Factors Affecting Methanogenesis and Associated Anaerobic Processes in the Sediments of a Stratified Eutrophic Lake

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Factors affecting methanogenesis in the sediments of a eutrophic lake were studied during late summer, a period during which CH_4 gas production slowed down dramatically or stopped completely. The most active methanogenesis occurred in the surface sediments and the temperature optimum for the process in these deeper sediments was 30 °C. Addition of H_2 or formic acid to sediment slurries stimulated CH_4 production to a greater extent than did acetic or pyruvic acid. Analysis of the kinetics of the conversion of H_2 to CH_4 suggested that the sediments were severely limited in H_2 , the concentration being considerably less than $2.5 \mu\text{mol l}^{-1}$, the K_m for the process. Methanogenesis was not stimulated by the addition of trace quantities of Ni^{2+} , Co^{2+} , MoO_4^{2-} or Fe^{2+} ions but was inhibited by $0.5 \text{ mmol SO}_4^{2-} \text{ l}^{-1}$. Under natural conditions the sediments were also limited in SO_4^{2-} and sulphate reducers acted as net H_2 donors to the methanogens; addition of SO_4^{2-} allowed the sulphate reducers to compete effectively for H_2 . The addition of $20 \text{ mmol Na}_2\text{MoO}_4 \text{ l}^{-1}$ to sediments inhibited methanogenesis but this was not due entirely to its effect in the H_2 transfer from sulphate reducers; it also inhibited CO_2 uptake by sediments and the production of CH_4 from CH_3COOH and CO_2 by cultures of methanogens. It is therefore inadvisable to use MoO_4^{2-} at this concentration as a specific inhibitor of sulphate reducers in such freshwater sediments. Experiments with other inhibitors of methanogens suggested that they may interact with sulphate reducers, acetogens or anaerobic bacteria involved in fatty acid decomposition. Small, sealed sediment cores, which were used to reproduce natural conditions, particularly of available H_2 concentration, were injected with trace quantities of $\text{H}^{14}\text{CO}_3^-$ and $^{14}\text{CH}_3\text{COOH}$. The results suggested that more than 75% of the CH_4 was derived from CO_2 and the remainder from CH_3COOH . The overall rates of methanogenesis in the small cores agreed well with results from the field.

INTRODUCTION

Whereas, in the past, decomposition processes in lakes have been considered largely in terms of aerobic respiration and the oxygen consumption of the sediment (Hargrave, 1969; Wetzel *et al.*, 1972; Jones, 1976), more recent attention has focused on anaerobic processes. SO_4^{2-} reduction is an important component of carbon turnover in marine sediments (Fenchel & Blackburn, 1979; Jørgensen, 1980) but it plays a less significant role in fresh water (Jones & Simon, 1980, 1981), where denitrification and methanogenesis are quantitatively more important. In certain North American lakes more than half the total carbon input to the sediment may be converted to CH_4 (Rudd & Hamilton, 1978; Fallon *et al.*, 1980) but this pathway is quantitatively less important in temperate lakes of the English Lake District (Jones & Simon, 1980). The interaction of methanogens with other anaerobes (particularly sulphate reducers) in freshwater sediments was first studied experimentally by Cappenberg (1974*a, b*) and Cappenberg & Prins (1974) who concluded that sulphate reducers converted

lactate to acetate which, in turn, was used by the methanogens. They calculated that most (70%) of the CH_4 generated in freshwater sediments was derived from acetate and that accumulation of sulphide inhibited methanogenesis. The demonstration by Bryant *et al.* (1977) that, in the absence of SO_4^{2-} , *Desulfovibrio* could act as a net H_2 donor to methanogens provided a possible alternative explanation for events in SO_4^{2-} -limited freshwater sediments. The addition of SO_4^{2-} to such sediments inhibits methanogenesis (Winfrey & Zeikus, 1977) and under these circumstances it is possible to demonstrate competition between the anaerobic bacteria for H_2 and acetate. This is in marked contrast to the marine system where, in the presence of excess SO_4^{2-} , the sulphate reducers compete successfully with methanogens for H_2 (Abram & Nedwell, 1978).

Some investigations of the relative importance of acetate and H_2/CO_2 as precursors of CH_4 in freshwater sediments have been conducted on sediment slurries incubated under artificial atmospheres (Cappenberg & Prins, 1974; Winfrey & Zeikus, 1979) but evidence of limitation of methanogenesis by H_2 has been reported (Winfrey *et al.*, 1977; Strayer & Tiedje, 1978). Therefore experimental procedures which alter the natural concentration of H_2 (an extremely insoluble substrate) could introduce errors in estimates of the importance of the H_2/CO_2 pathway. However, results are being obtained which suggest that a greater proportion of CH_4 is produced by this pathway (Winfrey & Zeikus, 1979; Zaiss, 1981) than originally suggested by Cappenberg & Prins (1974).

Recent studies with gas traps in Cumbrian lakes (Jones & Simon, 1980, 1981; Jones *et al.*, 1980) have shown that the CH_4 release rate was low compared with the more eutrophic American waters. In addition, gas release often decreased dramatically or stopped completely in late summer, before the lakes overturned. Methanogens (Zeikus & Winfrey, 1976; Zaiss & Kaltwasser, 1979), like sulphate reducers (Abdollahi & Nedwell, 1979), are temperature limited in such environments, but in a stratified lake the temperature in the profundal zone is relatively constant (6 °C to 8 °C) at this time of year. The purpose of this investigation was threefold: to determine what limited methanogenesis in late summer, to use selective inhibitors to investigate the interactions of methanogens with sulphate reducers and other anaerobes, and to determine the relative importance of CO_2 and acetate as precursors of CH_4 in such a way that the natural H_2 concentration was not altered.

METHODS

Sampling. Sediment and water samples were taken from the profundal zone and the hypolimnion of Blelham Tarn in the English Lake District during summer stratification, 1980. The site is described in more detail by Jones & Simon (1980, 1981). Sediment samples were taken with a Jenkin surface-mud sampler and the sealed cores were returned to the laboratory and sampled within 2 h. Water samples were taken with a peristaltic pump sampler at fixed distances from the sediment–water interface determined by an opto-electronic detector (Cunningham & Davison, 1980). Water samples to be analysed for gas content were taken directly into ground glass-stoppered bottles which were flushed with at least three times their own volume of water. The gas traps and sampling procedure were those described by Jones & Simon (1980).

Physical and chemical analyses. Dissolved CO_2 and CH_4 in the water and sediment and the contents of the gas traps were analysed by the methods described by Jones & Simon (1980). SO_4^{2-} was determined according to Tabatabai (1974) and S^{2-} according to Rees *et al.* (1971). Dissolved O_2 and temperature were measured in the field with a combination oxygen meter and thermistor (Model 57, Yellow Springs Instruments Co., Yellow Springs, Ohio, U.S.A.). Low molecular weight fatty acids (C_1 to C_4) were determined gas chromatographically by direct, on-column injection of 2 μl of untreated aqueous samples into a Perkin Elmer F30 gas chromatograph connected to an LDC 308 computing integrator. The 2 m (1.75 mm i.d.) glass column was packed with 5% H_3PO_4 and 15% Free Fatty Acid Phase on 60/80 mesh Chromosorb W.AW (Phase Separations Ltd, Queensferry, U.K.). The oven was isothermal at 150 °C and the injector and flame ionization detector temperature was 200 °C. The carrier gas was N_2 (40 ml min^{-1} , 552 kPa) and was mixed with H_2 (117 kPa) and air (138 kPa) at the detector. Although day-to-day performance of the column varied, the detection limit for individual fatty acids was never higher than 5 to 10 $\mu\text{mol l}^{-1}$.

Sample preparation. Wherever possible, samples were handled according to Hungate procedures to maintain strictly anaerobic conditions. The use of serum bottles and the purification of gases followed the general methods

described by Latham & Wolin (1978). Subsamples of sediment were taken as follows: (a) by extrusion of the sediment core using the device described by Jones (1976) except that the head of the core was enclosed in oxygen-free gas (N_2/CO_2 , 95:5, v/v); (b) by slow suction of surface sediment and overlying water into a Buchner flask which had been previously evacuated and filled several times with N_2/CO_2 ; (c) by insertion of small cores (Jones & Simon, 1981) which were stoppered under water and removed. The slurry samples collected according to (a) and (b) were dispensed into serum bottles. The small cores which did not contain a gas phase were used in an attempt to determine rates of methanogenesis under conditions as close as possible to those which occur in the field.

Experiments in serum bottles. Samples of sediment slurry (usually 5 ml) were dispensed into 30 ml serum bottles. Substrates, mineral nutrients and inhibitors were added as $\times 100$ strength aqueous solutions to give the final concentrations described in the text. The bottles were incubated in a shaking water bath. Modification of the gas phase was achieved by injection of a known quantity of gas through the butyl rubber septum or, if complete replacement was required, by flushing before the septum was sealed in place.

Experiments in small cores. In an attempt to maintain natural conditions, and to prevent loss of H_2 from surface sediment samples, rates of conversion of $\text{H}^{14}\text{CO}_3^-$ and $^{14}\text{CH}_3\text{COOH}$ to $^{14}\text{CH}_4$ were determined in sealed small cores, by a method analogous to that of Jørgensen (1978) for SO_4^{2-} reduction. No additions of gas were made to these cores and it was assumed that the concentration of H_2 was unaltered by this handling procedure. Cores were inoculated at 1 cm intervals with either 20 μl $^{14}\text{CH}_3\text{COOH}$ solution (equivalent to 0.25 μCi , specific activity 59 mCi mmol $^{-1}$, final concentration of added substrate 0.93 $\mu\text{mol l}^{-1}$) or 50 μl $\text{NaH}^{14}\text{CO}_3$ solution (equivalent to 0.76 μCi , specific activity 57.3 mCi mmol $^{-1}$, final concentration of added substrate 13.3 $\mu\text{mol l}^{-1}$) (1 mCi = 37 MBq). These additions of $^{14}\text{CH}_3\text{COOH}$ and $\text{NaH}^{14}\text{CO}_3$ were equivalent to approximately 2.5% and 0.4%, respectively, of the natural concentrations of these methanogenic substrates. They could therefore be treated as trace additions, and the calculation of the turnover rate was identical to that used by Jørgensen (1978) for SO_4^{2-} reduction. When required, solutions of Na_2MoO_4 (45 μl) and K_2SO_4 (10 μl) were added with the radioactive substrates giving final concentrations of 20 mmol l^{-1} and 0.5 mmol l^{-1} respectively. The small cores were incubated for 24 h at 10 °C and then frozen at -20 °C. The frozen sediment cores were extruded and sawn into segments at depth intervals of 1 cm. The $^{14}\text{CH}_4$ and $^{14}\text{CO}_2$ were then recovered by a method based on that of Zehnder *et al.* (1979). The frozen sediment segments were placed in 30 ml Teflon open top septum vials (Tuf Tainers, Pierce & Warriner Ltd, Chester, U.K.) and 0.5 ml 10 M-NaOH was added to retain $^{14}\text{CO}_2$ and $^{14}\text{CH}_3\text{COOH}$ in solution. The sediment was thawed and a subsample of the headspace containing $^{14}\text{CH}_4$ was injected into a scintillation vial which contained 20 ml of the toluene-based fluor. The recovery with this procedure was consistently about 76% and results were corrected for this. The total $^{14}\text{CH}_4$ content of the septum vial was calculated from Henry's law and published values of the constant K . To determine $^{14}\text{CO}_2$, 2 ml of 1.5 M- H_2SO_4 was then added to the sediment to which $^{14}\text{CH}_3\text{COOH}$ had been added and further subsamples of head space, now also containing $^{14}\text{CO}_2$, were injected into a serum bottle containing 0.25 ml phenethylamine, and left overnight. Excess $^{14}\text{CH}_4$ was flushed out for 1 min with N_2 , and the phenethylamine was incorporated in 10 ml of a fluor containing 100 g naphthalene, 4 g PPO and 0.05 g POPOP in 1 l 1,4-dioxan, to which a further 100 ml of phenethylamine was added. The total $^{14}\text{CO}_2$ content in the septum vial was calculated from Henry's law as described above. The efficiency of all separation and absorption procedures was checked with known quantities of CH_4 , $^{14}\text{CO}_2$ and $^{14}\text{CH}_3\text{COOH}$. The radioactivity in the samples was then measured in a Tracor Analytic Mark III liquid scintillation counter (Nuclear Chicago Ltd). Samples were corrected for quench with a ^{133}Ba external standard, by plotting the inverse of the relative pulse height against efficiency. This procedure gives better linearity than the channels ratio method.

Enumeration of anaerobic bacteria. Numbers of sulphate reducers were determined by a most probable number (MPN) procedure (Jones & Simon, 1981) using the medium of Postgate (1963) except that acetate- as well as lactate-utilizing bacteria were counted. Tubes in which a black precipitate of FeS developed were scored as positive. The MPN of acetate-utilizing and H_2/CO_2 -utilizing methanogens was determined in serum bottles using the liquid medium described below. Precautions to maintain strictly anaerobic conditions were observed during all stages of dilution and inoculation. Production of CH_4 in the bottles was determined gas chromatographically. The MPN tubes for sulphate reducers were incubated at 30 °C for 34 d and the serum bottles were checked for the production of CH_4 after 12, 19 and 26 d incubation at 30 °C. The contents of the most dilute positive tubes were used to culture methanogens for inhibitor studies.

Culture media for methanogens. The medium was essentially that of Patel *et al.* (1978). The final concentrations of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, NiCl_2 , $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (each 1 mg l^{-1}) and FeSO_4 (150 mg l^{-1}) were high enough to ensure adequate supplies of these micronutrients (Schonheit *et al.*, 1979) as well as Fe^{2+} and SO_4^{2-} (Patel *et al.*, 1978; Hoban & van den Berg, 1979). Acetate-utilizing methanogens were grown in a medium containing 2.5 mg $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O l}^{-1}$ under an atmosphere of N_2/CO_2 (95:5). The medium for hydrogen-utilizing methanogens contained no acetate and the gas phase was H_2/CO_2 (95:5). Primary enrichments of the two types of methanogens were obtained in liquid medium and then subcultured on to the same medium solidified with 1.5% agar and allowed to set as a slope in 50 ml serum bottles. Individual colonies were then returned to liquid medium.

and this process was repeated twice further before the cultures were assumed to be pure and used for experimental purposes. The cultures were checked for purity under phase-contrast illumination and for the presence of coenzyme F_{420} under ultraviolet epifluorescence microscopy.

Analysis of data. Where possible, 95% confidence limits of variables have been calculated, but when there were insufficient data to provide information on the parent distribution of any given variable, the results are presented as means with standard deviations.

RESULTS AND DISCUSSION

The field data obtained in 1980 are summarized in Fig. 1; the concentrations of dissolved gases were those found in water samples taken from 0.25 m above the mud. A rapid decrease in dissolved O_2 was observed during May and concentrations of dissolved CH_4 and CO_2 increased throughout the summer. The slight increase in the rate of dissolved CH_4 accumulation in August coincided with a decrease in CO_2 concentration, and immediately preceded a large increase in the rate of CH_4 bubble release from the sediment (Fig. 1a). The rate of release was low in 1980, probably reflecting the poor summer (monthly rainfall values are provided in Fig. 1), and therefore the decrease which occurred at the end of August was less marked than in previous seasons. Much of the experimental work on factors affecting methanogenesis was performed during this period. The build-up of dissolved CH_4 in the water column was parallel with that which occurred in the sediment (Fig. 2). There was no evidence of significant methanogenesis in the water column. Although the highest CH_4 concentrations were found at depths of 10 cm in the sediments, the surface sediment samples were more actively methanogenic than those taken from below the surface after anoxic conditions had developed in the hypolimnion (Table 1). The optimum temperature for methanogenesis was 30 °C, which was slightly lower than that found for Lake Mendota sediments (Zeikus & Winfrey, 1976). The energy of activation was 130 kJ mol⁻¹, 50% higher than that reported for SO_4^{2-} reduction in saltmarsh sediment (Abdollahi & Nedwell, 1979), and was the same for surface sediment as for that taken from a depth of 8 cm. One might speculate that the possession of identical temperature characteristics suggests that the populations at these depths were of the same origin, and that the one which developed in the surface layer during the summer originated at greater depths. The rate of methanogenesis, as measured in serum bottles, appeared to be cold sensitive, decreasing very rapidly below 15 °C. Whether this was true cold sensitivity (Morris, 1974) or merely reflected an inadequacy of the experimental procedure, was uncertain.

Addition of H_2 (as a H_2/CO_2 mixture) to serum bottles caused an immediate stimulation of CH_4 production in slurries of surface and deeper sediments (Table 1) which was greater than that caused by equivalent quantities of acetate and pyruvate, and comparable with that obtained with formate. Rapid utilization of formate as a H_2 donor has been reported in other freshwater sediments (Strayer & Tiedje, 1978; Winfrey *et al.*, 1977). Stimulation by H_2 was always observed and ranged between 35% and 750%, but addition of acetate to sediment samples taken during the period July to November (1980) did not produce such consistent stimulation when acetate was added. On occasions, particularly during September, slight inhibition was observed, but this could not be explained in terms of seasonal changes in substrate concentrations. The concentration of acetate in the sediment interstitial water during this period varied between 20 and 70 $\mu\text{mol l}^{-1}$, which was similar to that reported by Strayer & Tiedje (1978) but almost an order of magnitude less than that found in the hypereutrophic Wintergreen Lake (Molongoski & Klug, 1980). Results of further investigations into the effect of H_2 concentration on the rate of methanogenesis showed that the process conformed to Michaelis–Menten kinetics (Fig. 3a) with a V_{max} of 94 $\mu\text{mol CH}_4 \text{ g}^{-1} \text{ d}^{-1}$ and a K_m of 2.5 $\mu\text{mol l}^{-1}$. Gas chromatographic analysis of the sediments failed to detect H_2 although the limit of detection for the method used was about 0.1 $\mu\text{mol l}^{-1}$. The K_m value was similar to that obtained by Strayer & Tiedje (1978), and the failure to detect free

Fig. 1. Seasonal changes in the concentration of gases in the profundal water of Blelham Tarn during summer stratification, 1980. Monthly rainfall figures are also shown. (a) Dissolved O_2 concentration 0.25 m above the sediment (-----), and methane bubble release from the sediment into gas traps (——). (b) Dissolved CO_2 (-----) and CH_4 (——) concentrations 0.25 m above the sediment.

Fig. 2. Depth distribution of dissolved CH_4 in profundal sediments of Blelham Tarn on June 30 (○), September 8 (●) and October 14 (□) 1980.

H_2 indicated that the sediment methanogens were limited by H_2 at this time of year. Addition of large quantities of H_2 (as a H_2/CO_2 , 95:5 mixture) to the sample could result in depletion of the CO_2 present (Fig. 3b) and therefore incubation times for such experiments were kept short.

The requirements of certain methanogens for Ni, Co and Mo ions (Schonheit *et al.*, 1979) and for optimum levels of Fe^{2+} for growth (Patel *et al.*, 1978) have been demonstrated in pure cultures, but no stimulation of methanogenesis was observed on addition of these ions at final concentrations of $1.0 \mu\text{mol l}^{-1}$ (Ni^{2+} , Co^{2+} , MoO_4^{2-}) and $500 \mu\text{mol l}^{-1}$ (Fe^{2+}) to sediment slurries sampled in late summer. Some stimulation occurred when Ni, Co and Mo were added to surface sediments in early July, but not with sediments from 8 cm depth. One

Table 1. *Effect of H₂ concentration and carbon sources on methanogenesis in samples of sediment taken from different depths*

Sample slurries were incubated in serum bottles at 30 °C for 22 h. The coefficient of variation on determinations made in triplicate was usually 5 %.

Conditions	Rate of methanogenesis [$\mu\text{mol (g dry wt)}^{-1} \text{d}^{-1}$]		
	Sample depth (cm)		
	0–1	2.5–3.5	7.5–8.5
Control	29.2	21.3	5.5
H ₂ /CO ₂ added to serum bottle			
0.7 ml	39.5	29.4	8.4
7.0 ml	67.1	47.0	13.1
Vial flushed	213.2	182.1	115.7
Acetate (100 $\mu\text{mol l}^{-1}$)	37.3	29.4	9.5
Formate (400 $\mu\text{mol l}^{-1}$)	78.4	66.8	17.6
Pyruvate (400 $\mu\text{mol l}^{-1}$)	36.2	33.4	9.2

Fig. 3. Effect of the partial pressure of H₂ on (a) methanogenesis and (b) the final concentration of CO₂ in the headspace of the serum bottle after incubation of sediment slurries at 30 °C for 21 h (O) and 45 h (●).

interpretation of these observations might be that the minor nutrients were more readily available at low E_h values. Ferrous ions did not stimulate methanogenesis but concentrations of Fe²⁺ are naturally high in anoxic profundal sediments (Jones, 1982). Addition of 500 $\mu\text{mol SO}_4^{2-} \text{l}^{-1}$ to the sediment slurries caused an immediate inhibition of methanogenesis, a result consistent with other findings from SO_4^{2-} -limited sediments (Winfrey & Zeikus, 1977; Zaiss, 1981; Zeikus, 1977). In late summer the concentration of SO_4^{2-} in profundal sediment interstitial water was less than 10 $\mu\text{mol l}^{-1}$. Under such circumstance the sulphate-reducing bacteria are limited by the electron acceptor concentration and become net hydrogen donors to the methanogens. Such an interaction was first described with pure cultures by Bryant *et al.* (1977). Addition of SO_4^{2-} allowed the sulphate reducers to compete effectively for H₂ once more (Winfrey & Zeikus, 1977; Abram & Nedwell, 1978), thus inhibiting methanogenesis.

Bearing in mind the H_2 -limited state of the sediment in late summer, we decided to investigate further the interactions of the methanogens with other potential H_2 -utilizing bacteria. At this time of year, estimates of the populations of methanogens and sulphate reducers in the sediment, as estimated by MPN procedures, were of about the same order of magnitude at 10^5 (g dry wt) $^{-1}$. Counts of acetate-utilizing methanogens were about 25 % of the counts of those which utilized H_2 and CO_2 , and lactate-utilizing sulphate reducers were six times more numerous than acetate oxidizers. The activation of SO_4^{2-} by ATP, and therefore the metabolism of sulphate reducers, is inhibited by molybdate (Peck, 1959) and this anion has been used quite extensively at a final concentration of 20 mmol l^{-1} as a specific inhibitor of sulphate-reducing bacteria in marine sediments (Oremland & Taylor, 1978). We decided to examine its effectiveness in SO_4^{2-} -limited freshwater sediments. Addition of molybdate at 20 mmol l^{-1} resulted in an 80 % inhibition of methanogenesis under a N_2/CO_2 atmosphere and 60 % inhibition under H_2/CO_2 . This might be expected in a system where sulphate reducers were acting as H_2 donors to the methanogens, but a similar inhibition in the presence of excess H_2 was unexpected. Addition of selenate (1 mmol l^{-1}), another inhibitor of sulphate reducers, had the same effect. A further examination of the effect with time showed that the inhibition was more marked, in all cases, if the inhibitor was added at the beginning of the experiment than if the addition was made after a period of incubation during which methanogenesis had started. Under an atmosphere of N_2/CO_2 and in the presence of added SO_4^{2-} , molybdate stimulated methanogenesis. This was consistent with the interaction between sulphate reducer and methanogen described above, in that the addition of SO_4^{2-} allowed the former to compete for H_2 once again. Addition of molybdate would therefore inhibit the competitor and allow the formation of more CH_4 . In the presence of excess H_2 , however, molybdate added at zero time inhibited methanogenesis whether excess SO_4^{2-} was added or not. This suggested that at the concentration used, molybdate might affect bacteria other than sulphate reducers. When H_2 was in excess, total CO_2 consumption, in both the presence and absence of excess SO_4^{2-} , was inhibited by molybdate. Analysis of these samples after storage for 7 d in the refrigerator showed that addition of molybdate had also caused an accumulation of fatty acids, particularly acetic acid. Incubation in the presence of excess H_2 stimulated the production of all fatty acids, particularly acetic, the concentration of which was at least an order of magnitude higher than those of the other acids. Lactic acid was not detected. In the presence of excess H_2 , molybdate inhibited the production of acetic, butyric and valeric acids, but not of propionic acid. The experiment was repeated with inhibitors of both sulphate reducers and methanogens, and the samples were analysed immediately. The results obtained were essentially the same and are summarized in Table 2. All the inhibitors suppressed methanogenesis, chloroform being the most effective, and caused the accumulation of low molecular weight fatty acids. The presence of excess H_2 stimulated methanogenesis as well as fatty acid production. Under an atmosphere of N_2/CO_2 , molybdate inhibited H_2 -utilizing organisms and fatty acids accumulated; a similar accumulation of fatty acids occurred in the presence of excess H_2 . These results might be explained in terms of inter-species hydrogen transfer between anaerobic bacteria which degrade fatty acids and the methanogens and sulphate reducers which act as H_2 sinks. The decomposition of the fatty acids becomes thermodynamically feasible only under the low partial pressures of H_2 created by the methanogens and sulphate reducers; similar syntrophic associations have been demonstrated in anaerobic digester sludge (Boone & Bryant, 1980; McNerney *et al.*, 1979). The inhibition of acetate formation at low temperatures by molybdate in the presence of a high partial pressure of H_2 is less easily explained, unless the anion affected acetogenic bacteria directly. The accumulation of large quantities of acetic acid at temperatures comparable to those found in the profundal zone suggested that acetogens might be more important in the Blelham Tarn sediments than in those studied by Braun *et al.* (1979), where viable counts were two orders of magnitude lower than those of the methanogens.

Further evidence of the non-specific nature of molybdate inhibition at this concentration

Table 2. *Effect of inhibitors on the production of CH₄ and the accumulation of low molecular weight fatty acids in profundal sediment slurries*

Samples were incubated at 30 °C for 24 h.

Conditions	Rate of production [$\mu\text{mol (g dry wt)}^{-1} \text{d}^{-1}$]			
	CH ₄	Acetic acid	Propionic acid	Butyric acid
N₂/CO₂ atmosphere				
Control	31.1	1.2	ND	ND
+Na ₂ MoO ₄ (20 mmol l ⁻¹)	13.5	11.3	1.3	0.09
+Na ₂ SeO ₄ (1 mmol l ⁻¹)	14.5	8.7	0.3	0.05
+CHCl ₃ (0.5 %, v/v)	0.2	7.0	0.9	0.05
+BESA* (100 mg l ⁻¹)	9.2	8.0	1.9	0.2
H₂/CO₂ atmosphere				
Control	50.0	5.9	3.6	0.6
+Na ₂ MoO ₄ (20 mmol l ⁻¹)	20.4	4.4	2.2	0.1
+Na ₂ SeO ₄ (1 mmol l ⁻¹)	40.1	2.9	3.4	0.3
+CHCl ₃ (0.5 %, v/v)	0.4	5.2	1.9	0.2
+BESA* (100 mg l ⁻¹)	16.5	7.5	3.6	0.6

ND, Not detected.

* BESA, 2-Bromoethanesulphonic acid.

Fig. 4. Effect of molybdate (final concentration 20 mmol l⁻¹) on the production of CH₄ by pure cultures of (a) a H₂/CO₂-utilizing methanogen, and (b) an acetate-utilizing methanogen. ●, Control cultures; ○, molybdate added at zero time and when arrowed. Incubation temperature 30 °C.

was obtained with pure cultures of methanogenic bacteria. Both acetate-utilizing and H₂/CO₂-utilizing methanogens were inhibited by the addition of molybdate (20 mmol l⁻¹) to the culture medium at zero time, although acetate utilizers were unaffected if the inhibitor was added after the cultures had started to produce CH₄ actively (Fig. 4). These results were similar, in some respects, to those obtained with sediment slurries, where the effect of molybdate was more marked and persistent if added at zero time. One possible explanation might be that a molybdo-sulphide complex was formed in both the sulphide-rich sediments and the sulphide-containing medium. Both sediments and medium turned a yellow-orange colour after addition of the molybdate. Similar coloration of a medium was reported by Wolin & Miller (1980) who also demonstrated that the complex could prevent the formation of hydrogenase in *Ruminococcus albus*, although preformed enzyme was not itself inhibited. The involvement of hydrogenases in methanogenesis, and in the activity of H₂-utilizing acetogens, suggests that further studies are required on the effect of concentration on the specificity of molybdate as an inhibitor of sulphate reducers in freshwater sediments such as these. This

Table 3. *Effect of sulphate and molybdate on methanogenesis and CO₂ production in small sediment cores*

Cores were incubated at 10 °C for 24 h. Standard deviations are shown in parentheses.

Sediment depth (cm) ...	Rate of methanogenesis [nmol (g dry wt) ⁻¹ d ⁻¹]				Rate of CO ₂ production [nmol (g dry wt) ⁻¹ d ⁻¹]	
	From acetate		From H ₂ /CO ₂		From acetate	
	0-1	1-2	0-1	1-2	0-1	1-2
Conditions						
Control	166 (18)	162 (3.6)	516 (158)	748 (236)	34 (2.1)	19 (5.3)
+K ₂ SO ₄ (0.5 mmol l ⁻¹)	128 (14)	184 (0.4)	188 (26)	123 (18)	116 (23)	37 (23)
+Na ₂ MoO ₄ (20 mmol l ⁻¹)	66 (4)	68 (20)	364 (58)	364 (55)	40 (29)	24 (12)
Proportion of CH ₄ produced (%)	24	18	76	82		
Inhibition by K ₂ SO ₄ (%)	23	0	64	84		
Inhibition by Na ₂ MoO ₄ (%)	60	42	29	51		

does not imply that the inhibitor cannot be used satisfactorily to interpret events in SO₄²⁻-dominated marine systems.

Hydrogen-utilizing processes such as methanogenesis are extremely dependent on the partial pressure of H₂, and it would be very difficult to maintain natural concentrations of the gas when sediment slurries were incubated for short periods in serum bottles. Practical solutions to this problem might include the following: (1) short-term radiotracer experiments in atmospheres which contain excess H₂, thus ensuring maximum velocity of methanogenesis but likely to overestimate the role of the H₂/CO₂ pathway if rates are based solely on the natural CO₂ concentration (likewise the relative importance of the pathway will be underestimated if the partial pressure of H₂ in solution is reduced by incubation under an atmosphere of N₂); (2) long-term experiments with slurries, in which the natural concentration of H₂ is re-established by the microflora and in which measurements are made over the linear part of the methane production curve; (3) short-term experiments with radiotracers using systems in which disturbance of the natural concentrations of all substrates is kept to a minimum. In an attempt to determine the relative importance of the methane precursors and to check the effect of inhibitors under natural conditions we adopted the last of the three solutions. Known concentrations of H¹⁴CO₃⁻, ¹⁴CH₃COOH, K₂SO₄ and Na₂MoO₄ were injected into small sealed cores of sediment with no overlying headspace, and rates of methanogenesis were calculated in a manner analogous to that used by Jørgensen (1978) for determining rates of SO₄²⁻ reduction with ³⁵SO₄²⁻. The results showed that more than 75% of the CH₄ generated in Blelham Tarn sediments in late summer was derived from CO₂ (Table 3). The generation of CH₄ from both CO₂ and CH₃COOH was again inhibited by sulphate and molybdate, the former diverting acetate metabolism to CO₂ by stimulating the activity of sulphate-reducing bacteria. Conversion of CH₃COOH to CO₂ was not affected significantly by the addition of molybdate. The results are in agreement with other recent findings (Zaiss, 1981) which illustrate the importance of the H₂/CO₂ pathway in certain freshwater environments. Whilst it is accepted that the above is an indirect method of calculating the relative importance of the two methanogenic pathways, we are relatively confident that the results are realistic, since the total amount of CH₄ generated on an area basis in the small cores was in reasonable agreement with three other, independent measures of methanogenesis. The rate in the small cores was equivalent to a production of 850 μmol CH₄ m⁻² d⁻¹, which compared with results of 235 to 1245 μmol m⁻² d⁻¹ (based on CH₄ accumulation in the hypolimnion over 3 years, Jones & Simon, 1981), 650 μmol m⁻² d⁻¹ (field rates in this study derived by extrapolation from 0.5 m and 0.25 m above, to the sediment surface) and 1090 μmol m⁻² d⁻¹ (measured accumulation of CH₄ above sediment in

a sealed Jenkin core). These calculated rates confirm earlier observations (Jones & Simon, 1980) that the amount of carbon turned over by methanogenesis in Blelham Tarn is significantly less than in many North American lakes (Fallon *et al.*, 1980). A similar treatment of the SO_4^{2-} reduction rates obtained by Jones & Simon (1981) from experiments with small cores showed that, in the presence of sufficient electron acceptor, sulphate reducers could produce up to $3.25 \text{ mmol CO}_2 \text{ m}^{-2} \text{ d}^{-1}$. Seasonal means were, however, much lower, as the profundal sediments rapidly became limited in available SO_4^{2-} (Jones, 1982).

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