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Carbon and Electron Flow in Mud and Sandflat Intertidal Sediments at Delaware Inlet, Nelson, New Zealand

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An investigation of carbon and electron flow in mud and sandflat intertidal sediments showed that the terminal electron acceptor was principally sulfate and that the carbon flow was mainly to CO_2. Studies with thin layers of sediment exposed to H_2 showed that methane production accounted for virtually none of the H_2 utilized, whereas sulfate reduction accounted for a major proportion of the gas uptake. At all sampling sites except one (site B7), rates of methanogenesis were low but sulfate concentrations in the interstitial water were high (>18 mM). At site B7, the sulfate concentrations declined with depth from 32 mM at 2 cm to <1 mM at 10 cm or below, and active methanogenesis occurred in the low-sulfate zone. Sulfate-reducing activity at this site initially decreased and then increased with depth so that elevated rates occurred in both the active and nonactive methanogenic zones. The respiratory index (RI) [$RI = {}^{14}CO_2/({}^{14}CO_2 + {}^{14}CH_4)$] for [2-^{14}C]acetate catabolism at site B7 ranged from 0.98 to 0.2 in the depth range of 2 to 14 cm. Addition of sulfate to sediment from the low-sulfate zone resulted in an increase in RI and a decrease in methanogenesis. At all other sites examined, RI ranged from 0.97 to 0.99 and was constant with depth. The results suggested that although methanogenesis was inhibited by sulfate (presumably through the activity of sulfate-reducing bacteria), it was not always limited by sulfate reduction.

In anaerobic microbial ecosystems with sulfate present, methanogenesis is inhibited by sulfate-reducing bacteria which compete for the methanogenic precursors H_2 or acetate or both (1, 2, 12, 32). In marine sediments high concentrations of sulfate are naturally present, and it would be uncommon that sulfate reduction would be limited by lack of sulfate. In such environments it might be expected that sulfate reduction would lead to inhibition of methanogenesis. Oremland and Taylor (23) noted that slow rates of methanogenesis occurred in marine sediment, and Abram and Nedwell (1) concluded from their studies, using β-fluorolactate (an inhibitor specific for sulfate reducers), that sulfate-reducing bacteria competed against the methanogens for available H_2 in saltmarsh sediment. The latter workers also simulated the natural ecosystem by mixing cultures of H_2-utilizing sulfate-reducing bacteria, a methanogen, and an H_2-donating organism and found that methanogenesis was inhibited by active sulfate reduction (2). Other studies have also suggested that methanogenesis is limited by sulfate reduction. In anaerobic marine and saltmarsh sediments, methane concentrations were shown to increase with depth while sulfate reduction rates and sulfate concentrations decreased (10, 18, 21, 25).

In a recent paper, Abram and Nedwell (1) have challenged the view that sulfate reduction is implicitly suppressive to methanogenesis. They suggested that although competition for H_2 may occur, the availability of acetate could be increased by certain sulfate reducers, thus stimulating methanogenesis. Indeed, it is well known that sulfate-reducing bacteria can oxidize lactate and ethanol to acetate and use the reducing equivalents to reduce sulfate to sulfide (12, 24). Recently, F. Widdel and N. Pfennig (personal communication) have isolated a number of sulfate-reducing bacteria from marine and freshwater sediments which can produce acetate from the incomplete oxidation of long-chain fatty acids and propionate. Since acetate is a major precursor of methane, it is conceivable that methanogenesis could benefit provided that acetate-oxidizing, sulfate-reducing organisms similar to *Desulfotomaculum acetoxidans* (31) were not as competitive for the acetate.

The aim of this investigation was to examine methanogenesis and sulfate reduction in mud and sandflat intertidal sediments from Delaware Inlet in order to determine whether the former was always limited by the latter.

MATERIALS AND METHODS

Description of the sampling area. The location and distinctive features of the sampling sites are shown

in Fig. 1. For a general description of the study area, see Stanton et al. (28). The notation of the sites was based on that used by Bohlool and Wiebe (11). Per tidal cycle (approximately 12 h), the sediment surface of site B7 was the least exposed to seawater (<3 h, and no cover at neap tide), whereas the surface of site A5 was the most exposed (>10 h). At sites A5 and A7, the sediment consisted of sand at all depths examined (2 to 20 cm), whereas at sites B1 and B7, the sediment consisted of mud for the same depth range. The sediment at site A6 consisted of mud to a depth of 6 to 7 cm and a hard sandy clay pan below this depth. A detailed description of the sediment types will be published later (L. Belser and E. Mays, manuscript in preparation). Infiltration was negligible at the muddy sites B1, B7, and A6 but was high at the sandy sites A5 and A7. Site B7 was located near the outlet of a freshwater stream draining pasture grazed by cattle. At low tide there was a high density of snails (*Amphibola*) feeding on the surface of site A6. Eelgrass (*Zostera*) and cockles (*Chione*) were abundant in the 0- to 5-cm depth of site A5. The temperature of the sediment ranged from a minimum of 11°C (early October) to a maximum of 30°C (late January); the mean was 15°C.

Sampling procedure. Sediment samples were collected between October 1978 and April 1979. Samples were routinely removed with a large perspex core sampler (internal diameter, 8.5 cm; length, 42 cm) with 16-mm holes drilled at 2-cm intervals down the side. Side holes were sealed with Scotch 3M pressure-sensitive tape before sampling. Cores were taken up to 20 cm in depth and processed approximately 1 h after collection. Sediment subsamples were removed through side holes with 5-ml plastic disposable syringes with the distal ends sawn off, and were transferred either into vessels for incubation experiments or into the barrel of 10-ml syringes for interstitial water analysis. For the latter, the base of the syringe contained a copper grid overlaid with nonabsorbant cotton wool. The interstitial water was removed by penetrating the septum stopper of an evacuated Bellco anaerobic culture tube (16 by 125 mm) with the needle of the syringe loaded with sediment. Pressure exerted on the plunger also assisted in squeezing the interstitial water out of the sediment and into the tube. Interstitial water samples were stored frozen at −18°C for chemical analysis.

Incubation techniques. For incubations without [^{14}C]acetate, samples of sediment (approximately 5 g, wet weight) were transferred to 21-ml reaction vessels previously gassed with oxygen-free nitrogen. Unless stated otherwise, sediment was diluted 50% by weight with deoxygenated water. Filtered seawater was normally used to dilute the sediment. However, because the salinity of the interstitial water was lower at site B7 than at the other sites (as low as 4.9‰ compared with 15 to 31‰ for the other sites), deoxygenated distilled water was used. Vessels were sealed with recessed butyl rubber stoppers, and slurries were prepared by shaking on a gyratory shaker. Stoppers were then removed and vessels were reflushed with oxygen-free nitrogen (or appropriate mixture of H_2/N_2 in the case of experiments on H_2 utilization) for 30 s before being resealed. This procedure decreased methane in the headspace resulting from outgassing from the slurry. Slurries were incubated at 30°C, and measurements of methane were corrected for methane in the headspace at zero time. In experiments where H_2 was added, vessels were incubated using a radial shaker based on that described by Strayer and Tiedje (29). This minimized the limitation posed by the insolubility

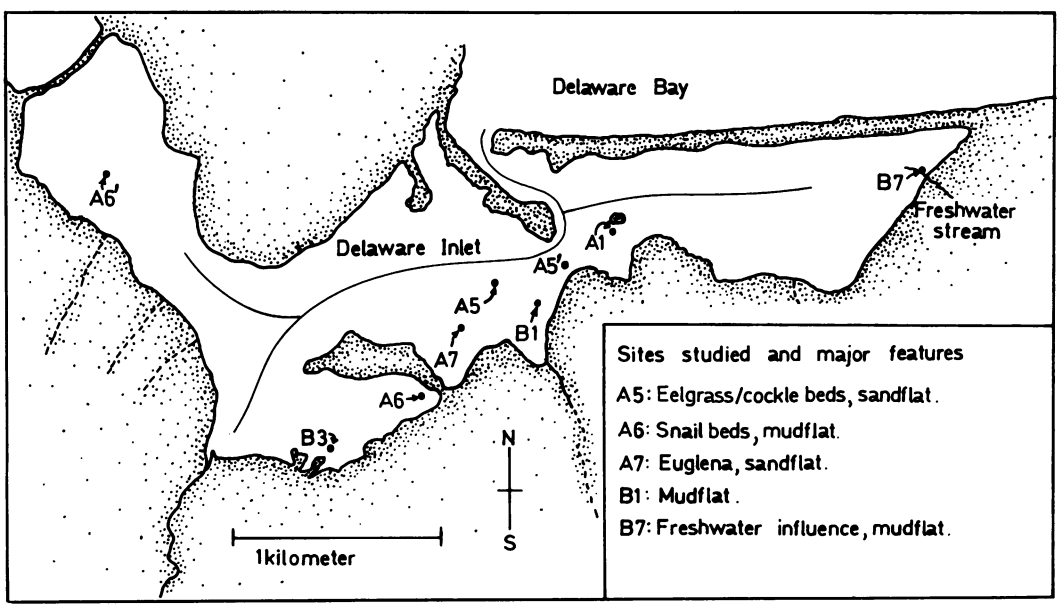

FIG. 1. *Location and major features of sampling sites at Delaware Inlet. Sites A5' and A6' shared the same features as sites A5 and A6, respectively. Sites A1 (sandflat) and B3 (Juncus on mudflat) were not studied.*

of H_2 and its slow diffusion from the gas phase into the sediment. A feature of this incubation technique was that the sediment accumulated on the sides of the reaction vessel to form a film (<2 mm), thus maximizing contact between the sediment and the gas atmosphere. In experiments where fluoroacetate was used, the inhibitor was added to give a final concentration of 0.1% (wt/vol) in the interstitial water of diluted sediment.

For radioactive incubations using [2-^{14}C]acetate, unless stated otherwise sediment samples (approximately 3.5 g, wet weight) were added to the reaction chambers of 25-ml Warburg vessels equipped with a center well. Sediment was prepared as a slurry as described above. [2-^{14}C]acetate was then added to each flask (0.57 μCi/g [dry weight] of sediment), and flasks were sealed with butyl rubber stoppers and incubated at 30°C. Incubation was terminated by injection of 0.3 ml of 50% H_2SO_4 into the reaction chamber immediately followed by addition of 0.4 ml of 5 N KOH to the center well. Flasks were stored at 2°C for 2 h to allow complete absorption of CO_2, and analyses were then performed.

In experiments to determine sulfate-reducing activity, plastic syringes (2.5 ml) containing 1 to 2 g (wet wt) of sediment subsample were sealed with butyl rubber caps. Two microcuries of $Na_2{}^{35}SO_4$ was injected as evenly as possible throughout the core of each sample. Syringes were then placed in 28-ml Universal bottles under nitrogen, and samples were incubated at 26°C for 48 h, during which time the production of $^{35}S^{2-}$ was linear. At the end of incubation, samples were frozen in an ethanol/dry ice mixture and stored at −18°C for analysis.

Analyses of [2-^{14}C]acetate incubations. After absorption of $^{14}CO_2$ in the center well of Warburg flasks, the resulting vacuum was relieved by injection of CO_2-free nitrogen, using a glass piston syringe to allow quantitative gas sampling. Flasks were maintained at 2°C, and the total volume of gas was determined by recording the excess gas forced into the syringe. For analysis of radioactive methane, 1-ml volumes of gas were injected into scintillation vials sealed with butyl septum stoppers and counted in 20 ml of toluene-based scintillant as described by Zehnder et al. (34). To ensure that a maximal amount of methane was dissolved, vials were agitated vigorously. Assay of the gas atmosphere above the scintillation fluid revealed that 80 ± 3% of the methane added was absorbed and therefore counted. Corrections were made for methane not absorbed and for quenching. Counting efficiency was 86 ± 2%.

$^{14}CO_2$ in the center well was counted as previously described (19). The proportions of [2-^{14}C]acetate converted to $^{14}CO_2$ and $^{14}CH_4$ are presented by an expression of Winfrey and Zeikus (33) termed the respiratory index (RI), where RI = $^{14}CO_2/(^{14}CO_2 + {}^{14}CH_4)$.

Radioactivity in the sediment pellet was determined by wet combustion, using a method similar to that described by Belly and Brock (8). Solid sediment (approximately 0.3 g) was washed once with distilled water and then taken up to a volume of 5 ml. The suspension was then added to the reaction chamber of a boiling flask. Potassium dichromate (1 g) was then added, followed by 5 ml of concentrated H_2SO_4/H_3PO_4 (3:2, vol/vol) mixture. The flask was boiled, and the $^{14}CO_2$ evolved was flushed under a stream of air into three successive traps, each containing 10 ml of cocktail consisting of 10% phenylethylamine and 10% methanol in toluene-based scintillant.

Analysis of $^{35}SO_4^{2-}$ incubations. A frozen core of $^{35}SO_4^{2-}$ incubated sediment was transferred from a syringe to the reaction chamber of a sulfide distillation apparatus (17). After the apparatus was flushed for several minutes with oxygen-free nitrogen, 20 ml of deoxygenated 3 N HCl was added to the reaction chamber, and $^{35}S^{2-}$ was distilled into 1% zinc acetate trapping solution for 30 min. The trap was then replaced by another, and 40 ml of reducing agent (7) was added to the reaction chamber. Contents were heated (115°C), and the $^{35}SO_4^{2-}$ remaining in the sample was distilled as sulfide into the trap for 1 h. Portions of distillate representing the $^{35}S^{2-}$ and $^{35}SO_4^{2-}$ components of the sample were counted in Packard Insta-Gel scintillation fluid. Recovery of total radioactivity ($^{35}SO_4^{2-}$ + $^{35}S^{2-}$) was 85%. In most incubations, less than 10% of the initial $^{35}SO_4^{2-}$ was converted to $^{35}S^{2-}$. From experiments with control sediments which had been killed with Formalin (12%[vol/vol] in the interstitial water), it was estimated that >95% of the added SO_4^{2-} label occurred in the interstitial water. Sulfate in the interstitial water was chemically determined as described below, and the pool size did not alter appreciably throughout the incubation period.

In calculating the rate of sulfate reduction, we assumed that the sulfate-reducing bacteria only reduced sulfate in the interstitial water. The equation is based on that described by Sorokin (27) and is expressed as follows:

$$\Delta SO_4^{2-} = \frac{SO_4^{2-} \cdot {}^{35}S^{2-} \cdot 1.06}{{}^{35}SO_4^{2-} \cdot t}$$

where SO_4^{2-} is the interstitial water sulfate content of the sediment in nanomoles per gram (dry weight), $^{35}S^{2-}$ is the radioactivity of sulfide, $^{35}SO_4^{2-}$ is the average of the initial and final radioactivity of sulfate, t is the incubation time, and 1.06 is a correction factor for microbial isotopic fractionation. The rate of sulfate reduction (ΔSO_4^{2-}) is expressed as nanomoles of SO_4^{2-} per gram (dry weight) per hour.

Gas measurements. Methane was determined by gas chromatography at 70°C, using a 2-m Porapak Q column connected to a flame ionization detector. Nitrous oxide was analyzed at 75°C, using a 4-m Porapak Q column connected to an electron capture detector. Injection was by sample loop. Analysis of H_2 was carried out on a Fisher-Hamilton gas partitioner equipped with a thermal conductivity detector. Gas was fractionated on a 2-m Molecular Sieve 13X column at room temperature. Argon was the carrier gas.

Chemical analysis of sediment. Sulfate in the interstitial water was determined by turbidometry (3). Soluble (water-extractable) sulfate in the sediment was determined after extraction of 1 part of sediment with 4 parts of water by weight. Chloride in the interstitial water was determined by the mercuric thiocyanate colorimetric method (4); nitrate was determined by the cadmium reduction method (3). Sulfide was

determined by the methylene blue method (30). Acetate in the interstitial water was determined by acidifying to pH 2.5 with HCl and analyzing directly by gas-liquid chromatography as previously described (19).

Dry weight of sediment. The dry weight of sediment was determined after heating a weighed portion of sediment in a crucible at 105°C to a constant weight. The dry-matter content of sediment ranged from 70 to 80%, depending on the sampling site. All rate data are presented on a gram (dry weight) of sediment basis, and concentrations are expressed either on the same basis or as millimolar in the interstitial water.

Chemicals. All chemicals were obtained from commercial sources and were of reagent grade. The radioisotopes [2-^{14}C]-acetate (specific activity, 56 mCi/mmol) and $Na_2{}^{35}SO_4$ (specific activity, ≤104 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Rates of methanogenesis. Rates of methanogenesis were determined over 48 h of incubation, during which time methane production was linear. Active methanogenesis occurred only at site B7 (Fig. 2). The fastest rate of methanogenesis at this site occurred at a depth of 12 to 14 cm. At site B7, sampling below 14 cm was difficult because of the presence of rock. Low but measurable rates of methanogenesis were observed at site A5 for depths of 2 and 20 cm. At all other sites examined, methanogenesis was nil. Sites A5 and B7 were also the only sites to show methane in the interstitial water collected by a diffusion sampler (15).

Proportions of methane accounted for by acetate and H_2/CO_2. To determine the proportions of methane accounted for by acetate and H_2/CO_2, studies were carried out at site B7 for convenience because this was the only site where active methanogenesis occurred. With increased depth, fluoroacetate, an inhibitor specific for methanogenesis from acetate (14), increasingly inhibited methanogenesis (Table 1). The inhibition at 12 cm indicates that acetate accounted for a major percentage (65%) of the methane formed at this depth. Previous studies (9, 16, 19, 26) have indicated that methane not accounted for by acetate in anaerobic ecosystems is mainly accounted for by H_2/CO_2. Thus, for the 12-cm depth about 30% of the methane may be assumed to have originated from H_2/CO_2, whereas at 2 cm H_2/CO_2 would have accounted for all the methane formed. The data from the fluoroacetate studies indicate that the proportions of methane accounted for by acetate and H_2/CO_2 altered with depth.

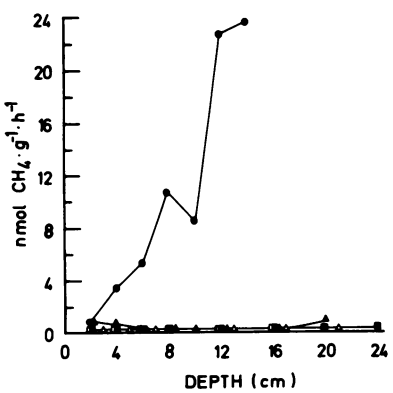

FIG. 2. *Rates of methane production at different sampling sites. Rates were determined from the first 24 h of incubation at 30°C. Symbols: (▲) site A5; (□) site A6; (△) site A7; (■) site B1; (●) site B7. Experiments were carried out with undiluted sediment collected in midsummer.*

Fates of acetate and hydrogen. [2-^{14}C]acetate was added to sediment to determine whether acetate was supporting methanogenesis or being oxidized. Preliminary experiments showed that <10% of the ^{14}C from [2-^{14}C]acetate degraded was incorporated into solid material (presumably cellular components), whereas >90% was converted into $^{14}CO_2$ + $^{14}CH_4$. The proportion of the methyl group oxidized to CO_2, expressed as RI, is shown in Table 2. An RI of 1.0 indicates oxidation of acetate to CO_2, whereas lower values indicate increasing amounts of methane formed from the methyl group of acetate. RI values did not alter significantly with time (0 to 5 h) after [2-^{14}C]acetate addition; therefore, for convenience, estimates were made at 3 h. It is evident that except for the lower depths of site B7 (>8 cm), the methyl group of acetate was mainly oxidized to CO_2. At site B7, with increased depth the proportion of the methyl group converted to methane increased, so that at the 14-cm depth (RI = 0.22) more than 75% of the methyl group was converted to methane.

Examination of hydrogen utilization at sites A5, A6, and B1 (2 to 12 cm) indicated that H_2 failed to effectively stimulate methanogenesis. At all three sites, the ratio of H_2 utilized to methane produced [(nanomoles of H_2/gram)/(nanomoles of CH_4/gram)] was ≥500 for H_2 in the headspace ranging from 0.005 to 0.1 atm. Addition of CO_2 to the headspace (0.2 atm) failed to stimulate methanogenesis above that which occurred in its absence. If all of the H_2 utilized were accounted for by methane production, a value of 4 for the H_2/CH_4 ratio would be expected. To establish whether sulfate reduction could account for H_2 utilization, sulfate concentrations were determined in sediment samples after incubation in the presence and absence of H_2. In H_2-incubated sediments, sulfate concen-

trations were lower than in the controls without added H_2 (Table 3). By assuming that the difference was due to sulfate reduction, it may be deduced that sulfate reduction accounted for a major proportion of the H_2 used.

Sulfate levels, sulfate-reducing activity, and effect on methanogenesis. We determined the sulfate concentrations in the interstitial water from undiluted sediment samples taken from sampling sites A5, A6, A7, B1, and B7 at the same depths as were examined for methane analysis in Fig. 2. Except for the deeper zone at site B7, the concentrations of sulfate were >18 mM but ≤32 mM at all sites. At site B7, the highest concentration of sulfate occurred at the 2-cm depth but decreased with depth so that at 14 cm it was <1 mM (Fig. 3). The data in Fig. 2 and 3 suggest that methanogenesis was predominant only when the interstitial concentrations of sulfate were low.

To determine whether sulfate inhibited methanogenesis, slurries of sediment were prepared from the low-sulfate zone (10-cm depth) of site B7. The concentration of sulfate in the diluted interstitial water was 0.3 mM. Sulfate was added to give final added sulfate concentrations of 0.3 and 1.0 mM. Addition of these small quantities of sulfate resulted in complete inhibition of methanogenesis (Fig. 4). Methanogenesis partially recovered 60 h after addition of sulfate.

Since acetate was a major precursor of methane in the low-sulfate zone of site B7, we investigated whether the increased sulfate concentrations affected the catabolism of this precursor. Sulfate was added to give added concentrations of 1 mM in the interstitial water of sediment slurries; no sulfate was added to the controls. Those slurries which contained added sulfate showed inhibition of methanogenesis similar to that in Fig. 4. [2-^{14}C]acetate was then added, and the RI was determined. Addition of sulfate to sulfate-depleted sediment resulted in an increase in CO_2 formation from the methyl group of acetate and a decrease in methane (Table 4). Therefore, addition of sulfate stimulated the oxidation of the methyl group to CO_2. These results also provide an explanation for the high RI values for [2-^{14}C]acetate catabolism at sites A5, A6, and B1 and for the 2- to 6-cm depths at site B7, where high concentrations of sulfate were encountered.

The results in Tables 2 and 4 indicated that the sulfate-reducing bacteria were likely to be the major agents competing against the methanogens for acetate. Therefore, one would expect

TABLE 1. *Effect of fluoroacetate addition on methanogenesis from sediment taken at different depths at site B7*[a]

Depth (cm)	Methane production (nmol·g^{-1}·h^{-1})		Inhibition by fluoroacetate[b] (%)
	− Fluoroacetate	+ Fluoroacetate	
2	0.32	0.45	Nil
6	2.17	1.89	13
12	22.7	7.91	65

[a] Sediment was collected in early autumn.
[b] Values are means of duplicate determinations. The error in determining the means was <5%.

TABLE 2. *RI for [2-^{14}C]acetate catabolism*[a]

Depth (cm)	RI[b]			
	Site A5	Site A6[c]	Site B1	Site B7[d]
2–3	0.98	0.980 ± 0.02	0.99	0.984 ± 0.003
4–5			0.99	0.990 ± 0.003
6–7	0.99	0.989 ± 0.003	0.99	0.964 ± 0.006
8				0.960 ± 0.005
10				0.521 ± 0.134
12–13	0.98	0.990 ± 0.005	0.99	0.454 ± 0.062
14				0.223 ± 0.017
17–25			0.99	

[a] Sediment was collected from early summer to midautumn.
[b] Determined after sediment had been incubated at 30°C for 3 h after injection of [2-^{14}C]acetate (0.57 μCi·g^{-1} [dry weight] of sediment).
[c] Values are means of triplicate determinations ± 1 standard deviation.
[d] Values are means of duplicate determinations ± 1 standard deviation.

TABLE 3. *Percentage of H_2 utilization accounted for by sulfate reduction for different depths at site B1*[a]

Depth (cm)	Initial H_2 (atm)	H_2 consumed (μmol·g^{-1})	SO_4^{2-} (mg·g^{-1})		Δ SO_4^{2-} (μmol·g^{-1})	H_2 used in SO_4^{2-}[b] reduction (%)
			−H_2	+H_2		
2	0.1	19.46	2.21	1.85	3.6	74
6	0.1	15.43 ± 1.91	2.24	1.89 ± 0.007	3.65 ± 0.15	95 ± 9[c]
12	0.1	7.75	2.6	2.50	1.04	53.6

[a] Sediment was collected in late summer and incubated for 48 h.
[b] Calculated assuming that the decrease in sulfate in the H_2-treated system was due to the formation of S^{2-} from SO_4^{2-} according to the following equation: $4H_2 + SO_4^{2-} \rightarrow S^{2-} + 4H_2O$.
[c] Mean of duplicate determinations ± 1 standard deviation.

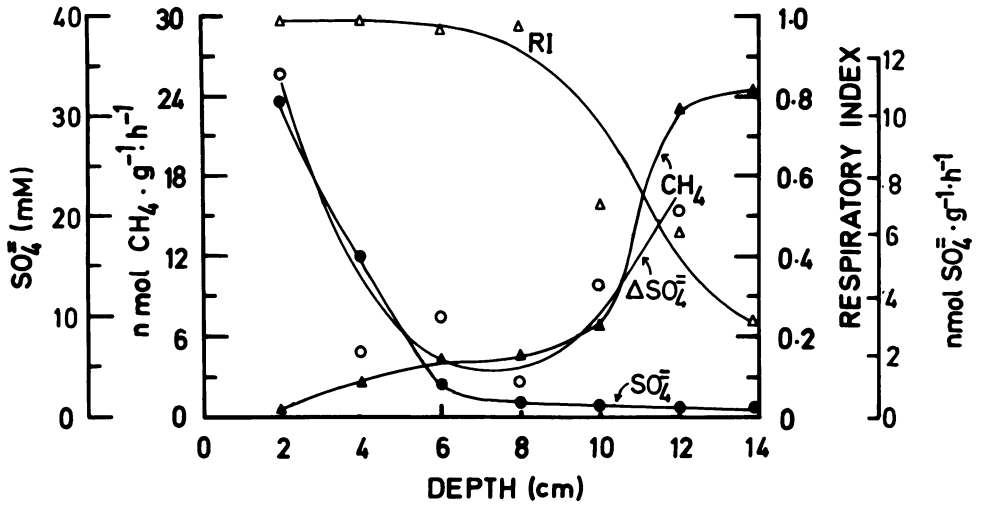

FIG. 3. *Depth profile of sulfate levels, sulfate reduction, methanogenesis, and RI at site B7. Symbols:* (▲) *rate of methane production;* (○) *rate of sulfate reduction;* (●) *concentration of sulfate in interstitial water;* (△) *RI from [2-^{14}C]acetate catabolism. All values are means of duplicate determinations. In determining the sulfate reduction rate, the standard deviation of the means ranged from 0.08 to 1.7 nmol·g^{-1}·h^{-1}. Sediment was collected in autumn.*

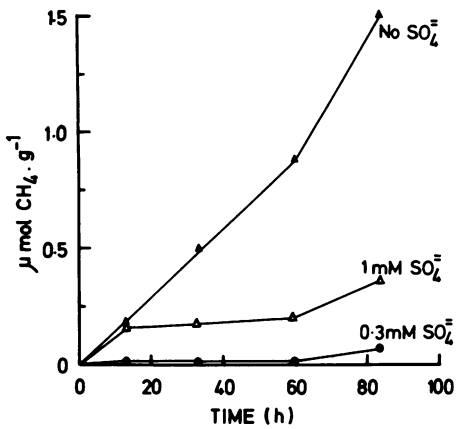

FIG. 4. *Effect of added sulfate on methanogenesis in sediment taken from a 10-cm depth at site B7. Symbols:* (▲) *control to which no sulfate was added;* (●) *0.3 mM added sulfate;* (△) *1 mM added sulfate. In the control, the sulfate concentration in the interstitial water was 0.3 mM. Incubation temperature was 30°C. Sediment was collected in midsummer.*

in a depth profile of sulfate-reducing and methane-producing activity at site B7 that low rates of the former would be encountered where high rates of the latter occurred. However, we found that although the highest rate of sulfate reduction occurred in the high-sulfate zone (2 cm), elevated rates also occurred in the low-sulfate zone (>10 cm) where methanogenesis was most active (Fig. 3). Furthermore, the rates of sulfate reduction in the latter corresponded with low values for interstitial sulfate and RI. At sites A6 and B1, where high sulfate concentrations were encountered, sulfate reduction ranged between 3 and 10 nmol·g^{-1}·h^{-1} in the depth range 2 to 20 cm (data not tabulated), but there was no observable trend with depth.

Effect of addition of acetate and formate to sediments low in methanogenic activity. Addition of acetate to sediment taken from a 5-cm depth at site A6, giving added levels in the interstitial water of 0 to 25 mM in diluted sedi-

TABLE 4. *Effect of sulfate addition on RI for sediment samples taken from site B7*[a]

Depth (cm)	Added sulfate[b] (mM)	$^{14}CH_4$[c] (10^5 dpm·g^{-1} [dry wt])	$^{14}CO_2$[c] (10^6 dpm·g^{-1} [dry wt])	RI[d]
10	0	1.99 ± 0.57	0.28 ± 0.23	0.58 ± 0.16
	1.0	0.28 ± 0.004	1.04 ± 0.03	0.97 ± 0.001
12	0	5.21 ± 1.28	0.66 ± 0.22	0.56 ± 0.15
	1.0	0.37 ± 0.11	1.05 ± 0.06	0.96 ± 0.007
14	0	7.86 ± 0.85	0.43 ± 0.10	0.35 ± 0.09
	1.0	0.34 ± 0.06	1.15 ± 0.10	0.97 ± 0.007

[a] Sediment was collected in autumn.

[b] Concentration of added sulfate in the interstitial water was 1 mM. The background level of interstitial sulfate in sediment slurries was <0.6 mM.

[c] Determined after slurries had been incubated at 30°C for 3 h after injection of [2-^{14}C]acetate (0.57 μCi/g [dry weight] of sediment). Values are means of duplicate determinations ± 1 standard deviation.

[d] Values are means of duplicate determinations ± 1 standard deviation. For each depth, RI values determined in the presence and absence of added sulfate were significantly different ($P < 0.05$).

ment, failed to convincingly stimulate methanogenesis (data not presented). Similar results were obtained for sediment taken from the 6- and 12-cm depths of site B1. These results contrasted with those obtained for sediment from the 8-cm depth at site B7, which showed that methanogenesis was stimulated by addition of acetate so that at 25 mM added acetate the rate of methane production (45.4 nmol·g^{-1}·h^{-1}) was four times that of the control (10.5 nmol·g^{-1}·h^{-1}). The concentration of 25 mM acetate was near saturating for methane stimulation. At sites A6, B1, and B7, the level of acetate in the interstitial water of diluted sediment was in the range of 0.1 to 0.2 mM. Addition of formate to sediment taken from 6 cm at site B1, giving added levels of 0 to 100 mM, failed to convincingly stimulate methanogenesis.

Effect of nitrate (NO_3^-) on methanogenesis. Nitrate from external sources is a potential electron acceptor in anaerobic ecosystems, and nitrate-reducing bacteria could compete against the methanogens for available reducing power. To test whether nitrate could inhibit methanogenesis, we added nitrate to sediment samples taken from depths of 2, 6, and 12 cm at site B7 to give added concentrations of 0.3, 1.0, and 3.0 mM in the interstitial water. At all depths, methanogenesis was inhibited after addition of 1 and 3 mM NO_3^-, but not by 0.3 mM No_3^-. Inhibition of methanogenesis after addition of 1 or 3 mM NO_3^- was associated with a peak of N_2O production such as is shown in Fig. 5 for 3 mM NO_3^- at 2 cm. As N_2O disappeared, methanogenesis recovered. For 3 mM NO_3^- (3.4 μmol·g^{-1}), at all depths examined, N_2O concentrations were highest (80 to 390 nmol·g^{-1}) 20 to 25 h after nitrate addition and completely disappeared after 45 h. After addition of 1 mM NO_3^- (1.14 μmol·g^{-1}), N_2O concentrations were highest (0 to 3.4 nmol·g^{-1}) 7 h after NO_3^- addition and completely disappeared after 27 h. Again methane production recovered as soon as N_2O disappeared.

Although NO_3^- could inhibit methanogenesis at >1 mM, the concentrations of nitrate in the interstitial water from undiluted sediment never exceeded 0.02 mM at any of the sites examined for methane production in Fig. 2. Since addition of 0.3 mM NO_3^- failed to inhibit methanogenesis and this concentration was much greater than that naturally encountered, it is unlikely that nitrate was an important factor governing methanogenesis during the course of this study.

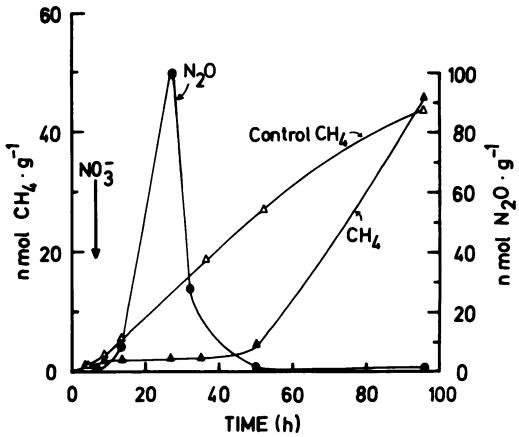

FIG. 5. *Effect of added nitrate on methanogenesis in sediment taken from a 2-cm depth at site B7. Nitrate was added at the time indicated by the arrow to give an added level in the interstitial water of 3 mM. The level of nitrate in the control was 0.002 mM. Symbols:* (●) *nitrous oxide;* (▲) *methane;* (△) *control methane. All values are means of duplicate determinations. In the control, no N_2O could be detected throughout the incubation. Incubation temperature was 30°C. Sediment was collected in early autumn.*

DISCUSSION

The results of this investigation leave little doubt that sulfate is a major factor controlling methanogenesis in mud and sandflat intertidal sediments. Suppression of methanogenesis when sulfate is present is well documented (1, 2, 12–14, 18, 32). Cappenberg (13, 14) has suggested that this is due to the sensitivity of the methanogens to H_2S produced by the sulfate-reducing bacteria. However, methanogens have been shown to tolerate quite high levels of sulfide. Bryant et al. (12) showed that *Methanobacterium* strain M.o.H. was tolerant to levels of sulfide of up to 20 mM at pH 6.6 to 6.8, and Mountfort and Asher (20) showed that *Methanosarcina barkeri* could tolerate sulfide levels of 7.5 mM at pH 7.0, although growth rate and growth yield declined. In the study reported here, it seems unlikely that free sulfide inhibited methanogenesis because the levels in the interstitial water never exceeded 0.05 mM. Sulfate toxicity is also unlikely to be a factor inhibiting methanogenesis. Sulfate is regularly used in culture media for growth of pure cultures of methanogens. Furthermore, studies in our laboratory have shown that increasing the sulfate levels up to 20 mM did not alter the growth rate of *Methanosarcina barkeri* growing on methanol (D. O. Mountfort and R. A. Asher, unpublished data).

The view that sulfate-reducing bacteria compete against the methanogens for available H_2 or acetate in anaerobic ecosystems has received considerable support in recent years (1, 2, 12, 32). We have attempted to thoroughly investigate the fate of both H_2 and acetate in anaerobic

intertidal sediments. In zones with active methanogenesis where methane was mainly accounted for by acetate, the metabolism of the precursor was examined in detail.

We observed that acetate was an important precursor of methane only in the low-sulfate zone of site B7, whereas in the high-sulfate zone little or none of the methane formed was accounted for by the substrate (Table 1). For other sampling sites we were unable to determine the proportions of methane accounted for by acetate because of very low rates of methane production. At site B7, in the high-sulfate zone, most of the [2-^{14}C]acetate was converted to CO_2, and little of the acetate was used in methane production. However, in the low-sulfate zone, most of the [2-^{14}C]acetate was converted to methane, and most of the methane was accounted for by the precursor. Methanogenesis was active in this zone. In the high-sulfate zone, the conversion of acetate to CO_2 would partially explain the low levels of methanogenesis. This would also be the case for sites A5, A6, and B1. The oxidation of acetate to CO_2 was most likely facilitated by the sulfate-reducing bacteria. Indeed, Widdel and Pfennig (personal communication) have isolated a number of sulfate-reducing bacteria from marine and freshwater sediments which can completely degrade fatty acids (chain length, C_1 to C_{14}) to CO_2. These include species of *Desulfobacter, Desulfococcus, Desulfosarcina,* and *Desulfonema. Desulfotomaculum acetoxidans* is perhaps the best-known fatty acid-oxidizing sulfate reducer and has been shown to oxidize acetate to CO_2 (31).

From the results on sulfate levels, methanogenic activity, and RI for [2-^{14}C]acetate degradation at site B7, it might have been expected that the sulfate-reducing bacteria would be least active in the zones with highest methanogenic activity. However, this was not the case, since elevated rates of sulfate reduction corresponded with high rates of methanogenesis, although the highest rate occurred in the high-sulfate zone (2 cm). These results appear to be at variance with studies on marine and salt-marsh sediments which suggest that an absence of sulfate reduction is required before active methanogenesis can take place (1, 18). However, recent observations by other workers may provide an explanation for our results. Bryant et al. (12) showed that strains of *Desulfovibrio* could convert lactate or ethanol to acetate and use the reducing equivalents in the reduction of SO_4^{2-} to sulfide. More recently, Pfennig and Widdel (personal communication) have isolated sulfate-reducing bacteria that degrade propionate to acetate and CO_2 (*Desulfobulbus propionicus*), long-chain even-numbered fatty acids to acetate, and long-chain odd-numbered fatty acids to propionate and acetate (vibrios of the sapovorans group). Again, reducing equivalents from the oxidation of fatty acids would be used in the reduction of sulfate to sulfide. Relating these findings to our results, we propose that with increased depth at site B7, the sulfate-reducing bacteria might increasingly participate in the production of acetate rather than in its oxidation to CO_2. In such a system, the role of the sulfate reducers would be determined by the sulfate levels. The consequence of the differential activity of the sulfate reducers on the methanogens would be beneficial in the low-sulfate zone and detrimental in the high-sulfate zone, the latter being due to competition for acetate.

Addition of H_2 to sediment with high sulfate levels resulted in little or no stimulation of methanogenesis. As shown in Table 3, sulfate reduction accounted for a major proportion of the H_2 utilized. Another mechanism which could have accounted for the remaining proportion involves the formation of acetate from H_2 (CO_2) by organisms similar to *Clostridium,* isolated by Ohwaki and Hungate (22) from sewage sludge, or *Acetobacterium,* isolated by Balch et al. (5) from sediments.

Our studies suggest that nitrate reduction was not normally an important event governing methanogenesis in sediments at Delaware Inlet because of the very low levels of nitrate present. However, inhibition of methanogenesis by added nitrate suggests that if high levels of nitrate were naturally present, methanogenesis could be governed by nitrate reduction. The results also suggest that N_2O, an intermediate of nitrate reduction, inhibited methanogenesis, thus supporting the previous studies of Balderston and Payne (6).

The results of this investigation indicate that sulfate-reducing bacteria are normally important agents in carbon and electron flow in mud and sandflat intertidal sediments where high concentrations of sulfate are usually encountered. However, in the unusual situation where low sulfate concentrations occur, the methanogens may have a more important role in the disposal of carbon and electrons and may benefit from the activity of the sulfate reducers.

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