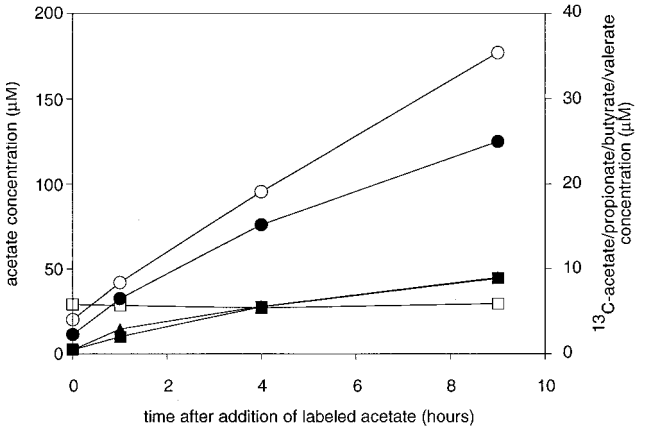

FIG. 5. Chloroform inhibition of the uptake of $^{13}CH_3COO(H)$ and the release of volatile fatty acids in a mixed section (2 to 7 cm) of Grosser Jasmunder Bodden sediment. ○, total pool; □, [^{13}C]acetate; ●, propionate; ■, butyrate; ▲, valerate.

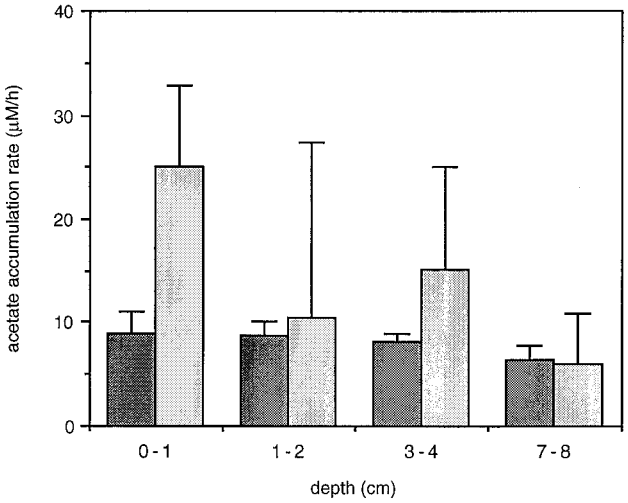

FIG. 6. Profiles of acetate production following injection of chloroform in replicate cores from Lake Vechten.

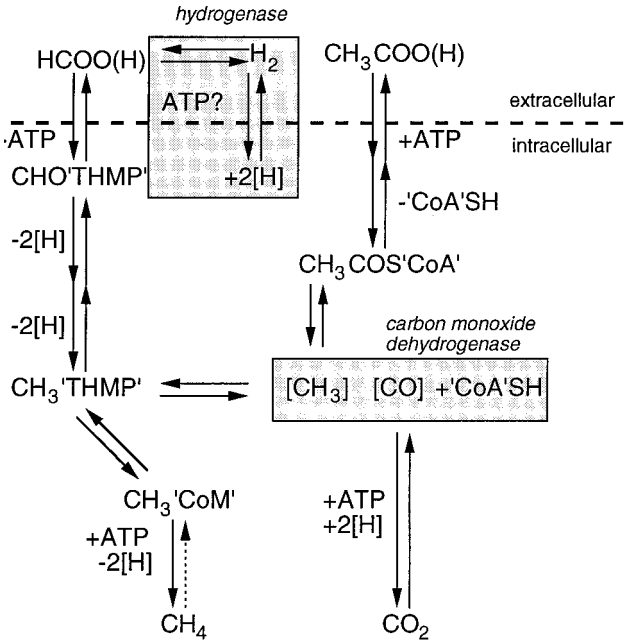

FIG. 7. Scheme of acetate metabolism by the C_1 pathway in methanogens. [H], hydrogen carrier (e.g., NADH and ferrodoxin); CoA, acetyl carrier coenzyme A; CoM, methyl carrier coenzyme M.

mixed sediments. Chloroform was injected in a line through the center of the core. The rates of increase of pore water acetate at specific depth intervals were calculated by linear regression in replicate subcores after 0, 1, 2, and 4 h of incubation (Fig. 6). A replicate experiment with an additional core showed that results can be variable.

DISCUSSION

Isotopic exchange of labeled acetates. The appearance of acetates with a different isotope labeling pattern from that of the initially added acetate demonstrates that isotopic exchange occurs during acetate metabolism in Lake Vechten sediment. The predominant exchange processes are (i) *D-H* exchange of the methyl group (Fig. 1) and (ii) ^{13}C-^{13}C exchange of the carboxyl group (Fig. 2). These exchanges are most probably caused by the activity of the enzyme complex carbon monoxide dehydrogenase (CODH) and subsequent methyl group dehydrogenation by tetrahydromethanopterin (THMP) or a related enzyme (Fig. 7). Acetate is actively transported into microbial cells as acetyl coenzyme A, and in this form the hydrogens of the methyl group are slightly acidic and thus prone to exchange with hydrogens in the medium. However, the absence of *D-H* exchange under sulfate-reducing conditions indicates that isotopic exchange most probably occurs later, during either the methyl-THMP dehydrogenation process or the methyl transfer to coenzyme M in methanogens (Fig. 7).

The observed ^{13}C-^{12}C-exchange of the carboxyl group of acetate (Fig. 2) is most probably due to a reversible exchange of bonded ^{13}CO with $^{12}CO_2$ from the medium via CODH (Fig. 7). Such an exchange has previously been observed in a culture of *Methanosarcina thermophila* (13). The observation of isotypically exchanged forms of acetate in pore water in these experiments indicates that the biochemical mechanism of acetate metabolism to CO_2 and CH_4 is partly reversible.

Despite the D-H exchange of the methyl group and ^{13}C-^{12}C exchange of the carboxyl group of acetate, the methyl carbon is almost stoichiometrically transformed (89%) to methane (Fig. 3). Less than stoichiometric conversion is consistent with the presence of the competing THMP and additional pathways for metabolism of the methyl group (Fig. 7), which accounted for 11% of the ^{14}C-labeled methyl group forming $^{14}CO_2$ in these experiments. The relative importance of this pathway depends upon the hydrogen concentration. For example, hydrogen uptake by sulfate- or ferric ion-reducing bacteria can change the metabolism of the acetate methyl group completely to carbon dioxide (1). If the D-H exchange is indeed a result of the THMP pathway, the possibility arises that methane production from acetate and H_2/CO_2 takes place in the same organism. Hence, the 13% of methane from H_2/CO_2 measured in this study does not necessarily indicate the activity of a separate population of H_2/CO_2-utilizing methanogens.

Further opportunity for isotopic exchange in methanogens may occur via reversal of the methane formation pathway (reduction of acetyl coenzyme M), although this accounts for a maximum of only 2% of methane formation (19).

Despite the presence of the CODH pathway in sulfate-reducing bacteria (18), the lack of carboxyl ^{13}C-^{12}C exchange or methyl D-H exchange indicates that the acetate metabolism pathway in sulfate-reducing bacteria may differ slightly from that in methanogens or that the flow through this pathway may be sufficiently rapid to prevent significant isotopic exchange.

Calculation and comparison of rates of acetate turnover. As there is isotopic exchange of the carboxyl ^{13}C and methyl D of labeled acetates, it would be incorrect to determine turnover rates from the depletion of the initially added labeled acetate (Table 1). To account for this isotopic exchange, the turnover rate should be based on the metabolism of the sum of all labeled acetates.

The various estimates of acetate metabolism to methane are compared in Fig. 8, with total methane production minus methane production from H_2/CO_2 considered to be independent estimate of acetate turnover to methane (dotted line).

Overestimation of the turnover when using stable isotopes is due to the incorrect use of pseudo-first-order kinetics (rate constant × pool size [Table 1]), because the decrease in pool size during incubation (Fig. 1 and 2) results in non-steady-state conditions. Application of the non-steady-state model demon-

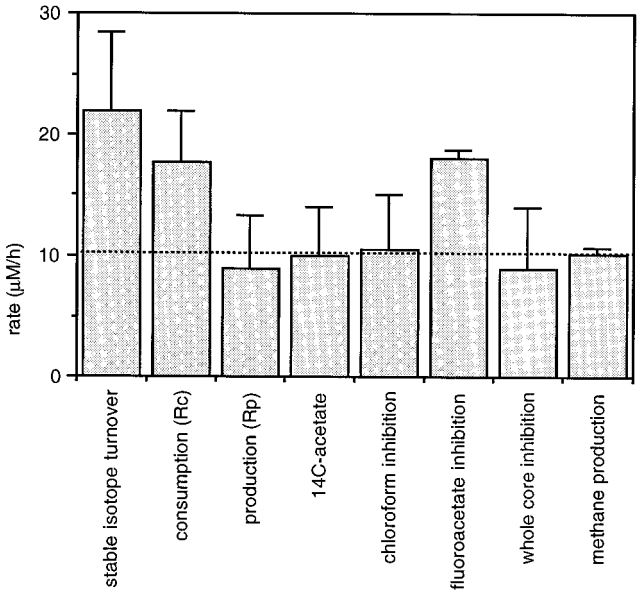

FIG. 8. Combined results of the estimation of acetate cycling by turnover and inhibition techniques in mixed sections and whole cores from the bottom sediment of Lake Vechten, taken on 25 October 1994.

strates the stimulation of acetate consumption as a result of the elevation of the acetate pool size (Fig. 8, R_c). In contrast, calculated production rates yield reasonable estimates of the acetate turnover (Fig. 8, R_p). The accuracy of this determination is impaired by the large scatter in the pool size data. Similarly, estimates of acetate turnover based on ^{14}C-labeled tracers are dependent upon accurate determination of the small pool size. The rate of acetate accumulation after inhibition with chloroform gives a good, direct estimate of the acetate production rate and is easy to measure by GC (Fig. 8). This approach works equally well in intact sediment cores, in which the diffusion of chloroform is apparently complete well within the first hour of incubation. Conversely, acetate turnover is overestimated following inhibition with fluoroacetate.

Conclusions. (i) The uptake of acetate in methanogenic freshwater sediment is associated with isotopic exchange of methyl hydrogen and carboxyl carbon, although the methyl carbon is transformed mainly into methane.

(ii) Radioisotope or stable isotope labeling of the methyl group of acetate is useful for the estimation of its turnover rate, although both methods have drawbacks: the former method is hampered by the estimation of the small free-pool size, and the high acetate additions required for the latter cause deviations from pseudo-first-order kinetics. The use of a non-steady-state model overcomes this problem, but the problem of accurately measuring the free pool of acetate remains.

(iii) Inhibition of acetate uptake by chloroform provides a reasonable estimate of the acetate production rate. It can be applied to intact cores, and the method also works well when sulfate reduction is the dominant acetate-consuming process.

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