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Methanogenesis from Methanol and Methylamines and Acetogenesis from Hydrogen and Carbon Dioxide in the Sediments of a Eutrophic Lake†

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^{14}C-tracer techniques were used to examine the metabolism of methanol and methylamines and acetogenesis from hydrogen and carbon dioxide in sediments from the profundal and littoral zones of eutrophic Wintergreen Lake, Michigan. Methanogens were primarily responsible for the metabolism of methanol, monomethylamine, and trimethylamine and maintained the pool size of these substrates below 10 μM in both sediment types. Methanol and methylamines were the precursors for less than 5 and 1%, respectively, of the total methane produced. Methanol and methylamines continued to be metabolized to methane when the sulfate concentration in the sediment was increased to 20 mM. Less than 2% of the total acetate production was derived from carbon dioxide reduction. Hydrogen consumption by hydrogen-oxidizing acetogens was 5% or less of the total hydrogen uptake by acetogens and methanogens. These results, in conjunction with previous studies, emphasize that acetate and hydrogen are the major methane precursors and that methanogens are the predominant hydrogen consumers in the sediments of this eutrophic lake.

Hydrogen metabolism and acetate metabolism by methanogens in freshwater sediments have been studied extensively, but several other potential mechanisms for methane production and hydrogen consumption in lake sediments have received little attention. These reactions which have been demonstrated with pure cultures (1, 2, 4, 9, 12, 14, 18, 19) are shown in Table 1. Potential methane precursors other than hydrogen and acetate include methanol (reactions 1 to 3) and methylamines (reactions 4 to 6). It has been suggested that the failure to measure the contribution of these substrates to methane production may have resulted in errors in proposed carbon budgets for sediments (13). [^{14}C]methanol is metabolized to [^{14}C]methane in sediments (13, 20), and the potential for methylamines to serve as methane precursors in sediments has been demonstrated in artificial sediment-slurry systems (4, 13). However, the significance of these substrates to total methane production in freshwater sediments has not been determined. Studies on hydrogen metabolism in sediments have suggested that populations other than methanogens may consume hydrogen (16, 20). Although sulfate reducers can be important in the hydrogen uptake in sediments of lakes with low rates of organic input to the sediment (7), they metabolize a minor fraction of hydrogen produced in productive lakes (5, 6). Another potential hydrogen-consuming population is the hydrogen-oxidizing acetogenic bacteria which use hydrogen to reduce carbon dioxide to acetate (reaction 7). Hydrogen-oxidizing acetogens are present in sediments (3), but the importance of acetogenesis in consuming hydrogen in situ has not been quantified. The purpose of this study was to determine the significance of hydrogen uptake by hydrogen-oxidizing acetogens and the metabolism of methanol and methylamines by methanogens in the sediments of eutrophic Wintergreen Lake in an attempt to make our previous model of carbon and electron flow in these sediments (6) more complete.

MATERIALS AND METHODS

Surface sediments were collected during summer stratification with an Eckman dredge from two sites in Wintergreen Lake, Michigan (10). The profundal sediments (depth 6.5 m) receive a highly proteinaceous input of sedimenting blue-green algae (cyanobacteria). Methanogenesis is the predominant terminal process in the sediments (5, 6, 11). The littoral sediments were

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TABLE 1. Equations for the conversion of methanol and methylamines to methane and the consumption of hydrogen by acetogens

Reaction[a]	$\Delta G^{0\prime}$(kJ)
1. $4\ CH_3OH \rightarrow 3\ CH_4 + HCO_3^- + H^+ + 2\ H_2O$	−315
2. $4\ CH_3OH + CH_3COO^- \rightarrow 4\ CH_4 + 2\ HCO_3^- + H^+$	−346
3. $CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−113
4. $4\ CH_3NH_3^+ + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 4\ NH_4 + H^+$	−225
5. $2\ (CH_3)_2NH_2^+ + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3 + 2\ NH_4^+ + H^+$	−220
6. $4\ (CH_3)_3NH^+ + 9\ H_2O \rightarrow 9\ CH_4 + 3\ HCO_3^- + 4\ NH_4^+ + 3\ H^+$	−670
7. $4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O$	−105

[a] Stoichiometry and free energy change for reactions 1 to 6 and 7 are from references 8 and 1, respectively.

sampled at a depth of 0.5 to 1 m. Aquatic macrophytes comprise the major input to these sediments (P. Salvas, M.S. thesis, Michigan State University, East Lansing, 1979). Oxygen, nitrate, and sulfate are depleted from the water immediately overlying the littoral sediments due to the high rates of sediment decomposition (Klug, unpublished data), and thus methanogenesis is considered to also be the terminal step of decomposition at this site.

The sediments were transported to the laboratory in sealed canning jars. Aliquots (10 ml) were transferred to anaerobic pressure tubes (Bellco Glass, Inc.) under a flow of nitrogen that had been passed through a column of heated copper filings to remove oxygen. The tubes were sealed with a butyl rubber stopper and an aluminum crimp. Profundal and littoral sediments were incubated at 10 and 18°C, respectively, to approximate in situ temperatures.

To examine the effect of sulfate on the metabolism of methanol and methylamines, 500 ml of profundal sediments was incubated at 10°C in 1-liter reagent bottles with slow rolling on a bottle roller (Bellco Glass). Ferrous sulfate was added to give a final concentration of 20 mM. Sediments were transferred to pressure tubes as above before the addition of ^{14}C-substrates.

Total methane production rates were calculated from the increase in methane concentration over time in the same sediments that were used for the tracer studies. Nitrogen-flushed solutions of ^{14}C-substrates (0.25 ml) were added to the sediments, and the sediments were shaken vigorously for 15 to 30 s. The substrates added were: 78.5 nCi of [^{14}C]methanol (56.9 mCi mmol^{-1}, Amersham Corp.), 648.6 nCi of [^{14}C]monomethylamine (51.8 mCi mmol^{-1}, New England Nuclear Corp.), and 95.7 nCi of [^{14}C]trimethylamine (5.0 mCi mmol^{-1}, New England Nuclear). The incubations were stopped by adding glutaraldehyde (8% final concentration). First-order turnover rate constants, k, were calculated from $k = f/t$ where f is the fraction of added label metabolized to product over an incubation time of t. The substrate concentration, which equalled the sum of the in situ pool and the amount of substrate added with the ^{14}C-substrate solution, was multiplied by k to estimate the rate of substrate metabolism.

To determine the fraction of total acetate production derived from the reduction of carbon dioxide, 0.3 ml of a nitrogen-flushed solution containing 5 μCi of [^{14}C]bicarbonate (7.8 mCi mmol^{-1}, New England Nuclear) was injected into tubes of sediment. The specific activity of methane and carbon dioxide was measured after an initial equilibration period of 30 min and again after a 24-h incubation period. ^{14}C disintegrations per minute in the acetate pool (determined as outlined below) were combined with measurements of the acetate pool (see below) to determine the specific activity of acetate. The specific activity of acetate on a per-carbon basis was divided by the specific activity of the carbon dioxide pool to calculate the fraction of acetate produced from carbon dioxide. The fraction of methane produced from carbon dioxide was similarly determined by comparing the specific activity of the methane produced during the incubation period with the specific activity of carbon dioxide.

Analytical techniques. Methanol, methylamines, and acetate were analyzed by gas chromatography on a Varian 3700 gas chromatograph equipped with dual-flame ionization detectors. Interstitial water for pool size analysis was collected by centrifugation. Interstitial water (100 ml) was distilled at 98°C, and 1.0 to 1.5-ml fractions of the distillate were collected for methanol analysis (13). Methanol was separated on a 2-m glass column of Porapak Q at 125°C with a helium carrier. Recoveries of methanol added to interstitial water were 80%, and pool size estimates were corrected for this factor.

For the analysis of methylamines, 200 to 500 ml of interstitial water was acidified (pH < 3) with phosphoric acid and evaporated at 70°C under a stream of air in a 120-ml serum bottle. Ammonium sulfate (20 mg) was added to the bottles which were then sealed with a Teflon-coated septum (Supelco, Inc.) and an aluminum crimp. Sodium hydroxide (10 ml, 10 N) was injected into the bottles which were then placed on a rotary shaker at 350 rpm for 3 h at room temperature. Samples of the headspace gas (1 ml) were separated on a 2-m glass column of Carbopack B–4% Carbowax 20 M–0.8% KOH (Supelco) at 85°C with helium as the carrier. Recoveries of monomethylamine and trimethylamine added to interstitial water were 65 and 84%, respectively, and pool size estimates were corrected accordingly.

To analyze acetate concentrations, potassium hydroxide was added to 5 ml of sample in a 10-ml serum bottle to raise the pH above 9. The samples were evaporated to dryness at 80°C under a stream of air. Dried samples were reconstituted with 0.5 ml of 10% phosphoric acid, and the volatile fatty acids were extracted with a vacuum distillation procedure adapted from Sørensen et al. (15). Several Teflon boiling chips were added to the bottle which was then sealed with a silicone stopper and aluminum crimp and connected to a similarly capped bottle with a bent glass

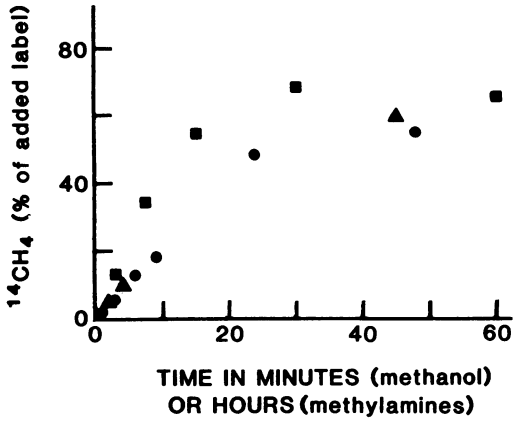

FIG. 1. Representative examples of the metabolism of [^{14}C]methanol (■), [^{14}C]methylamine (●), and [^{14}C]trimethylamine (▲) to [^{14}C]methane in Wintergreen Lake sediments.

tube (3-mm inside diameter, 5-mm outer diameter). A vacuum was placed on the system with a pump connected to a needle that pierced the cap of the stopper on the empty serum bottle. The needle was removed, and the empty bottle was placed in an ethanol-dry ice bath. The bottle containing the sample was placed on a hot plate, and the volatile fatty acids and water were distilled over and frozen in the other bottle. The recovery of acetate was greater than 90%. The distilled samples were separated on a 2-m glass column packed with 15% SP1220–1% H_3PO_4 on Chromosorb W (Supelco) at 125°C with helium as the carrier.

For determination of radioactivity in the acetate pool, the interstitial water was filtered through 0.22-μm pore size filters (Millipore Corp.). Carrier acetate solution (100 μl) was added to 1 ml of the filtered water to give a final concentration of approximately 18 mM. Fatty acids were separated on a HPX-187 column (Bio-Rad Laboratories) with 0.01 N H_2SO_4 as the solvent at a flow rate of 0.75 ml/min. The acid peaks were detected by measuring absorbance at 210 nm with a flow cell detector (Hitachi). Individual acid peaks were collected in 0.1 ml of 1 N NaOH in a scintillation vial. A 15-ml amount of Instagel (Packard, Inc.) was added, and the vials were counted in a Beckman LS 8500 scintillation counter.

Methane and carbon dioxide were separated at 45°C on a 3-m column of Porapak N with a helium carrier. The mass analyses were made on a Varian 3700 equipped with a thermal conductivity detector which was connected in series with a proportional counter for detection of radioactivity. Total inorganic ^{14}C was corrected for dissolved inorganic ^{14}C with an empirical factor based on the distribution of radioactivity after the injection of [^{14}C]bicarbonate into tubes of sediment.

RESULTS

Methanogenesis from methanol and methylamines. The metabolism of added [^{14}C]methanol to [^{14}C]methane was relatively rapid and was complete within 0.5 to 1 h (Fig. 1). Monomethylamine and trimethylamine were metabolized to methane more slowly (Fig. 1). The production of [^{14}C]methane was linear over the initial three time points (including zero time), and thus the fraction of label evolved as methane at time points less than 5 min for methanol and less than 5 h for methylamine and trimethylamine was used for rate estimates in these and replicate experiments. The recoveries of added label as [^{14}C]methane and [^{14}C]carbon dioxide in long-term incubations were greater than 80% for all three substrates, which indicated that most of the added label was available for bacterial uptake and was not consumed by abiological processes.

The pool size of each substrate was less than 10 μM in both the profundal and the littoral sediments (Table 2). The concentrations in the littoral sediments were generally less than those in the profundal sediments. The production of methane from all three substrates was low rela-

TABLE 2. Methanol, monomethylamine, and trimethylamine pool sizes, rates of metabolism, and relative importance to total methane production in the profundal and littoral sediments of Wintergreen Lake

Substrate	Site	Pool size[a] (μM)	Substrate conversion to methane (μmol/liter per h)	% of total methane production
Methanol[b]	Profundal	0.2–3.1	3.7 ± 0.4	4.0
Methanol	Littoral	0.04–0.7	0.8 ± 0.3	2.8
Monomethylamine[c]	Profundal	3.3–8.9	0.3 ± 0.1	0.4
Monomethylamine	Littoral	2.1–2.4	0.07 ± 0.02	0.3
Trimethylamine[d]	Profundal	0.2–2.2	0.09 ± 0.01	0.2
Trimethylamine	Littoral	0.2–0.2	0.04 ± 0.01	0.1

[a] Pool sizes are ranges observed over a period from 25 May 1982 to 24 July 1982.

[b] Methanol rates are the mean ± the standard error of the means of replicate determinations (n = 3, two dates; n = 2, one date) on three separate dates.

[c] Monomethylamine rates are the mean ± the standard error of the means of triplicate determinations on two separate dates.

[d] Trimethylamine rates are the mean ± the standard error of triplicate determinations on one date.

TABLE 3. Metabolism of methanol and methylamines in untreated and sulfate-amended profundal sediments

Substrate	% of substrate metabolized to methane[a]			
	6 days[b]		14 days[b]	
	Control	Sulfate	Control	Sulfate
Methanol	76 ± 9	58 ± 3	82 ± 10	63 ± 2
Methylamine	73 ± 1	59 ± 2	76 ± 6	62 ± 1
Trimethylamine	52 ± 2	45 ± 0.1	45 ± 1	42 ± 1

[a] $^{14}CH_4$ production as a percentage of total $^{14}CH_4$ and $^{14}CO_2$ production. The mean ± the standard error of duplicate determinations is given.

[b] Sediments were incubated for 6 and 14 days with no additions (control) or with 20 mM ferrous sulfate added (sulfate) before the addition of labeled compounds.

tive to total methane production (Table 2). The estimates of the rates of conversion of methanol and methylamines to methane are probably higher than the actual rates in the sediments since the addition of the ^{14}C-labeled compounds increased the in situ pool from approximately 10% with the [^{14}C]methanol additions to profundal sediments to ninefold with the [^{14}C]trimethylamine additions to littoral sediments. The increased pool size was taken into account for the rate estimates, and thus the reported rates are for metabolism at a higher degree of substrate saturation than at the observed in situ concentrations.

Addition of sulfate to the sediments inhibited total methane production as previously demonstrated (5). [^{14}C]methane production from [^{14}C]methanol and [^{14}C]methylamines was slightly lower in sulfate-amended sediments than in controls with a corresponding increase in [^{14}C]carbon dioxide production (Table 3). The partitioning of the added label between [^{14}C]methane and [^{14}C]carbon dioxide in sulfate-amended sediments remained nearly constant even after an additional week of incubation (Table 3).

H_2 uptake by acetogens. The percentage of total acetate that was produced from the reduction of carbon dioxide was less than 2% at both sediment sites (Table 4). From the percentage of methane produced from hydrogen and carbon dioxide (Table 4) and with the assumption that methane not produced from hydrogen is derived from acetate, a maximum estimate of the rate of total acetate production could be made from the equation, MP × (1 − HM) ÷ 0.8, where HM is the fraction of total methane produced from hydrogen and MP is the total methane production rate. Division by 0.8 is necessary since only 80% of the acetate pool is metabolized to methane in these sediments (6). The rate of hydrogen consumption by acetogens could thus be calculated by multiplying the total acetate production rate by the fraction of acetate produced from carbon dioxide reduction and multiplying this by the 4 mol of hydrogen necessary for the production of 1 mol of acetate from carbon dioxide. Hydrogen consumption by methanogens could be calculated as HM × MP × 4, where 4 represents the number of moles of hydrogen required to produce a mole of methane. Comparison of the hydrogen uptake by the two processes indicated that hydrogen uptake by hydrogen-oxidizing acetogenic bacteria was 5% of the hydrogen uptake by methanogens in the profundal sediments and less than 1% in the littoral sediments (Table 4).

DISCUSSION

The results of this study fit our previous working model of decomposition in Wintergreen Lake sediments (6) in which acetate and hydrogen are the primary methane precursors and methanogens are the primary hydrogen consumers. Although methanol and methylamines were converted to methane, and carbon dioxide was fixed into acetate, the overall significance of these processes was minor in relation to total carbon and electron flow in sediments receiving a carbon input of primarily phytoplankton (profundal sediments) as well as in sediments with a large input of aquatic macrophytes (littoral sediments). The small contribution of methanol and methylamines to total methane production can be explained by the low availability of substrates that are metabolized to methanol and methyl-

TABLE 4. Acetogenesis from hydrogen and carbon dioxide and its importance in consuming hydrogen relative to methanogenesis in the profundal and littoral sediments of Wintergreen Lake

Site	Amt (%) produced from CO_2		Total production rate (μmol/liter per h)		Hydrogen consumption (μmoles/liter per h)	
	Acetate	Methane	Methane	Acetate[a]	Methanogenesis[a]	Acetogenesis[a]
Profundal	1.8 ± 0.5[b]	31 ± 1	43 ± 5	37	54	2.7
Littoral	0.5 ± 0.2	46 ± 3	17 ± 5	11	31	0.2

[a] Calculated as outlined in the text.

[b] Mean ± the standard error.

amines relative to the many substrates that can be fermented to hydrogen and acetate.

The distribution of [^{14}C]methane and [^{14}C]carbon dioxide produced from [^{14}C]methanol suggests that the methanogens in the sediments primarily metabolized methanol according to reaction 1 (Table 1) and that methanol was not significantly used as an electron acceptor for acetate (reaction 2, Table 1) or hydrogen oxidation (reaction 3, Table 1). The stoichiometry of [^{14}C]methylamine metabolism in untreated sediments (Table 3) closely approximated the 75% methane/25% carbon dioxide ratio predicted from pure cultures (reaction 4, Table 1). However, less methane was produced from trimethylamine than has been observed in cultures of methanogens (reaction 6, Table 1), which indicates that the metabolism of trimethylamine by the methanogens in the sediments was different or that other bacterial populations metabolized a portion of the trimethylamine by as yet to be described pathways.

Metabolism of methanol and methylamines may help methanogens maintain their population during periods of sulfate intrusions into the sediments. Although sulfate reducers can outcompete methanogens for hydrogen and acetate when sulfate concentrations are increased in freshwater sediments (5, 7, 21), sulfate reducers were poor competitors for methanol and methylamines in sulfate-amended sediments. The slightly greater production of carbon dioxide from methanol and methylamines in sulfate-amended sediments probably reflected the need for methanogens to oxidize more of these substrates to generate reducing equivalents when hydrogen and acetate were not available rather than indicating the utilization of the substrates by sulfate reducers. The metabolism of methanol and methylamines to carbon dioxide should have increased between 6 and 14 days of incubation in sulfate-amended sediments if sulfate reducers were capable of metabolizing these substrates. Thus, the observation that methanogens survive in sulfate-amended freshwater sediments (5, 21) may be explained by continued methane production from methanol and methylamines. Oremland and co-workers (13) have suggested that methanol and methylamines are important methane precursors in marine sediments in which sulfate reduction is the dominant terminal process. We have observed that 35% of the added [^{14}C]methanol and 45% of the added [^{14}C]methylamine are converted to [^{14}C]methane even in the sediments of an oliogtrophic lake with oxygen and nitrate in the water overlying the sediments (unpublished results).

The relatively small amount of hydrogen metabolized by hydrogen-oxidizing acetogenic bacteria in Wintergreen Lake sediments compares well with results on the mesophilic anaerobic digestion of cattle waste (8) and with the finding that the number of acetogens in lake sediments is small relative to the number of hydrogen-utilizing methanogens (3). Kinetic analysis of hydrogen uptake had previously suggested that methanogens and sulfate reducers were the only two potentially important hydrogen-consuming populations in Wintergreen Lake sediments (5). Hydrogen utilization by hydrogen-oxidizing acetogens in Wintergreen Lake sediments was actually overestimated since bacteria that do not use hydrogen as an electron donor can also produce acetate from carbon dioxide (17). The predominance of methanogens in hydrogen uptake is expected from thermodynamic considerations. The acetate concentration in the sediments ranges from 10 to 300 μM (11). Based on best estimates of the dissolved inorganic carbon concentration (15 mM) and the hydrogen (1 Pa) and methane (150 kPa) partial pressures in the sediment, the energy available from the conversion of hydrogen to acetate ranges from a $\Delta G'$ of +1.2 kJ per mol of hydrogen consumed at 300 μM acetate to −0.8 kJ at 10 μM acetate. The estimated energy yield of methanogenesis from hydrogen is 4 kJ per mol of hydrogen. Thus, methanogens may be able to outcompete hydrogen-oxidizing acetogens by maintaining the hydrogen partial pressure too low for acetogens to obtain enough energy from the metabolism of hydrogen. Since sulfate reducers have a higher affinity for hydrogen than methanogens (5), the production of acetate from hydrogen and carbon dioxide is probably an even less important hydrogen-consuming process in freshwater sediments during periods of sulfate intrusions and in marine sediments.

In summary, studies to date have failed to identify a significant hydrogen-consuming population other than methanogens in Wintergreen Lake sediments. The importance of hydrogen and acetate (5, 6, 16) and the minor contribution of methanol and methylamines to methanogenesis have been demonstrated. As yet to be determined are the carbon flow through formate, the other major potential methanogenic precursor, and whether or not formate is utilized directly by methanogens in these sediments (16).

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