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Role of Sulfate Reduction Versus Methanogenesis in Terminal Carbon Flow in Polluted Intertidal Sediment of Waimea Inlet, Nelson, New Zealand

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An investigation of the terminal anaerobic processes occurring in polluted intertidal sediments indicated that terminal carbon flow was mainly mediated by sulfate-reducing organisms in sediments with high sulfate concentrations (>10 mM in the interstitial water) exposed to low loadings of nutrient (equivalent to $<10^2$ kg of N · day^{-1}) and biochemical oxygen demand ($<0.7 \times 10^3$ kg · day^{-1}) in effluents from different pollution sources. However, in sediments exposed to high loadings of nutrient ($>10^2$ kg of N · day^{-1}) and biochemical oxygen demand ($>0.7 \times 10^3$ kg · day^{-1}), methanogenesis was the major process in the mediation of terminal carbon flow, and sulfate concentrations were low (≤2 mM). The respiratory index [$^{14}CO_2/(^{14}CO_2 + ^{14}CH_4)$] for [2-^{14}C]acetate catabolism, a measure of terminal carbon flow, was ≥0.96 for sediment with high sulfate, but in sediments with sulfate as little as 10 μM in the interstitial water, respiratory index values of ≤0.22 were obtained. In the latter sediment, methane production rates as high as 3 μmol · g^{-1} (dry weight) · h^{-1} were obtained, and there was a potential for active sulfate reduction.

In recent years there have been many studies on the terminal anaerobic processes occurring in ecosystems in which sulfate is present. The two major processes concerned are sulfate reduction and methanogenesis, and there is much evidence to suggest that the latter is inhibited by the former because of competition for precursors such as H_2, acetate, or both (1, 2, 7, 22). This may explain why in certain marine and salt-marsh sediments, methane concentrations decrease in zones where sulfate concentrations and sulfate reduction rates increase (5, 11, 15, 17). King and Wiebe (10) have studied acetate catabolism in salt-marsh soils and shown that depletion of sulfate results in a shift in carbon flow from CO_2 to methane. Similarly, other investigations have shown a shift in carbon flow towards CO_2 production when sulfate is added to sediment depleted in sulfate (14, 22). Such studies indicate that in the presence of sulfate, acetate-oxidizing, sulfate-reducing bacteria similar to *Desulfotomaculum acetoxidans* (21) oxidize acetate to CO_2.

Several investigators have challenged the view that sulfate reducers always act to suppress methanogenesis; they suggest that the former may stimulate the latter through increasing the availability of acetate (2, 8, 14). Sulfate-reducing bacteria capable of producing acetate from the oxidation of lactate and ethanol are well known (7, 8, 16). Cappenberg (8) demonstrated that an acetate-fermenting *Methanobacterium* sp. benefits from the acetate released by a lactate-fermenting *Desulfovibrio* sp. in mixed continuous culture. Widdel (Ph.D. thesis, University of Göttingen, Göttingen, West Germany, 1980) has recently isolated sulfate-reducing bacteria from marine and freshwater sediments; these isolates can produce acetate from the incomplete oxidation of long-chain fatty acids and propionate. Mountfort et al. (14) proposed that similar organisms might inhabit some intertidal sediments where the sulfate concentrations were unusually low. Indeed, on the basis of the studies by Bryant et al. (7), it is conceivable that such organisms in sulfate-depleted sediments could rely on methanogenesis from $H_2 \cdot CO_2$ as the electron sink, rather than on the sulfate-reducing system, and still produce acetate.

There is comparatively little information on the factors affecting terminal anaerobic processes in intertidal sediments, which result in changes in available sulfate. The aims of this study were to examine sulfate reduction and methanogenesis in the mediation of carbon flow at different sampling sites in polluted intertidal sediments to determine the effects of pollution on the two processes and to investigate the relationship of these processes as a function of

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sulfate availability.

MATERIALS AND METHODS

Description of sampling area. Waimea Inlet (41°18′ S, 173°10′ E) is located at the base of Tasman Bay in the Nelson District, New Zealand. The total area of the inlet at the high-water line is 33 km^2, and the area at low tide is about 10 km^2. Five sampling sites were studied, four of which were located near the liquid effluent discharge from various pollution sources (see Table 1 for discharge rates and analyses of effluents). Per tidal cycle (ca. 12 h), the sediment surface of the sampling sites was exposed to seawater for ≤3 h. Our notation of the sampling sites was based on that used by Bohlool (6). Site B2 was located near the effluent discharge which came from log debarking in a wood-chipping mill. The effluent was held in a pond before it was discharged at the edge of the intertidal zone. A dark reddish-brown coloration characteristic of phlobaphenes (highly condensed tannins) stained the sediments in this area. The sediment of site B3 was of a sludgelike consistency and was visibly affected by the effluent discharge from a slaughterhouse, as indicated by a black color to depths of up to 12 cm. The sediment also gave off a strong odor of H_2S. Site B4 was located near the effluent discharge of untreated sewage from a town (population, 7,000). The sediment was of a sludgelike consistency to a depth of 6 cm and consisted of coarse particles, gravel, and small stones below this depth. Site B5 was located near a leakage in a pipe carrying effluent from an apple cannery. There were numerous cracks and leakages in the pipe. Site B6 was located in mud-flats near a phosphate fertilizer works. Windblown residue occurred on the surface of the sediments at this site. The pH of the sediments was approximately 7, except for site B3, which was 6.5. The temperature of the sediments ranged from a minimum of 4°C (September) to a maximum of 30°C (late January); the mean was 15°C.

Sampling procedure. Sediment samples were collected between September 1979 and April 1980 and during September 1980. 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 a depth of 24 cm 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. The interstitial water was removed from the syringe as previously described (14) and stored at −18°C for chemical analyses.

Chemical analysis of sediment. Sulfate in the interstitial water was determined by turbidometry (3). The method was optimized to measure as little as 10 nmol of SO_4^{2-} per ml of interstitial water (equivalent to ca. 16 nmol · g^{-1} [dry weight]). 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 thiocycanate colorimetric method (4), and acetate was determined as previously described (13). Biochemical oxygen demand (BOD), chemical oxygen demand, and total nitrogen of effluents were determined by standard procedures (3).

The dry weight of the sediment was determined after a weighed portion was heated in a crucible at 105°C to a constant weight. The dry-matter content of the sediment ranged from 30 to 77%, depending on the sampling site and depth. 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.

Incubation techniques. The sediment was incubated by previously described procedures (14). Unless stated otherwise, before incubation, the 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 sites B3 and B4 than at other sites (ca. 14‰, compared with 20 to 30‰ for other sites) and the sulfate concentrations in the interstitial water were low (see Table 3), deoxygenated distilled water containing 1.3% NaCl was used, with 0 mM (site B3) and 2 mM (site B4) Na_2SO_4 additions. For radioactive incubations with [2-^{14}C]acetate, the radioisotope was added at 0.57 μCi · g (dry weight) of sediment^{-1}. For studies on sulfate reduction, 0.5 μCi of $Na_2{}^{35}SO_4$ was injected as evenly as possible throughout a core of sediment (2 g, wet weight) contained in a plastic syringe (2.5 ml) sealed with butyl rubber caps.

Analysis of radioactive incubations. Analyses of $^{14}CO_2$ and $^{14}CH_4$ produced from [2-^{14}C]acetate incubations were performed as previously described (14). 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 (23) termed the respiratory index (RI), where RI = $^{14}CO_2/(^{14}CO_2 + {}^{14}CH_4)$. Analysis of $^{35}SO_4^{2-}$ incubations to determine sulfate reduction rate was performed as previously described (14), except that $^{35}S^{2-}$ was trapped in 1 M NaOH rather than in 1% zinc acetate. Trapped $^{35}S^{2-}$ was counted in toluene-based scintillant (20) in which Triton X-100 was replaced by methanol. Also, unreacted $^{35}SO_4^{2-}$ was not determined after chemical reduction to $^{35}S^{2-}$ but was counted directly after extraction with distilled water. Recovery of total radioactivity ($^{35}SO_4^{2-}$ + $^{35}S^{2-}$) was ≥85%. In experiments with control sediments which had been inactivated with Formalin (12% [vol/vol] in the interstitial water), 85% of the added label occurred in the interstitial water. Rates of sulfate reduction were calculated as previously described (14), using the formula of Sorokin (19), and it was assumed that sulfate-reducing bacteria only reduced sulfate in the interstitial water. Also, rates were determined for periods in which the production of $^{35}S^{2-}$ was linear. When sulfate concentration approached the limit of detection of the analytical technique employed (see above), values for sulfate reduction were approximated and expressed on a "less than" (<) basis. Under such circumstances, the potential for sulfate reduction was assessed by adding unlabeled sulfate in addition to $^{35}SO_4^{2-}$ to give initial concentrations in the interstitial water of 2 to 20 mM.

Gas measurements. Methane was determined by gas chromatography at 70°C, using a 2-m Porapak Q column connected to a flame ionization detector.

Chemicals. All chemicals were obtained from com-

mercial 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

Composition of, and chemical loadings in, effluents discharged from the various pollution sources. The highest values of chemical oxygen demand, BOD, and total nitrogen occurred in effluent discharged from the slaughterhouse (Table 1). The BOD for the slaughterhouse effluent was three to four times greater than those for the other effluents, and the BOD loading (BOD times discharge rate) was twice that for the sewage effluent and more than three times that for the wood-chipping mill and apple cannery effluents. Total nitrogen loading was highest for the effluent discharged into the zone of site B3, although there was a high nitrogen loading for the sewage effluent (discharged into the zone of site B4) compared with the loadings for the effluents from the wood-chipping mill and apple cannery. The values for nitrogen loadings indicate that the highest nutrient input was into the zone of site B3.

Rates of methanogenesis. Rate measurements for methanogenesis were made at five sites, only two of which had detectable rates. At these two sites, rates were determined over 24 h of incubation, during which time methane production was linear. The most active site was B3, where rates of up to 3 $\mu mol \cdot g^{-1} \cdot h^{-1}$ were observed (Fig. 1). Elevated rates of methanogenesis also occurred at a depth of 6 cm for site B4, and low but measureable rates also occurred at depths of 2 and 18 cm at this site.

Acetate metabolism. Measurements of acetate catabolism may be used to elucidate terminal carbon flow in anaerobic ecosystems. [2-^{14}C]acetate was added to two sediment samples

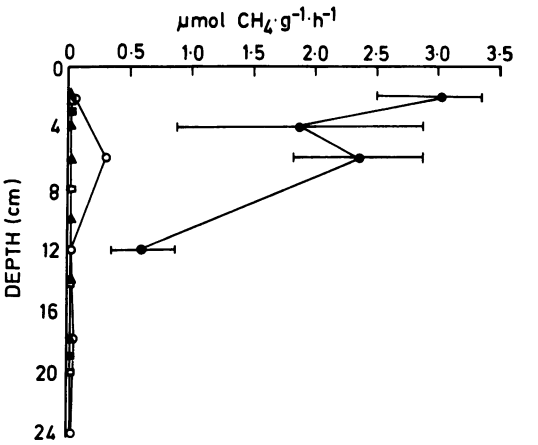

FIG. 1. *Rates of methane production at different sampling sites. Rates were determined over 24 h of incubation at 30°C. Symbols: ▲, site B2; ●, site B3; ○, site B4; □, site B5; ■, site B6. Experiments were carried out with undiluted sediment. Values for site B3 are means for three separate experiments conducted from late spring to midsummer. Bars represent ranges.*

with detectable methanogenesis (sites B3 and B4) and to two sediment samples with undetectable methanogenesis (sites B2 and B5) to determine the proportion of acetate converted to methane and CO_2. The amount of [2-^{14}C]acetate added was small (10 $nmol \cdot g^{-1}$) so as not to affect the pool size of acetate already present (≥2.6 $\mu mol \cdot g$). In most experiments, ≥90% of the [2-^{14}C]acetate degraded was converted to $^{14}CH_4$ and $^{14}CO_2$. The proportion of the methyl group oxidized to CO_2, expressed as the RI, is shown in Table 2. An RI of 1.0 indicates oxidation of [2-^{14}C]acetate to $^{14}CO_2$, whereas lower values indicate increasing amounts of $^{14}CH_4$ formed from the methyl group. The results showed that the RI was ≥0.95 for all depths examined at sites B2

TABLE 1. *Chemical analyses, discharge rates, and chemical loadings of liquid effluents discharged into various sampling sites*

Site	Effluent						
	Source	COD[a] (10^2 mg·liter^{-1})	BOD (10^2 mg·liter^{-1})	Total nitrogen (mg·liter^{-1})	Discharge rate (10^6 liter·day^{-1})	BOD loading[b] (10^3 kg·day^{-1})	Total nitrogen loading[c] (10^2 kg of N·day^{-1})
B2	Wood-chipping mill	5.58	1.50	6.1	3.00	0.45	0.18
B3	Slaughterhouse	7.70	6.20	68.0	2.50	1.55	1.70
B4	Sewage	3.28	1.95	31.0	3.80	0.74	1.17
B5	Apple cannery	ND[d]	1.56	6.9	2.16	0.34	0.15

[a] Chemical oxygen demand.
[b] BOD times discharge rate.
[c] Total N × discharge rate.
[d] ND, Not determined.

and B5, indicating that the methyl group was mainly converted to CO_2. For site B3, the very low RI values (≤0.22) indicated that most of the methyl group of acetate was converted to methane (≥78%). For site B4, RI values decreased from 0.62 at 12 cm to 0.37 at 2 cm, indicating a decreased proportion of the methyl group converted to methane at increased depth.

Sulfate levels, sulfate-reducing activity, and effect on carbon flow. The concentrations of sulfate in the interstitial water from undiluted sediment taken from the various sampling sites are shown in Table 3. Except for the 2- to 12-cm-depth range at site B4 and all depths at site B3, elevated sulfate concentrations occurred in the intertidal sediments. Comparison of the data with the results on methanogenesis (Fig. 1) suggested that methanogenesis was predominant only when the interstitial concentrations of sulfate were low.

We investigated whether decreased methane at elevated sulfate concentrations related to a change in the pathway for acetate catabolism favoring CO_2 formation from the methyl group. Sulfate was added to sediment taken from a depth of 6 cm at site B3 to give an initial added concentration of 8 mM in the diluted interstitial water; no sulfate was added to the controls. Methane production, sulfate concentration, and RI were monitored over 24 h. The results (Fig. 2) showed that sulfate addition inhibited methanogenesis. The RI increased to 0.74 soon after sulfate addition (2 to 5 h) and then declined so that at 21 to 24 h, it was close to that of the control, which had no sulfate added (RI, <0.2). Sulfate concentrations in the sulfate-treated sediment also declined to control levels within the same period. Additional experiments in which sediment was incubated in the presence of Formalin (12% [vol/vol] in the interstitial water) showed no loss of added sulfate over 24 h (data not shown).

Because of the very low concentrations of sulfate in the interstitial water sediment from site B3 (<16 nmol · g^{-1}, measured at a depth of 6 cm), accurately determining the basal rate of sulfate reduction with $^{35}SO_4^{2-}$ was not possible. The maximum rate that could be obtained, assuming the above sulfate concentration, was <22 nmol · g^{-1} · h^{-1} (Table 4). Table 4 also shows that when the sulfate was added to the sediment, the rate of sulfate reduction increased dramatically, indicating the potential of this site for active sulfate reduction.

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

Depth (cm)	RI[b]			
	Site B2	Site B3[c]	Site B4[c]	Site B5
2	0.99	0.22 ± 0.11	0.37 ± 0.23	0.99
6	0.99	0.15 ± 0.11	0.40 ± 0.01	ND
8	ND[d]	ND	ND	0.96
12	0.99	0.13 ± 0.03	0.62 ± 0.15	ND
16–24	ND	ND	ND	0.99

[a] Sediment was collected in the summer.
[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 duplicate determinations ± 1 standard deviation.
[d] ND, Not determined.

TABLE 3. *Concentrations of sulfate in the interstitial water of undiluted sediment*[a]

Site	Depth range (cm)	Sulfate (mM)
B2	2–18	22.0–26.5
B3	2–12	<0.01
B4	2–12	≤2.0
B4	12–24	2.0–12.0
B5	2–20	10.4–13.0
B6	3–19	>30.0

[a] Determined between spring and early autumn.

DISCUSSION

There seems little doubt that the conversion of the methyl group of acetate to carbon dioxide in these sediments was mediated by sulfate-re-

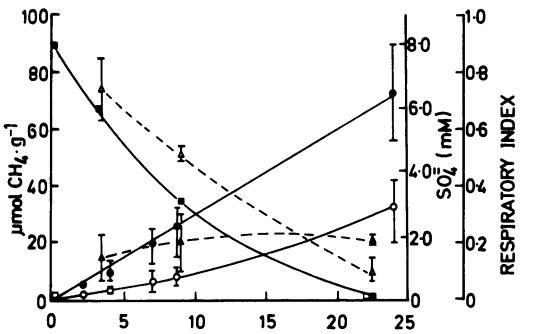

FIG. 2. *Effect of sulfate addition on methanogenesis and RI for [2-^{14}C]acetate catabolism in sediment taken from a depth of 6 cm at site B3. Symbols: ●, methanogenesis in the absence of added sulfate (control); ○, methanogenesis in the presence of added sulfate; □, sulfate concentration in control; ■, sulfate concentration in sulfate-treated sediment; ▲, RI for control; △, RI for sulfate-treated sediment. Values for RI and sulfate concentration are means of duplicate determinations, and values for methanogenesis are means of triplicate determinations. Bars represent ranges. RI values are presented as the midpoint of a 3-h incubation with [2-^{14}C]acetate. Incubation temperature was 30°C. Sediment was collected in midsummer.*

TABLE 4. *Potential for sulfate reduction for sediment from site B3*[a]

Depth (cm)	Sulfate[b]		Sulfate reduction[c] (nmol·g^{-1}·h^{-1})
	(mM)	(nmol·g^{-1})	
6	0	0	<22
6	2.67	4.54	2.30×10^3
2-8	6.25	10.36	6.80×10^3
2-8	22.50	38.30	3.08×10^3

[a] Sediment was collected in spring and incubated at 30°C.

[b] The concentration of added sulfate in sediment is expressed as the amount in millimolars in the interstitial water and the equivalent nanomoles per gram (dry weight). The level of sulfate in sediment to which no sulfate was added was <10 nmol·ml^{-1} or 16.5 nmol·g^{-1}.

[c] Determined during the period in which the production of $^{35}S^{2-}$ from $^{35}SO_4^{2-}$ was linear. Values are means of duplicate determinations. In the presence of added sulfate (2.67 to 22.5 mM), the standard deviation of the means was $<1.5 \times 10^3$ nmol·g^{-1}·h^{-1}.

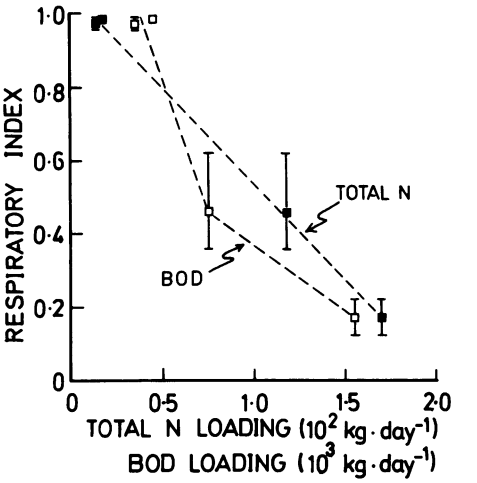

FIG. 3. *Effect of BOD and total nitrogen loadings on the RI for [2-^{14}C]acetate catabolism. Symbols: □, BOD loading; ■, total nitrogen. Values for BOD and total nitrogen are for the various effluents (data from Table 1), and values of RI are for the corresponding sampling sites (data from Table 2). RI values are averages for the 2- to 12-cm-depth range (taken as representative) of each sampling site. Bars represent ranges, and, for the data from sites B3 and B4, ranges of the means.*

ducing organisms. Widdel (Ph.D. thesis, 1980) has isolated several sulfate-reducing bacteria from marine and freshwater sediments which can degrade fatty acids (chain length, C_1 to C_{14}) to CO_2. A fatty acid-oxidizing sulfate-reducing bacterium which oxidizes acetate to CO_2 has been documented (21).

This study indicates that exposure of intertidal sediment to high loadings of readily degradable organic matter and nutrient (ascertained from BOD and total nitrogen loadings, respectively) results in a shift from sulfate reduction to methanogenesis in the mediation of terminal carbon flow (Fig. 3), and the former process may become limited because of exhaustion of available sulfate (Table 4). Limitation in sulfate-reducing activity would have allowed methanogenesis to proceed without competition from the sulfate-reducers for available substrates. The balance between the two processes would be expected to have also been affected by the state of the tides and freshwater influence. With the former, at high tide, sulfate availability to the sediment from seawater (28 mM sulfate) would be increased, and the events expected to occur with replenishment of sulfate in sediment exposed to high concentrations of nutrients may be similar to those described in Fig. 2 for site B3. The freshwater influence, however, may have contributed to decreased sulfate concentrations in some sediments but could not have explained the very low sulfate levels, such as those observed in the sediment from site B3, since the salinity of the interstitial water was 14‰, close to half that of seawater.

For site B3, the rates of methanogenesis were striking, and as far as the authors are aware there have been no previous reports of comparable rates in intertidal or marine sediments. The rates reported here for site B3 were comparable to those obtained for various sludge digestors. In the 2- to 12-cm-depth range, mean rates calculated on a basis of milliliters of wet sediment with the data from Fig. 1 (1 g [dry weight], equivalent to 1.3 ml of undiluted sediment) ranged from 0.46 to 2.3 μmol·ml^{-1}·h^{-1}. Smith and Mah (18) obtained a rate of 1.98 μmol of CH_4·ml^{-1}·h^{-1} for sewage sludge digestion at 35°C, and Mountfort and Asher (13) obtained a rate of 0.6 μmol·ml^{-1}·h^{-1} for digestion of cattle waste at 37°C. The comparison between the various sludge systems must be viewed with some caution because of differences in the temperature used (30°C in this study) and likely variations in dry-matter content.

The studies on the effects of sulfate addition to the sediment from site B3 (Fig. 2; Table 4) indicated the presence of sulfate-reducing organisms capable of active reduction of added sulfate with concomitant oxidation of acetate to CO_2. However, under conditions of low sulfate normally encountered at this site in which the basal rate of sulfate reduction was low (<22 nmol·g^{-1}·h^{-1}) and in which little acetate was oxidized to CO_2, it is conceivable that sulfate-reducing

bacteria could be involved in the production of acetate rather than in oxidation and could rely on the methanogens as the electron sink. Such a mechanism would be analogous to that described by McInerney et al. (12) for an acetogenic bacterium which degrades fatty acids to acetate and H_2 in syntrophic association with an H_2-utilizing methanogen. Bryant et al. (7) demonstrated that strains of *Desulfovibrio* could convert lactate or ethanol to acetate and could depend on an H_2-utilizing methanogen as the electron acceptor in low-sulfate media. More recently, Widdel (Ph.D. thesis, 1980) has isolated from marine and freshwater sediments 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 (*Desulfovibrio sapovorans*). However, it remains to be determined whether these organisms can be coupled to a methanogenic system acting as the electron sink.

Terminal electron flow has not been a principal aim of this investigation, since previous studies on intertidal sediments indicated that this is mediated principally by the sulfate-reducing bacteria (14). However, in sediment with low sulfate levels (site B3), this may not be the case. The rate of sulfate reduction at a depth of 6 cm at site B3 was <22 nmol $\cdot g^{-1} \cdot h^{-1}$, and the minimum rate of methanogenesis was 1.7 μmol $\cdot g^{-1} \cdot h^{-1}$ (Fig. 1). On the basis of previous studies on anaerobic ecosystems, which were essentially sulfate-free or had low concentrations of sulfate (9, 13, 14, 18), approximately 30% of the methane produced may be expected to have been derived from $H_2 \cdot CO_2$. From the above data and on the basis of eight electrons required for the reduction of 1 mol of SO_4^{2-} to sulfide or the production of 1 mol of methane from $H_2 \cdot CO_2$, it may be tentatively deduced that the latter process accounted for >90% of the electrons consumed by the two processes. A detailed account of electron flow in these sediments will be presented in a later communication (D. O. Mountfort and R. A. Asher, manuscript in preparation).

This investigation indicates that the balance between sulfate reduction and methanogenesis in the mediation of terminal carbon flow in polluted intertidal sediment may be influenced by the effluent discharge into the tidal zone, and where there is a high nutrient input, methanogenesis becomes the dominant process.

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