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SULFATE REDUCTION IN THE SEDIMENTS OF A EUTROPHIC LAKE

By

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ABSTRACT

SULFATE REDUCTION
IN THE SEDIMENTS OF A EUTROPHIC LAKE

by

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Concentrations of various sulfur compounds ($SO_4^=$, H_2S, S^o, acid volatile sulfide (AVS), and total sulfur) were determined in the profundal sediments and overlying water column of a shallow eutrophic lake. Low concentrations of sulfate relative to those of AVS and total sulfur and a decrease in total sulfur with sediment depth implied that the contribution of dissimilatory sulfate reduction to H_2S production was relatively minor. However addition of 1.0 mM $Na_2{}^{35}SO_4$ to upper sediments in laboratory experiments resulted in the production of $H_2{}^{35}S$ with no apparent lag time. ^{35}S-Tracer experiments indicated an average turnover time of the sediment sulfate pool of 1.5 hours. Total sulfate reduction in a sediment depth profile to 15 cm was 15.3 mmoles sulfate reduced m^{-2} day^{-1}, which corresponds to a mineralization of 30% of the particulate organic matter entering the sediment despite low sediment sulfate concentrations. Reduction of $^{35}S^o$ occurred at a lower rate.

Most probable number estimates of sediment sulfate-reducing bacteria grown on a variety of sole carbon sources were between 4.5×10^3 and 3.3×10^6 cells/gram dry weight sediment. This represented up to 60% of the total anaerobic heterotrophic bacterial population

present in the lake sediments. Isolates of sulfate-reducing bacteria obtained from similar enrichments were able to utilize a variety of carbon sources for growth. Included among these were acetate, propionate, and butyrate while lactate, pyruvate, and casamino acids supported growth of all strains isolated. Each substrate tested supported growth of at least one isolate, establishing that a number of carbon sources can potentially serve as electron donors for sulfate reduction in lake sediments.

Sediment reactor systems were designed and constructed within which a continual low-level input of sulfate was maintained, thereby simulating sulfate diffusion into freshwater sediments. Potential rates of sulfate reduction in sediments contained within reactors dropped with time and with increasing sulfate additions while effluent acetate concentrations increased in sulfate amended reactors. Addition of H_2, lactate, and glucose decreased the effluent acetate concentration in sulfate amended reactors relative to controls and stimulated potential rates of sulfate reduction. Acetate production was attributed to sulfate reduction, suggesting that substrates which can be oxidized to acetate must be included among the primary electron donors for sediment sulfate reduction. Decreases in the effluent acetate concentration coupled with increases in potential rates of sulfate reduction indicated that acetate may also be an electron donor for sulfate reduction at increased sulfate concentrations.

Mineralization rates of ^{14}C-substrates were determined in the presence and absence of Na_2MoO_4, an inhibitor of sulfate reduction. Na_2MoO_4 inhibited sulfate reduction at all concentrations tested (0.2 mM-200mM), while methane production was inhibited at Na_2MoO_4

concentrations greater than 2 mM. Initial mineralization rates of glucose were unaffected by Na_2MoO_4, however the presence of Na_2MoO_4 decreased the mineralization rates of lactate (58% inhibition), propionate (52% inhibition), an amino acid mixture (85% inhibition) and acetate (14% inhibition). These decreases were attributed to the heterotrophic activity of sulfate-reducing bacteria. Hydrogen stimulated the reduction of $^{35}SO_4^=$ 2.5-2.8 fold, demonstrating potential hydrogen oxidation by sulfate-reducing bacteria. These results indicate that sulfate reducers in freshwater sediments utilize an array of substrates as electron donors and are of potential significance to the in situ mineralization of lactate, propionate, and free amino acids in these sediments. Thus sulfate-reducing bacteria appear to be involved with several major steps of anaerobic carbon metabolism in freshwater sediments.

To Elaine, for dancing with every mountain she meets.

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I would certainly be remiss without expressing my heartfelt appreciation to Elaine, my wife, whose devotion, sacrifice, and willing assistance were the mainstay of this effort, and to my parents for exemplifying the continual need to grow and understand.

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INTRODUCTION

The decomposition of organic matter in the sediments of productive freshwater lakes is typically anaerobic and is characterized by the production of reduced metabolic endproducts. These include both organic and inorganic compounds such as ammonia, hydrogen sulfide, volatile fatty acids, and methane (33). Mineralization of initial fermentation endproducts in these sediments, and in other anaerobic habitats, depends upon the activity of various terminal microbial processes, which serve to complete the metabolism of sedimenting organic matter. These processes include nitrate reduction, sulfate reduction, and methanogenesis. The relative role of each of these terminal processes is often inferred from the concentrations of the various electron acceptors involved (e.g. 10, 33, 54), though this may be misleading if the turnover time of the natural pool is rapid (52).

Much of the understanding of sulfate reduction comes from studies in the marine environment where sulfate levels are relatively high. In such systems sulfate reduction appears to dominate anaerobic carbon mineralization in the sediments (21). Sulfate concentrations in freshwater lake sediments are very low in comparison to marine systems and consequently the contribution of sulfate reduction to total carbon mineralization has been inferred to be insignificant (33, 54).
The rate of sulfate reduction is dependent upon both electron donor and electron acceptor availability (18, 32). The sulfate concentration is

the limiting factor for sulfate reduction in eutrophic lake sediments since carbon is readily available and sediment sulfate concentrations are low (33). However if the rate of sulfate diffusing into the sediments is the rate limiting step, significant rates of sulfate reduction may occur even when the concentration of sulfate in the sediments is virtually undetectable. Other oxidized sulfur compounds may also be utilized for dissimilatory reduction in freshwater sediments. Elemental sulfur reduction has been implicated (39, 55), though as yet has not been demonstrated despite reports that elemental sulfur concentrations can exceed sulfate concentrations in freshwater sediments (36). Hence although the potential exists in eutrophic freshwater sediments, the mineralization of organic carbon coupled to sulfur reduction has been poorly characterized.

CHAPTER I

LITERATURE REVIEW AND OBJECTIVES

Dissimilatory Sulfate Reduction. The utilization of sulfate as a terminal electron acceptor is called dissimilatory sulfate reduction. It is an obligately anaerobic process carried out by bacteria of two distinct genera. Desulfotomaculum sp. are sporeforming, heterotrophic, motile rods (9) while Desulfovibrio sp. are nonsporulating, heterotrophic, motile, spiral-shaped organisms (42). Other reported genera, though not widely recognized, include Desulfomonas (34), Desulfobulbus (26), Desulfobacter, Desulfococcus, Desulfosarcina, and Desulfonema (see 35). As a group these organisms can tolerate a wide range of environmental conditions including temperature, pressure, and salinity (41). They are present in many habitats, including freshwater and marine sediments, sewage, rumen, and human intestinal tracts (19, 27, 28).

While this group of bacteria is characterized by the ability to reduce sulfate, some organisms possess the capability to utilize sulfite, thiosulfate, or tetrathionate as electron acceptors (50). Recently Biebl and Pfennig (5) demonstrated that certain strains can also reduce elemental sulfur. Pure cultures of Desulfovibrio desulfuricans and D. gigas are able to grow in the absence of oxidized inorganic sulfur compounds by reducing fumarate to succinate (28). Sulfate-reducing bacteria were also shown to produce H_2 as an electron

sink when grown with ethanol or lactate in the absence of sulfate, provided a low partial pressure of hydrogen was maintained (7). The energy obtained by the reduction of these various electron acceptors as compared with the energy obtained from electron acceptors utilized by other organisms is shown in Table 1. While sulfate is not the most energetically favorable electron acceptor for sulfate-reducing bacteria it is the species most frequently encountered in nature. The significance of these other electron acceptors to sulfate-reducing bacteria in sediments has yet to be demonstrated.

Carbon metabolism of sulfate-reducing bacteria is generally incomplete, with the production of fatty acids and CO_2 (41). In general the range of substrates which these organisms can utilize as electron donors is limited (8) with lactate, pyruvate, ethanol, and formate reported to be the preferred electron donors (50). Hydrogen and sulfate can also be utilized as the sole energy source (4); however, an organic carbon source is required for growth (3, 45). This latter reaction may allow the sulfate reducers to more effectively compete for electron donors in sediments if they can participate in interspecies hydrogen transfer similar to that proposed for methanogens (48). Such a transfer was demonstrated in mixed cultures of Desulfovibrio with H_2-producing, acetogenic bacteria (6, 31). The narrow range of known carbon substrates may be the ramification of isolating most sulfate reducers using lactate as the electron donor.

Widdel and Pfennig (53) recently isolated a sulfate-reducing, acetate-oxidizing strain of Desulfotomaculum from acetate enrichments. Other strains of this genus are described as lacking the capability to oxidize acetate (9). Acetate-oxidizing, sulfate-reducing activity has

Table 1. Standard free energies for the reduction of selected electron acceptors[1].

Electron Acceptor	Product	$\Delta G^{o'}$ (kcal/mole H_2)
O_2	H_2O	-56.7
NO_3^-	N_2	-53.6
fumarate	succinate	-20.6
$SO_3^=$	H_2S	-13.8
$S_2O_3^=$	H_2S	-10.4
$SO_4^=$	H_2S	- 9.1
HCO_3^-	CH_4	- 8.1
S^o	H_2S	- 6.7
H^+	H_2	+ 4.6

[1]Values from Thauer et al. (50).

also been reported to be present in sewage (17, 32), which indicates the natural occurrence of acetate-oxidizing sulfate reducers. Other investigators have reported isolating sulfate-reducing bacteria that can oxidize propionate and long chain fatty acids (26, see also 35).

Sulfate Reduction in Natural Habitats. Sulfate reduction plays an important role in carbon metabolism in anaerobic marine habitats due to the high concentrations of sulfate in seawater (21). Since the rate of sulfate reduction is independent of the sulfate concentration at concentrations greater than approximately 10 mM (18), sulfate concentrations are not likely rate limiting in the water column and upper sediments of most marine habitats. This implies that electron donor availability is the rate dependent factor for sulfate reduction (18, 43). Goldhaber and Kaplan (18) conclude that sulfate-reducing bacteria rely on a complex community of fermentative bacteria to supply the small range of organic molecules utilized by sulfate reducers. They also demonstrated that the rate of sulfate reduction was related to the sedimentation rate of organic material. The importance of sulfate reduction to carbon metabolism in marine sediments is supported by studies that report that sulfate reduction catalyzed over 50% of the carbon mineralized in a marine sediment model system (24) and 53% of the mineralization in a coastal marine sediment (21). In the latter case the sulfate reduction rate ranged from 25-200 nmole SO_4^{-2} cm^{-3} day^{-1} over an annual cycle, which corresponds to a turnover time of the sulfate pool of 4-5 months.

Stratified freshwater systems have much lower concentrations of sulfate present in the hypolimnion and the sediments, which limits sulfate reduction (43). As a result the activity of sulfate-reducing

bacteria in freshwater sediments has frequently been overlooked. Stuiver (49) found that sulfur transport by sedimentation of organic material was neglible relative to the sulfate concentration, as most of the sulfur present in the sediments was in the form of SO_4^{-2}, H_2S, and S^{-2}. Loss of sulfur from the hypolimnion was neglible as very little sulfate was exchanged between the hypolimnion and epilimnion by eddy diffusion, while the loss of H_2S to the epilimnion by both diffusion and ebullition was also small (49). Although sulfate reduction occurs in the water column, the maximum rate occurs in the sediments (47, 49, 52). Dunnette (16) found that H_2S production from (^{35}S) sulfate displayed saturation kinetics, while cysteine decomposition contributed between 5.1 and 53 percent of the total H_2S produced.

Although sulfate values may be low in freshwater sediments, drawing conclusions about reaction rates from pool sizes may be in error (21). van Gemerden (52) demonstrated that in mixed cultures of Desulfovibrio desulfuricans and Chromatium vinosum both organisms were able to grow exponentially in the presence of very low total sulfur concentrations. The turnover time of the total amount of sulfur was as short as 15 minutes. Thus a significant amount of sulfate reduction can occur even when sulfate concentrations are low, if a rapid turnover time is evident. Cappenberg (10) found up to $1x10^7$ sulfate-reducing bacteria per liter of wet mud in lake sediments where no sulfate was detectable.

Generally in a sediment depth profile significantly greater rates of sulfate reduction are found near the sediment surface and decrease exponentially with depth (2, 21, 35). In marine sediments an estimated 65% of the total sulfate-reducing activity occurred in the top 10 cm

(21), while sulfate concentrations (21) and the number of sulfate-reducing organisms (10, 21) displayed similar trends. The exact nature of the sulfate-reducing profile depends (at least in part) on the total electron donors and total electron acceptors available.

Current evidence indicates that sulfate reduction and methanogenesis are not compatible. The maximum number of methanogens are reported at sediment depths below that of the maximum number of sulfate reducers in both marine and freshwater sediments (10, 14). In laboratory experiments with marine sediments methane production did not occur until 90% of the sulfate was depleted (29, 30), while negligible methane production was observed in sewage sludge when high levels of sulfate were added (17). Winfrey and Zeikus (54) report that the addition of 0.2 mM sulfate to Lake Mendota sediments inhibited methanogenesis, though sulfate itself is not toxic to methanogenic bacteria (7). Cappenberg (12) concluded that methanogenic bacteria are inhibited by H_2S produced by sulfate reducers. He suggests a commensalistic relationship between the two bacterial groups whereby the sulfate reducers produce acetate, which diffuses down and is utilized by methanogenic bacteria (11, 12, 13). The inhibition of methanogenesis by sulfide appears unlikely however at natural sulfide concentrations (7, 14, 30, 54). While acetate production by sulfate-reducing bacteria and subsequent utilization by methanogenic bacteria is still a feasible hypothesis it must be reexamined in light of reports that sulfate-reducing bacteria can oxidize acetate (53). Recent evidence indicates acetate mineralization by both methanogens and sulfate reducers in freshwater and marine sediments (35, 53, 55). Thermodynamically the utilization of acetate by sulfate reducers is

favored, yielding 11.3 kcal/mole versus 6.8 kcal/mole yielded by the conversion of acetate to CO_2 and CH_4 (54). This compares with 38.2 kcal/mole available to classical sulfate-reducing bacteria when oxidizing lactate (50). Since the acetate-oxidizing, sulfate-reducing bacteria isolated to date can utilize only acetate, butyrate, butanol, or ethanol as electron donors (53) they face a competitive disadvantage when competing with lactate-oxidizing, sulfate-reducing bacteria for depleting sulfate concentrations. This appeared to be the case in New Zealand intertidal sediments where acetate oxidation by sulfate-reducing organisms and interstial sulfate values both decreased with depth despite elevated rates of sulfate reduction in the deeper sediments (35).

Studies by several investigators have indicated that sulfate-reducing bacteria in marine sediments can oxidize hydrogen (1, 35, 37). Oremland and Taylor (37) demonstrated that sulfate-respiring bacteria were primarily responsible for H_2 oxidation while additions of H_2 stimulated sulfate reduction with very little increase in methane production (1, 35). It appears that sulfate reducers can compete for hydrogen as well as acetate in marine sediments. However, hydrogen contributed little as an energy source when sulfate-reducing bacteria were grown on lactate and sulfate in culture (25), though active hydrogenase is produced (51). In addition the strain of Desulfovibrio which reportedly oxidizes hydrogen as the sole energy source also oxidizes lactate (3). Thus it appears that sulfate-reducing bacteria oxidize hydrogen only when electron donor availability is rate limiting, though hydrogen oxidation in sediments by sulfate-reducing bacteria at in situ hydrogen concentrations has not yet been

demonstrated. The hydrogen concentration in eutrophic freshwater sediments is less than 0.7 μmoles l^{-1} of wet sediment (48).

Methanogenic bacteria and sulfate-reducing bacteria can also interact by means of interspecies hydrogen transfer. Winfrey and Zeikus (54) demonstrated that the sulfate inhibition of methanogenesis in freshwater sediments could be reversed by additions of hydrogen. They concluded that sulfate altered the normal carbon and electron flow, which involved H_2 transfer to the methanogens in sulfate depleted Lake Mendota sediments. Desulfovibrio sp. can grow in sulfate-free media containing either lactate or ethanol in the presence of a methanogenic bacteria (7). In the absence of sulfate the Desulfovibrio produced H_2, which the methanogen utilized to produce methane. The methane-producing bacteria maintain a low partial pressure of H_2 in such a system, which allows the reaction to be thermodynamically favorable. Apparently the flow of electrons to methane via H_2 does not compete with their flow to sulfate as their data indicates that methane production was reduced by addition of sulfate in stoichiometric amounts. Bryant et al. (7) suggest that sulfate reducers may be important to carbon metabolism in anaerobic habitats containing little or no sulfate if their metabolism is coupled with H_2-utilizing methanogenic bacteria.

Several investigators have reported that sulfate-reducing organisms can oxidize methane while producing sulfide. Much of the evidence comes from the observed distribution of methane in marine environments (30) where a concave up curve is viewed as indicative of methane consumption in surface sediments (30, 44). Evidence indicates that Desulfovibrio desulfuricans can oxidize methane, but at a very

slow rate (15). Anaerobic methane oxidation was observed in hypolimnetic water samples from Lake Mendota (38), though additions of sulfate did not stimulate methane oxidation; acetate or lactate and sulfate were required for growth of enrichments. Zehnder and Brock (56) reported anoxic methane oxidation in the sediments of the same lake, attributing the activity to methanogens rather than sulfate-reducing bacteria. Apparently methane oxidation by sulfate reducers is relatively unimportant in sediments that have high amounts of readily utilizable organic carbon sources (30, 44).

Measurement of sulfate-reducing activity in natural sediments has been attempted using several different procedures. One of the most common is enumeration of sulfate-reducing bacteria. However, the number of sulfate-reducing organisms does not reflect the activity of sulfate reduction measured with $Na_2{}^{35}SO_4$ in either freshwater or marine sediments (16, 21). Jørgensen (23) reports that colony counts underestimate the true numbers of sulfate reducers by 1000 fold. As previously indicated pool sizes of sulfate are not reliable estimators of sulfate reduction. Dunnette (16) found that the contribution to total sulfide by sulfate reduction and organic putrefaction varied, while Sorokin (46) indicated that the maximum H_2S concentration and the rates of sulfate reduction rarely correspond.

The accumulation of sulfide is also not considered to be a useful measurement of sulfate reduction. The accumulation of reduced sulfur compounds was found to underestimate the in situ metabolism of coastal marine sediments 10-fold because it neglected diffusional losses of sulfide (23). A portion of the sulfide produced is also precipitated by heavy metals, of which iron is the most important (18). Initially

iron combines with free sulfide to form the intermediate iron sulfides of mackinawite (feS) and greigite (Fe_3S_4), however these are not stable under sedimentary conditions and slowly transform to pyrite (FeS_2). Pyrite is acid insoluable and difficult to quantify in sediments. Jørgensen (21) estimates that 10% of the sulfide produced in coastal marine sediments is transformed to pyrite, however pyrite formation can be very rapid in salt marsh sediments (20).

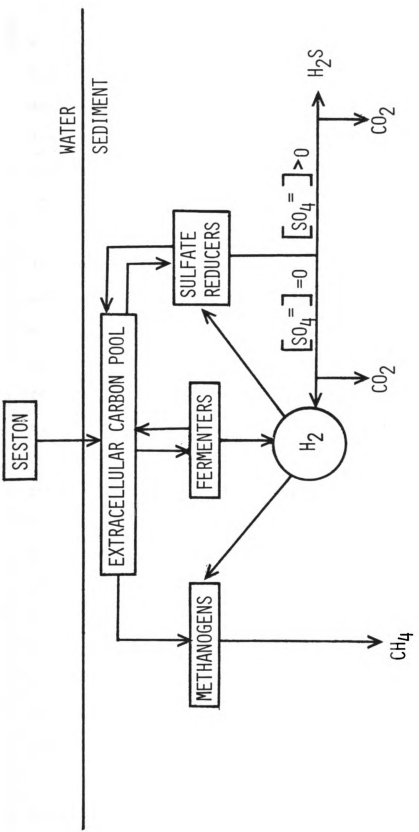
Determinations of stable sulfur isotope ratios have proven to be an important aid in examining the origin of the various sulfur pools and estimating the rates of bacterial sulfate reduction (21). The stable isotope ^{32}S reacts more rapidly than does ^{34}S during bacterial reduction. This results in sulfide, acid volatile sulfide, and pyrite pools enriched in ^{32}S, while the sulfate pool is enriched in ^{34}S (18). Rates of sulfate reduction may be calculated by measuring the depletion of total sulfate in pore water with depth, and hence with time (18). It is difficult to establish the dynamics of the sulfur cycle from stable isotope determinations (21) and therefore radiotracer techniques using ^{35}S have been developed to increase sensitivity when measuring sulfate reduction (22, 24, 46). This technique takes advantage of the availability of carrier-free $Na_2{}^{35}SO_4$, which can be amended to sediments without altering the *in situ* sulfate concentration.

Elemental Sulfur Reduction. Although it is found in many environments, very little is known about the reduction of elemental sulfur to sulfide. Postgate (40) was unable to demonstrate elemental sulfur reduction using six strains of *Desulfovibrio*. However cultures of *Desulfovibrio* growing on formate and reducing sulfate displayed large increases in the production of sulfide when elemental sulfur was

added (52), indicating that S^o may have been reduced to H_2S. As indicated previously Biebl and Pfennig (5) demonstrated S^o reduction to sulfide by several sulfate-reducing organisms. They have also isolated and described Desulfuromonas acetoxidans, which is capable of reducing elemental sulfur through acetate, ethanol, or propanol oxidation to CO_2. This organism is unable to utilize other inorganic sulfur compounds as electron acceptors. Previously the oxidation of acetate coupled to the reduction of elemental sulfur was not thought to yield enough energy to produce ATP ($\Delta G^{o'}$ = -4.0 kcal/mole). However, accounting for physiological conditions (HS^- = 10^{-2}M) the free energy change can be sufficient to produce ATP ($\Delta G^{o'}$ = -14.8 kcal/mole) (50). The organism does not possess hydrogenase and can not use hydrogen as an electron donor. The distribution of this organism in sediments and other natural habitats is unknown.

Overview and Objectives of Research. The proposed role of sulfate reducing bacteria in eutrophic freshwater lake sediments is depicted in Figure 1. The sulfate reducers compete with other organisms (for example methanogens) for low molecular weight organic carbon sources or hydrogen. These are utilized primarily as electron donors, and are either completely oxidized to CO_2 or only partially oxidized to CO_2 and a second lower energy containing carbon substrate. Alternatively if the sulfate concentrations are very low the hydrogenase containing sulfate-reducers may produce molecular hydrogen, which would be rapidly utilized by methanogens. This would make the process energetically favorable for the sulfate-reducers and allow them to remain metabolically active in the absence of sulfate.

Figure 1. The relationship between sulfate-reducing bacteria and methanogenic bacteria in freshwater sediments.


WATER
SEDIMENT
SESTON
EXTRACELLULAR CARBON POOL
FERMENTERS
METHANOGENS
SULFATE REDUCERS
H_2
$[SO_4^=]=0$
$[SO_4^=]>0$
CO_2
CO_2
H_2S
CH_4

Within this framework the purpose of this study was to investigate the relationship between carbon metabolism and sulfate reduction in eutrophic freshwater sediments. A multifaceted approach involving field investigations, tracer studies with ^{35}S sulfur compounds, a survey of growth substrates utilized by sulfate-reducing bacteria isolated from the sediments, laboratory experiments in sediment containing flow-through flasks, and mineralization experiments with ^{14}C-labeled substrates were utilized to examine these relationships.

The first objective (Chapter II) was to characterize the distribution of naturally occurring sulfur compounds in these sediments and to determine the magnitude of sulfur (both sulfate and elemental sulfur) reduction. This study demonstrated the nature of the available sulfur substrate in this sedimentary environment relative to sulfur reduction and provided an estimation of the amount of carbon mineralized by sulfate-reducing bacteria.

The second objective (Chapter III) was to investigate the potential range of substrates that can be utilized as electron donors by sulfate-reducing bacteria isolated from these sediments. This survey was based upon the contention that the reportedly limited range of electron donors utilized by sulfate-reducing bacteria is due to the isolation of most strains of sulfate-reducing bacteria with lactate. Enrichments and subsequent isolations of sulfate-reducing bacteria were attempted utilizing a variety of electron donors. The isolates obtained were in turn tested for growth upon the same array of potential electron donors.

The third objective (Chapter IV) was to examine the range of potential electron donors for sediment sulfate reduction by determining

the effects of added substrates upon sulfate-reducing activity as a function of time. In order to simulate low levels of sulfate diffusing into the sediments, flow-through flasks were designed and constructed which allowed continuous substrate inputs to mixed sediment without altering the sediment volume.

The fourth objective (Chapter V) was to establish and quantify the range of carbon substrates mineralized by sulfate-reducing bacteria in freshwater sediments. ^{14}C-substrates and Na_2MoO_4, a specific inhibitor for sulfate-reducing bacteria, were utilized to investigate this objective. The effect of Na_2MoO_4 upon other sediment processes was also investigated in order to characterize the specificity of Na_2MoO_4 inhibition in freshwater sediments.

The approach taken allowed for a comprehensive examination of the role of sulfate reduction as a process in the mineralization of organic substrates in freshwater sediments, and the potential capabilities and diversity of the organisms responsible.

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CHAPTER II

REDUCTION OF SULFUR COMPOUNDS IN THE SEDIMENTS OF A EUTROPHIC LAKE BASIN

INTRODUCTION

The hydrogen sulfide in anoxic hypolimnia and sediments of many aquatic habitats has two origins: microbial putrefaction of organic sulfur and reduction of oxidized inorganic sulfur compounds. Estimates of the relative contribution of organic sulfur to H_2S production (16) range from 3% in marine sediments (7) to over 50% in freshwater sediments (17; D. Dunnette, Ph.D. Thesis, University of Michigan, Ann Arbor, 1973).

In marine systems where high sulfate concentrations exist, sulfate reduction dominates anaerobic carbon mineralization in the upper sediments (7). The significance of sulfate reduction in sediments of inland waters has not been as extensively characterized. This is especially true for freshwater lakes where sulfate concentrations are low in comparison to marine systems.

The rate of sulfate reduction is dependent upon the availability of both organic carbon and sulfate (6,13). In eutrophic lake sediments carbon is readily available, however, if the redistribution of oxidized sulfur compounds back to the sediments is the rate limiting step, significant rates of sulfur reduction may be evident even though oxidized sulfur is virtually undetectable. In this paper we report the

distribution of natural sulfur substrates in eutrophic freshwater sediments and the occurrence of significant rates of sulfate reduction.

Materials and Methods

Sampling Site: Wintergreen Lake is a shallow (z_m = 6.5m) hypereutrophic lake located within the W. K. Kellogg Bird Sanctuary, Hickory Corners, Michigan. The hypolimnion is anaerobic approximately 7 months of the year with anoxic conditions extending to within 3 meters of the lake surface during summer stratification. Molongoski and Klug (14,15) have described the lake, characterized sedimenting particulate organic matter, and reported on the pool sizes of metabolites involved in anaerobic decomposition processes.

Sample Collection: Water column samples were pumped to the surface through Tygon tubing into a flask with a sidearm fitted with a septum. The collecting flask was flushed approximately 1.5 times with water from the depth sampled to insure removal of residual air. Subsamples were transferred from the collection flask by syringe for H_2S analysis; the remainder of the sample was analyzed for sulfate.

Sediment pore water collected with interstitial water dialysis samplers (15) was analyzed for H_2S and $SO_4^=$. Sediment samples were collected by 3 inch i.d. gravity cores, SCUBA, or by Eckman dredge (Wildlife Supply Co.), and processed to allow minimum exposure to air. Cores were obtained with Plexiglass® core tubes (3 inch i.d.), which contained a row of vertical holes centered 3 cm apart and sealed with pressure sensitive tape during coring. Subsamples of the core were obtained with cut-off 5 or 10 ml syringes and placed in 30 or 50 ml Wheaton serum bottles containing 5 ml of 2% $CdCl_2$. Bottles were flushed with O_2-free N_2 throughout the subsampling. Samples for S° and

total sulfur analysis were removed from the bottles and oven dried at 50°C while subsamples for acid volatile sulfide analysis were crimp sealed with Teflon lined rubber septa (Supelco, Inc.) and frozen at 0°C.

Chemical Analyses: Total sulfur was determined by a modified NaOBr oxidation to sulfate (20), as described by King and Klug (11), and subsequent turbidimetric determination of sulfate (19). Natural sulfate concentrations were also determined by the latter method. Sulfide was assayed by the methylene blue method (5). Water samples for sulfide analysis were transferred by syringe to preflushed (with O_2-free N_2) evacuated tubes containing the appropriate reagent concentration.

S^o was assayed by refluxing dried sediment samples with 15 ml of benzene for 1 hour at 90-100°C, followed by filtration. The extracts were assayed using a Varian 3700 gas chromatograph equipped with a Flame Photometer Detector. Analysis was made with a stainless steel column (50 cm x 0.3 cm OD) packed with 5% OV-101 on a solid support of chromosorb G.H.P. (100/200 mesh). Sample values were corrected for extraction efficiency as determined by amending sediment with a known amount of S^o prior to drying.

Acid volatile sulfide was determined with a trapping train similar to the one described by Jørgenson and Fenchel (9). O_2-free N_2 was flushed through a syringe needle into serum bottles containing frozen sediment samples and exhausted through a second syringe needle into two scintillation vials connected in series with nylon tubing and containing 10 ml of 2% $CdCl_2$. The scintillation vials were stoppered with Teflon coated rubber septa held in place by screw caps with holes in the center for the gassing lines. The headspace gas in the serum

bottle was flushed 2 min to purge the system of O_2 prior to addition of O_2-free 3N HCL (1x sediment volume). The serum bottle was placed in a hot water bath, and the gassing needle lowered into the sediment slurry. The samples were flushed 30 min, the appropriate reagent immediately added to each trap, and the sulfide concentration assayed as above. The efficiency of the trapping train was determined with standard solutions of Na_2S; all results were corrected accordingly.

Sulfur Reduction: Surface sediment was collected with an Eckman Dredge, mixed well in an anaerobic glovebox, and 5 or 10 ml subsamples transferred to 30 or 50 ml Wheaton serum bottles, which were sealed with Teflon lined rubber septa (Supelco, Inc.) and aluminum crimps. The sediment samples were removed from the glovebox, the headspace flushed with O_2-free N_2, and incubated at 10°C. Controls were "killed" by the addition of 50% glutaraldehyde to a final concentration of 2%. Pre-reduced carrier-free $Na_2{}^{35}SO_4$ (New England Nuclear, 1-2 μCi), diluted with unlabelled Na_2SO_4 when appropriate, was added (0.5 or 1.0 ml) with a syringe and needle to each sample. Bottles were vortexed and incubated with shaking at 10°C for appropriate periods. Sulfate reduction was terminated by quick freezing in a dry ice-acetone bath, and the samples stored at -10°C until analysis. The $H_2{}^{35}S$ produced was trapped with the flushing train described above, except a third trap was added and the volume of 2% $CdCl_2$ reduced to 5 ml in each trap. Upon completion of flushing 10 ml of Aqueous Counting Scintillant (Amersham Searle) was added to each scintillation vial and the radioactivity determined in a Beckman LS8000 liquid scintillation counter (Beckman Instruments). Counting efficiencies, which were determined with ^{14}C-toluene as an internal standard, varied with the

amount of CdS present in a given trap. The recovery of $Na_2{}^{35}S$ (New England Nuclear) standards for the freezing-flushing system varied from 82% if the samples were trapped immediately after freezing to 23% if frozen for 15 days or greater and corrections were made accordingly.

Elemental sulfur reduction was measured by placing 58 µg $^{35}S°$ (0.8 µCi, Amersham Searle) dissolved in toluene in 30 ml serum bottles, evaporating the toluene, and transferring 5 ml of mixed surface sediment to each bottle in the anaerobic glovebox. The headspace of the bottles was flushed with O_2-free N_2, and the samples incubated at 10°C with shaking for appropriate lengths of time. The reaction was terminated by freezing and $H_2{}^{35}S$ stripped and trapped as described above.

Results

Distribution of Sulfur Compounds: The distribution of sulfur compounds in Wintergreen Lake profundal sediments during summer stratification (Figs. 1, 2, and 3) indicated that concentrations of the primary oxidized species, $SO_4^=$, were less than 30 µmoles/l throughout the sediment and overlying hypolimnion (Figs. 1,2). During nonstratified periods sulfate concentrations were higher in the overlying water column, however concentrations in the sediments were relatively constant (Fig. 2B). Soluble H_2S concentrations were the inverse of those of sulfate in the water column, being high during stratification and low during nonstratification. Pool sizes of soluble sulfide in sediments remained higher than sulfate except in the top 4 cm during nonstratified periods due to the overlying oxygenated water. On an annual average the sulfate pool drops from 0.231 (se 0.030) mmoles $SO_4^=$/l 1.8 cm above the sediment-water interface to 0.034

Figure 1. Distribution of sulfur compounds in Wintergreen Lake profundal sediments. Data points represent the mean of sediment cores and interstitial water samples collected from 6-26-78 to 11-7-78, except S° for which samples were pooled prior to analysis. Error bars represent ± one standard error.

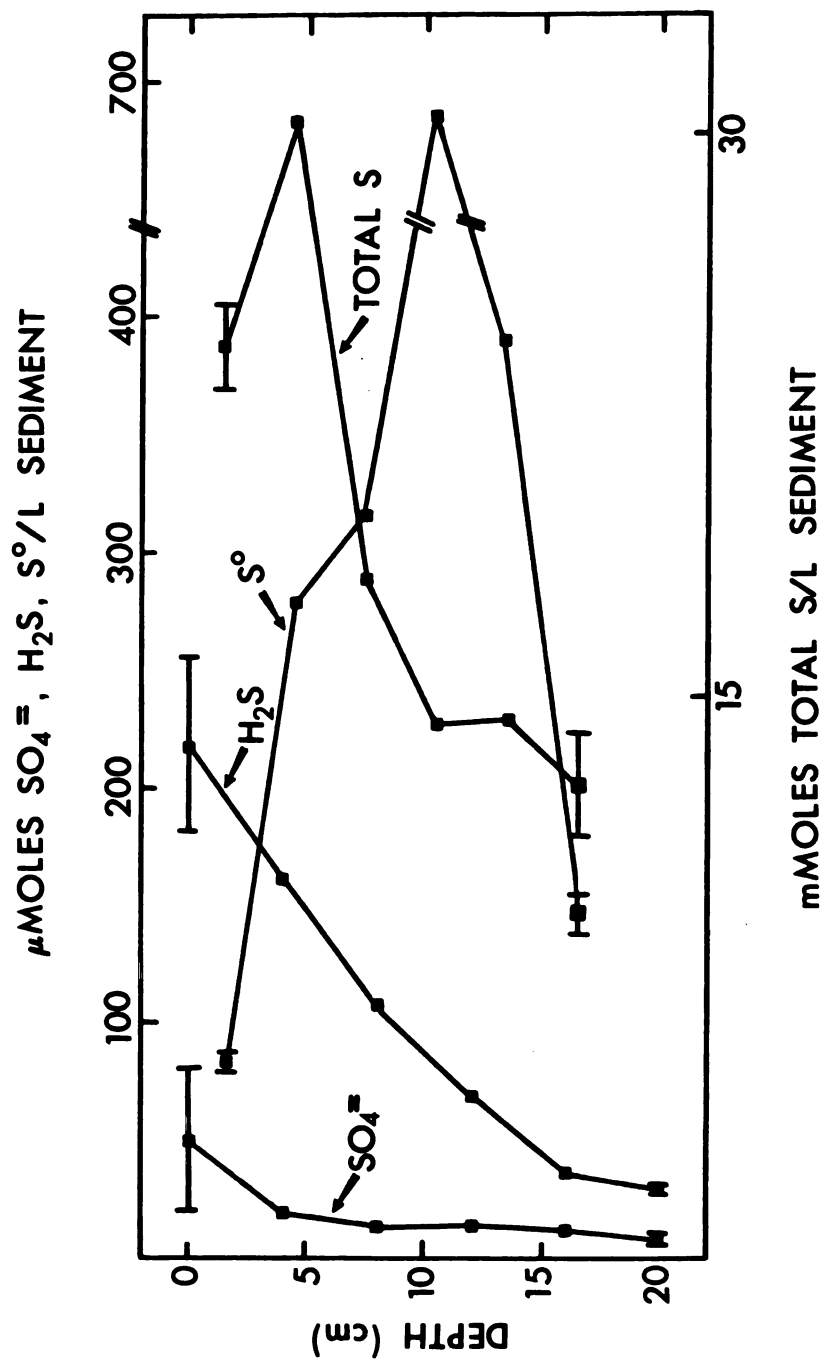

μMOLES $SO_4^=$, H_2S, S°/L SEDIMENT
mMOLES TOTAL S/L SEDIMENT
DEPTH (cm)
TOTAL S
S°
H_2S
$SO_4^=$
0
5
10
15
20
100
200
300
400
700
15
30

Figure 2. Distribution of $SO_4^{=}$ and H_2S in Wintergreen Lake profundal sediments and overlying water column on A) 8-17-78 and B) 11-7-78. Squares represent samples taken from the surface by pump and circles represent samples taken from an interstitial water sampler.

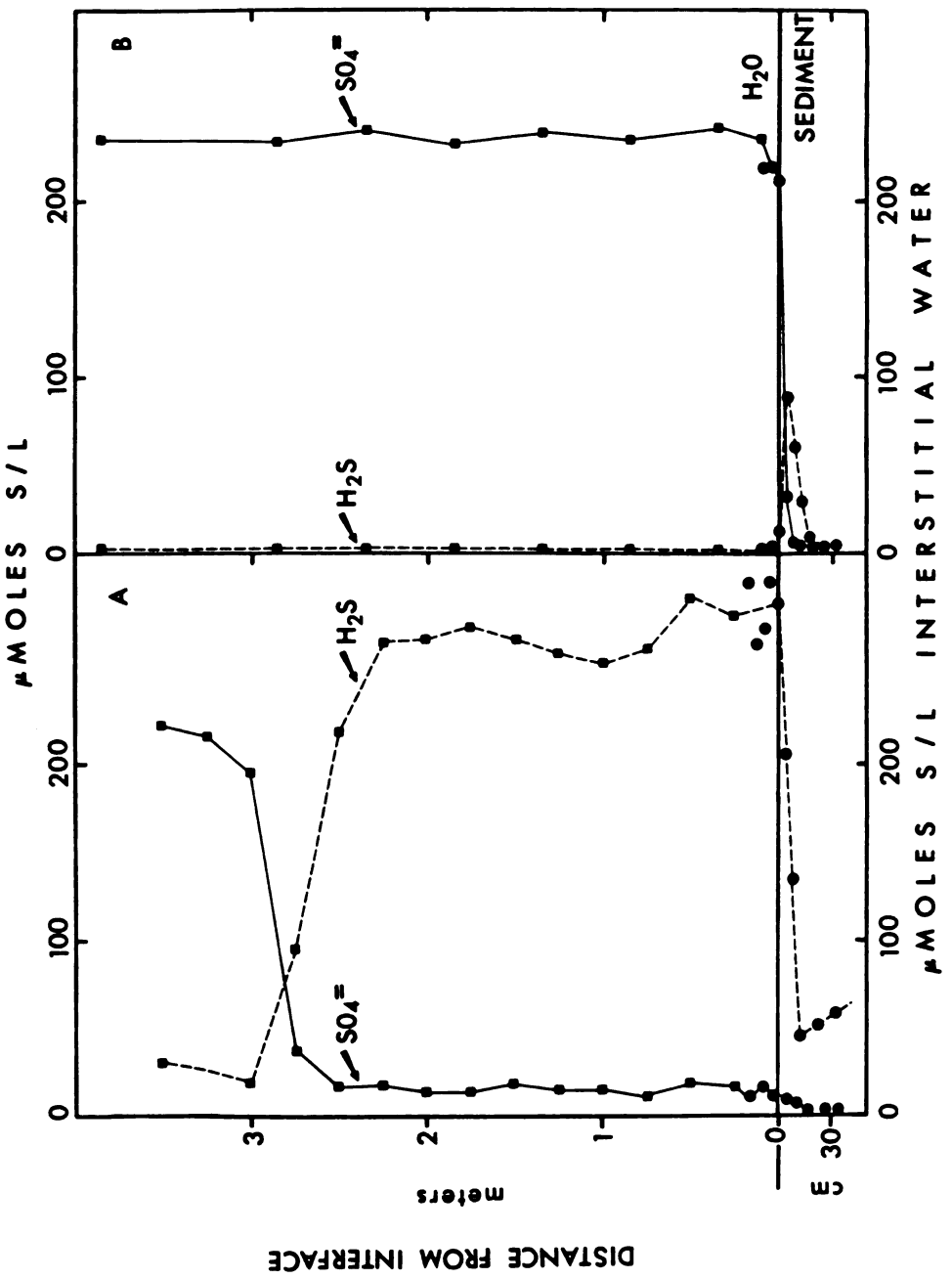
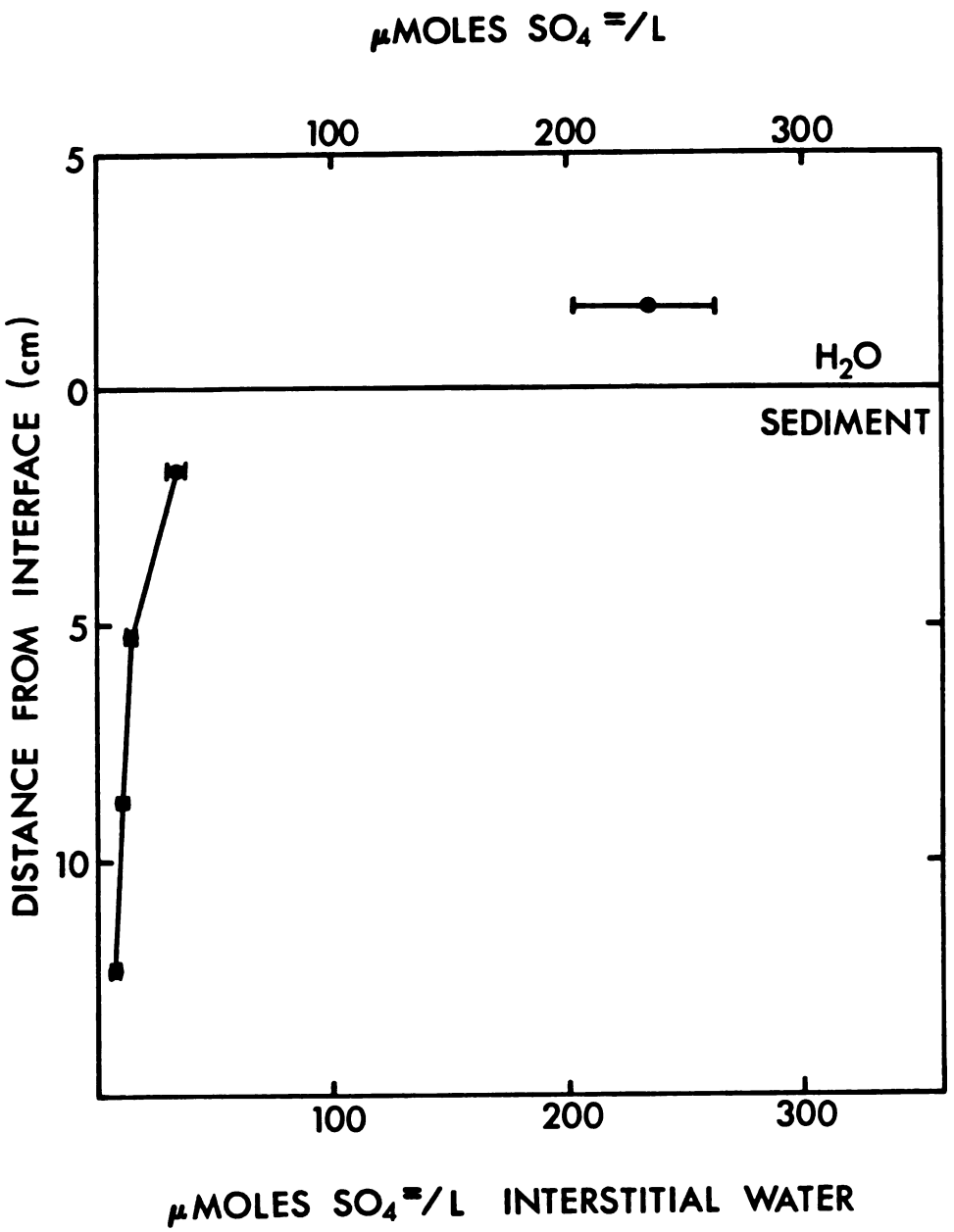

B
A
$SO_4^=$
H_2S
H_2O
SEDIMENT
µMOLES S/L
µMOLES S/L INTERSTITIAL WATER
0
100
200
3
2
1
0
30
meters
DISTANCE FROM INTERFACE

Figure 3. Average annual sulfate concentration in Wintergreen Lake profundal sediments. Samples taken biweekly in 1977. No curve is shown near the sediment-water interface to emphasize the unknown nature of the sulfate gradient in this region. Error bars represent $\pm$ one standard error.


μMOLES $SO_4^=$/L
100
200
300
5
0
5
10
H_2O
SEDIMENT
DISTANCE FROM INTERFACE (cm)
100
200
300
μMOLES $SO_4^=$/L INTERSTITIAL WATER

(se 0.004) mmoles $SO_4^=$/l interstitial water 1.8 cm below the interface (Fig. 3). Relative to an annual average the sulfate profile displayed in Figure 2A was minor.

Acid volatile sulfide (AVS) concentrations (primarily FeS and Fe_3S_4) were much higher in Wintergreen Lake sediments than those of H_2S (Figure 4). The AVS pool (0.8 mmoles/l) was constant in the top 10 cm of sediment while increasing markedly below 10 cm. This increase was visually confirmed by the presence of black bands or patches in sediment cores below 10 cm depths and accounts for the increase in the relative variability (Figure 4) of the AVS determinations below this depth.

Elemental sulfur concentrations in the sediments were greater than those of sulfate and a peak concentration was observed at 10 cm (Fig. 1). The total sulfur content was much greater than the combined concentrations of H_2S, $SO_4^=$, S°, and AVS (Fig. 1) and decreased with depth in the sediments.

Sulfate Reduction: The addition of $^{35}SO_4^=$ to profundal surface sediments to a final concentration of 1 mM resulted in the production of $H_2{}^{35}S$ with no apparent lag time (Fig. 5). The production was linear for the first hour followed by no further production from 1-8 hours. Ninety-nine percent of the radiolabel was recovered as $H_2{}^{35}S$ within 52 hours (data not shown). This production corresponds to a sulfate reduction rate of 19.1 µmoles $SO_4^=$ reduced/l sediment x hour, or a 4-fold increase in the rate of sulfate reduction from the first hour (4.7 µmoles $SO_4^=$ reduced/ 1 sediment x hour). Initial rates of sulfate reduction determined for surface sediment containing varied concentrations of added sulfate indicated that sulfate-reducing

Figure 4. Distribution of H_2S and total acid volatile sulfide (AVS) in Wintergreen Lake profundal sediments on 11-29-79. Error bars represent $\pm$ one standard error.

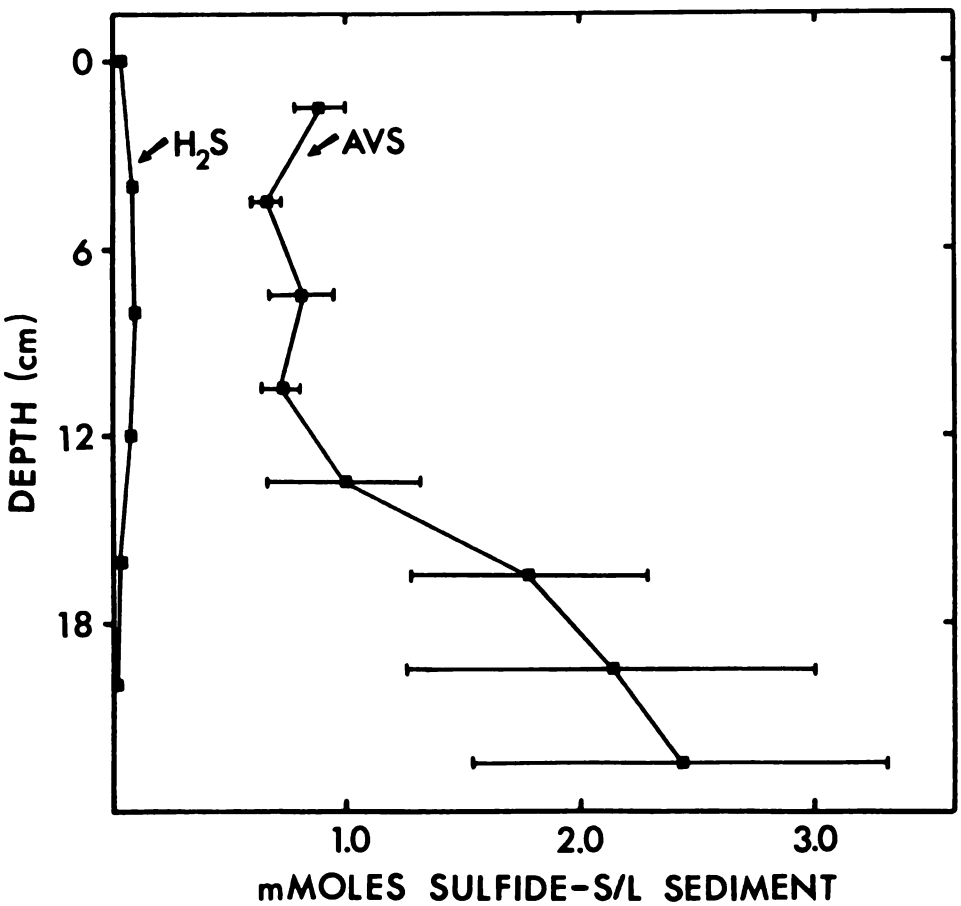
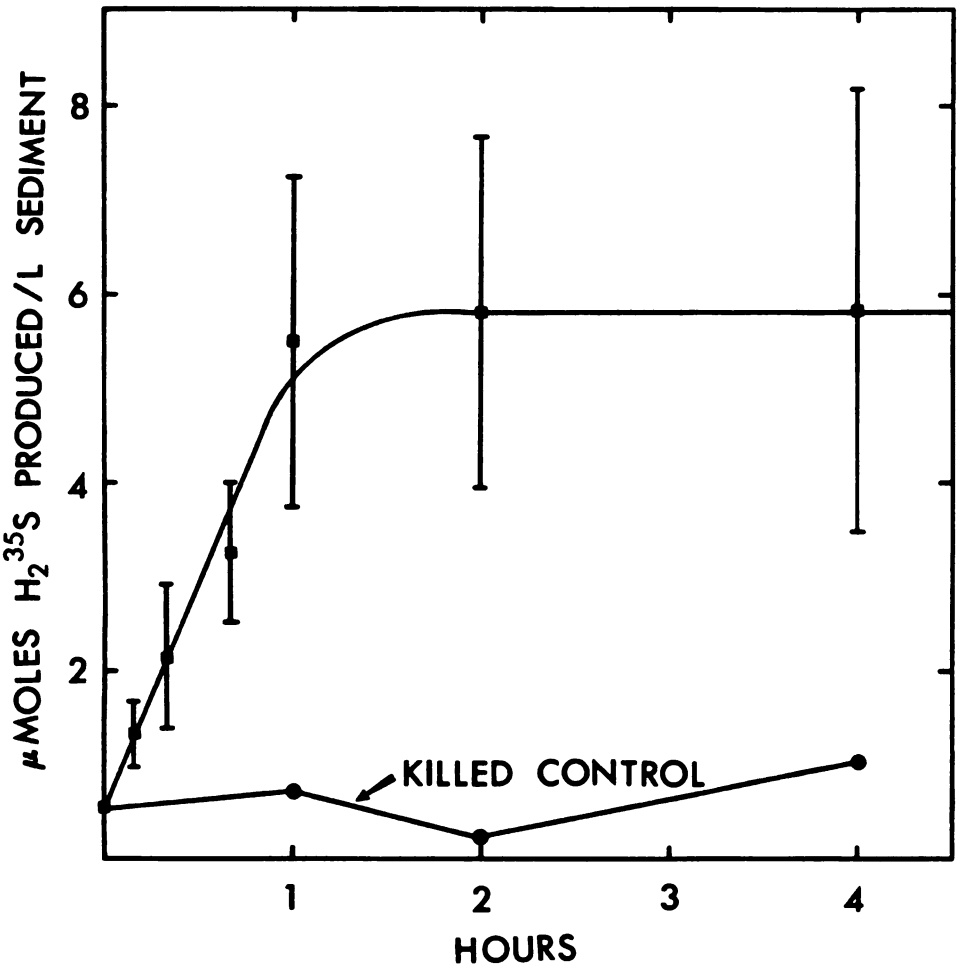

0
6
12
18
DEPTH (cm)
H_2S
AVS
1.0
2.0
3.0
mMOLES SULFIDE-S/L SEDIMENT

Figure 5. Time course of sulfate reduction at 10°C in profundal sediments amended with $^{35}SO_4^{=}$ to a final concentration of 1.0 mM. Controls were pretreated with glutaraldehyde. Error bars represent $\pm$ one standard error.


μMOLES $H_2{}^{35}S$ PRODUCED/L SEDIMENT
8
6
4
2
KILLED CONTROL
1
2
3
4
HOURS

activity displayed Michaelis-Menten kinetics (Fig. 6). Lineweaver-Burke transformations indicate an apparent K_M of 0.068 mmoles $SO_4^=$ /l sediment and a V_{max} of 13.2 μmoles $SO_4^=$ reduced/l sediment x hour. A turnover time of 5.4 hours for the in situ sulfate pool (at 10°C) can be estimated from the data shown in Figure 6 (minus the in situ $SO_4^=$ concentration) using the method of Wright and Hobbie (22).

Sulfate reduction rates at in situ sulfate concentrations obtained by introducing carrier-free $H_2{}^{35}SO_4$ to sediments (Fig. 7) were estimated to be 0.9 μmoles sulfate reduced/l sediment x hr. This rate corresponds to a turnover time of the in situ sulfate pool of 2.4 hours. Similar measurements run concurrently with the sulfate reduction kinetic experiment (Fig. 6) yielded a turnover time of 2.2 hours. The range of turnover times found in mixed surface sediments (0-5 cm) taken from March 1979 through February 1980 and incubated at 10°C was 0.1-5.8 hours (η=14) with a mean of 1.5 hr (s.e. 0.5). No seasonal trends were observed.

The depth profile of sulfate reduction for sediments collected in July (Table 1) shows maximum activity in the surface sediments, 0-9 cm, and a marked decrease below 10 cm. The total reduction rate to 15 cm was 15.3 mmoles sulfate/m^2 x day, with 51% of the activity occurring in the top 5 cm and 89% in the top 10 cm (Table 1).

Elemental Sulfur Reduction: $H_2{}^{35}S$ was produced with no apparent lag time after the addition of 30 mg $^{35}S^o$/l of sediment (Fig. 8). The activity was biological, since no activity was observed in killed controls, with a reduction rate of 8.8 μmoles S^o/l sediment x day. Similar rates were observed in sediments sampled in May and September.

Figure 6. Kinetics of sulfate reduction at 10°C in profundal sediments collected in March amended with $^{35}SO_4^=$. Values of $SO_4^=$ concentration account for <u>in situ</u> $SO_4^=$ present. Inset is the Lineweaver-Burke plot.

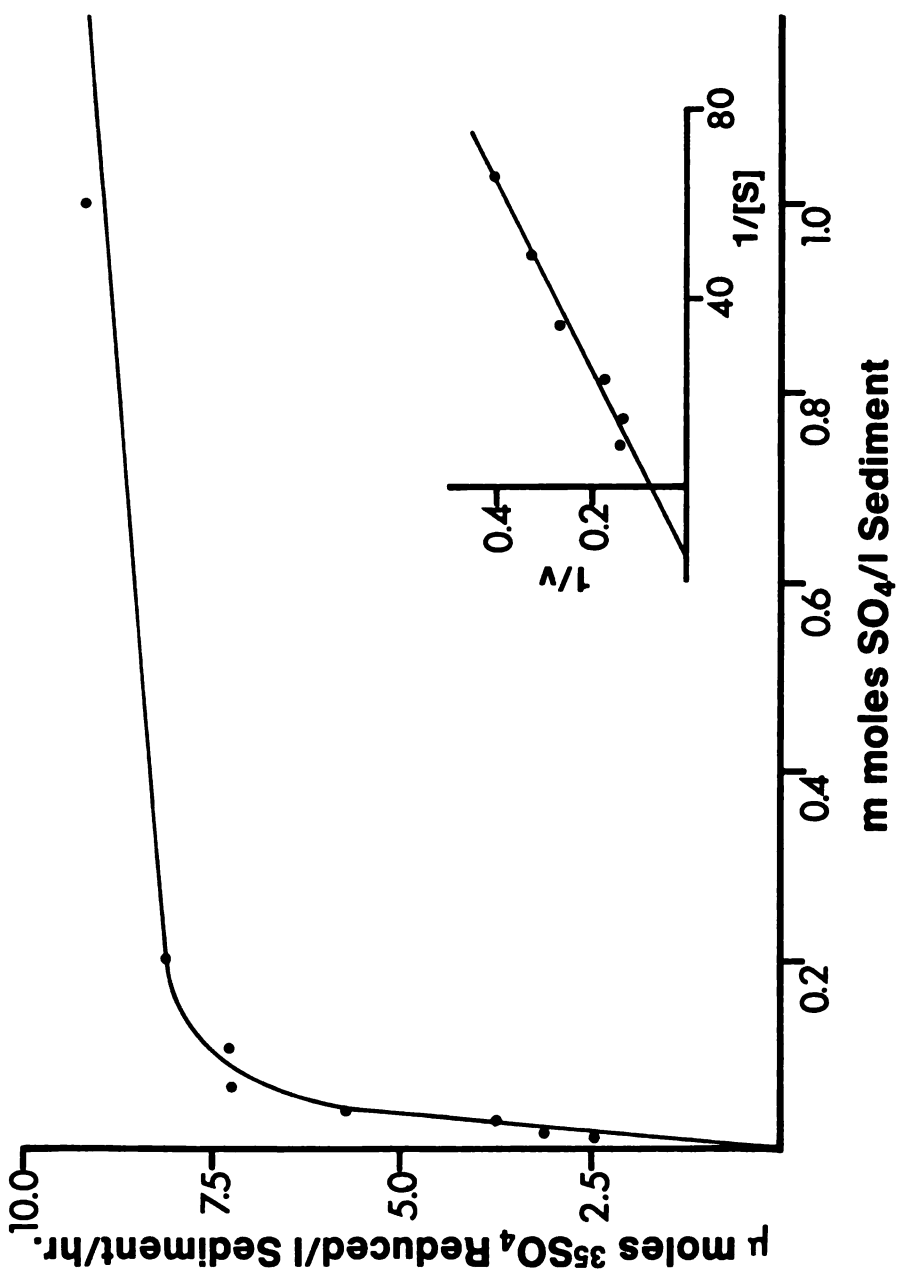

μ moles $^{35}SO_4$ Reduced/l Sediment/hr.
10.0
7.5
5.0
2.5
0.2
0.4
0.6
0.8
1.0
m moles SO_4/l Sediment
1/v
0.4
0.2
40
80
1/[S]

Figure 7. Reduction of $^{35}SO_4^=$ at 10°C in profundal sediments collected in March at *in situ* $SO_4^=$ concentrations. Controls were pretreated with glutaraldehyde. Error bars represent ± one standard error.

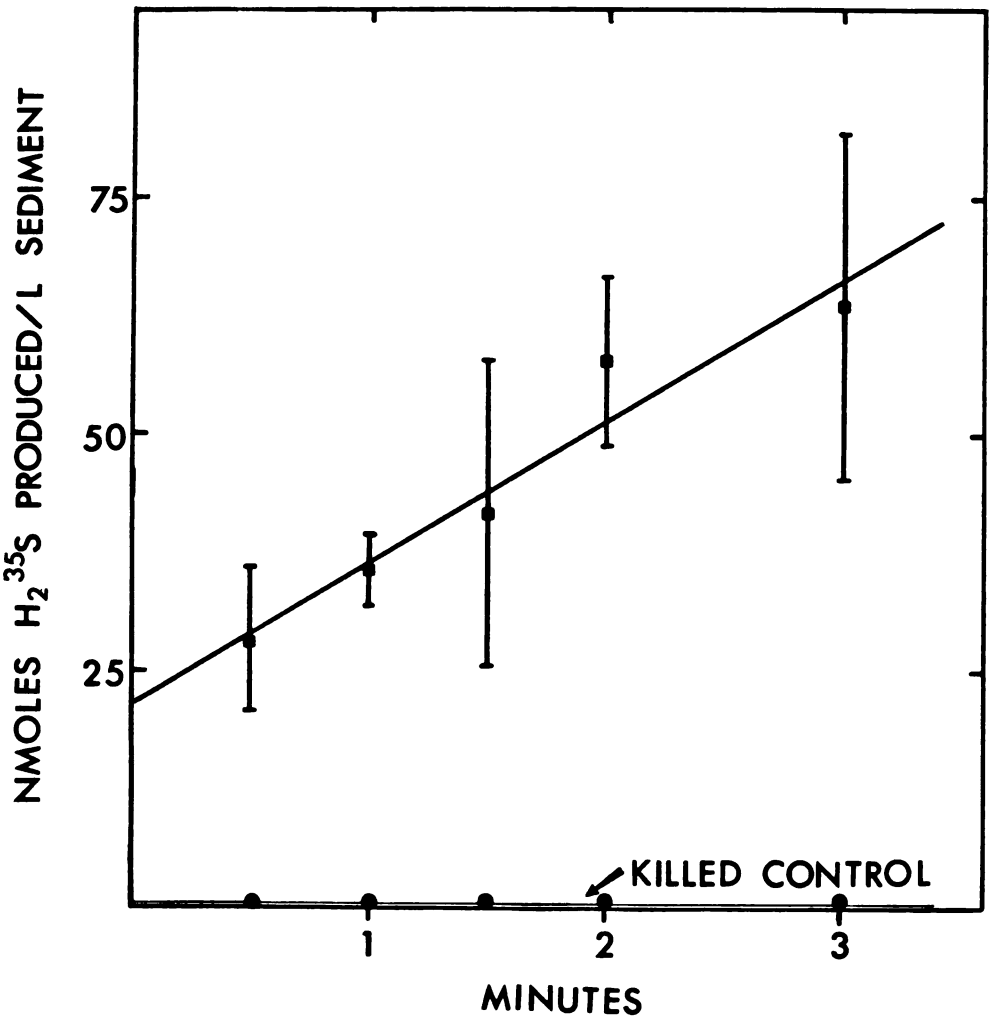

NMOLES $H_2{}^{35}S$ PRODUCED/L SEDIMENT
75
50
25
KILLED CONTROL
1
2
3
MINUTES

Table 1. Reduction of $^{35}SO_4$ with depth in Wintergreen Lake profundal sediments.[a]

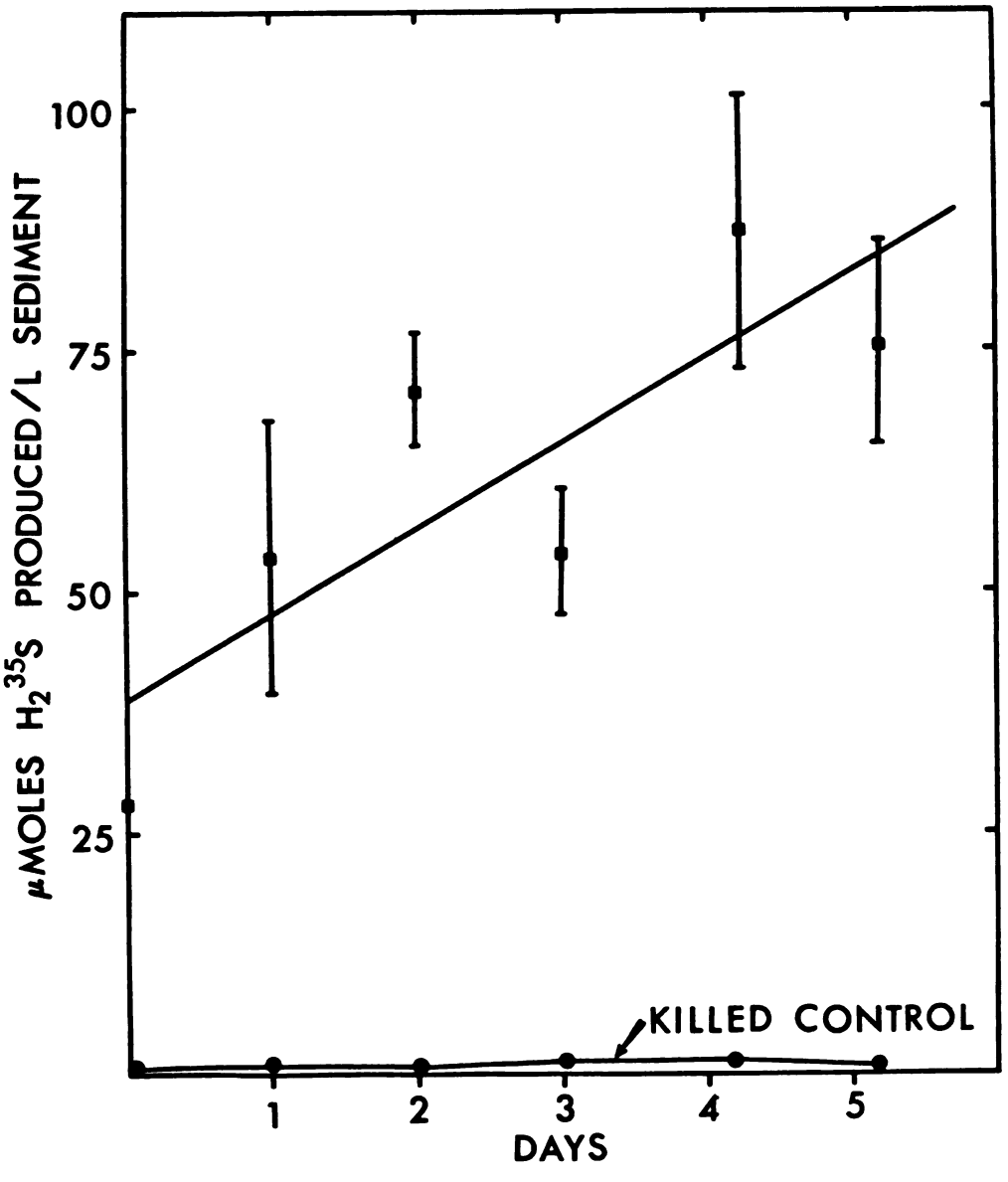
Depth (cm)	Rate of Sulfate Reduction	
	μmoles/l sed x day	mmoles/m^2 x day
0	97 (63)[b]	- [c]
3	100 (63)	2.9
6	171 (67)	7.0
9	119 (52)	11.4
12	64 (32)	14.1
15	18 (2)	15.3

[a] Sediments collected in July; incubated at 10°C.

[b] Brackets enclose standard errors.

[c] Cumulative total.

Figure 8. Time course of elemental sulfur reduction at 10°C in profundal sediments amended with 30 mg $^{35}S^{o}$/l sediment. Controls were pretreated with glutaraldehyde. Error bars represent $\pm$ one standard error.


100
75
50
25
μMOLES $H_2^{35}S$ PRODUCED/L SEDIMENT
KILLED CONTROL
1
2
3
4
5
DAYS

Discussion

The distribution of sulfur compounds in Wintergreen Lake profundal sediments indicates that the combined concentrations of inorganic sulfur compounds was much lower than the total sulfur content, suggesting that the total sulfur pool was primarily organic in nature. These values correspond with the reported concentrations of total sulfur, which was 32% organic ester sulfate and 15% protein sulfur in surface sediments of Wintergreen Lake (King and Klug, submitted). Pyrite was not measured, but in freshwater sediments containing similar concentrations of S^o very little pyrite was found (17). The low concentration of sulfate relative to the organic and total sulfur content, H_2S, and AVS concentrations all suggest that most of the sulfide production comes from organic sulfur. This is further substantiated by the decrease in the total sulfur content with depth and an increase in AVS, suggesting a mineralization of organic sulfur. These trends in pool sizes imply that sulfate-reducing activity is low in freshwater sediments, as has been frequently suggested (e.g. 21).

At sulfate concentrations above 10 mM rates of sulfate reduction are independent of sulfate concentration (6). Therefore sulfate is not likely rate limiting in the water column and upper sediments of most marine habitats where sulfate concentrations are 25 mM (7). This is quite the opposite in freshwater sediments where sulfate pools are very low (Fig. 1 and 2). In non sulfate limited systems the sediment sulfate profiles exhibit exponential decreases with depth as a function of diffusion, sedimentation, and sulfate reduction (8) and have been used accordingly to calculate rates of sulfate reduction. The profile of sulfate concentrations in Wintergreen Lake sediments indicates that

a steep gradient exists from the water column to the surface sediments (Fig. 3). Due to the steepness of the gradient, estimates of the sediment sulfate concentration are needed in much narrower sampling intervals (< 1 cm) to calculate the sediment diffusive uptake of sulfate since the concentrations below 2 cm sediment depth are near the limit of detection for the sulfate turbidimetric assay. The actual sulfate concentrations below 2 cm depth are probably between the reported value and zero. The gradient does indicate a large potential sulfate sink in Wintergreen sediments. The range of sulfate concentrations in the sediments was very constant on an annual basis, even though concentrations in the water immediately above the sediment displayed greater variability (Fig. 2a,b; 3).

Jørgensen (7) has shown that drawing conclusions about reaction rates from pool sizes may be in error. In addition van Gemerden (1967, Ph.D. Thesis, Leiden, Netherlands) demonstrated that in mixed cultures of Desulfovibrio desulfuricans and Chromatium vinosum both organisms were able to grow exponentially in the presence of very low total sulfur concentrations. The turnover time of the total sulfur pool was as short as 15 minutes. Thus significant rates of sulfate reduction can occur even when sulfate concentrations are low, if a rapid turnover time is evident. Such was the case in Wintergreen Lake sediments where rapid turnover times of the in situ sulfate pool and corresponding high rates of sulfate reduction were observed (Fig. 6,7). The rate of sulfate reduction found in the top 15 cm, 15.3 mmoles $SO_4^=$ reduced/m^2 x day, for sediments collected in summer even exceeds the 13.3 mmoles $SO_4^=$ reduced/m^2 x day reported by Jørgensen (7) for the upper 10 cm of a coastal marine sediment. The variability exhibited in Table 1 was

considered due to core heterogeneity. The lack of observed seasonality in the turnover time of the in situ sulfate concentrations could be considered due to sampling heterogeneity as well, although changes in the rate of sulfate reduction would be expected as a function of the available sulfate in a sulfate limited system. As indicated previously a lack of variation in the annual sediment sulfate concentrations was observed, which suggests a constant turnover time for the sediment sulfate concentration.

Other potential sources of sulfate to the sediment are organic sulfate esters, due to the presence of an active sulfhydrolase system (11), and bound sulfate potentially becoming available during mixing and handling of the sediment. KCl extractions of Wintergreen sediments have indicated no bound sulfate. King and Klug (submitted) report that 23% of the total S in Wintergreen sediments is in the form of ester sulfates, but that mineralization occurs only in the upper sediments. Of the annual ester sulfate sestonic input 42% is mineralized, which is the equivalent of 0.11 mmoles $SO_4^{=}/m^2$ x day. This is less than 1% of the rate of sulfate reduction found in the top 15 cm of sediment collected in July (Table 1) and would not appear to be a significant sulfate source even if all of the ester sulfate were mineralized during summer stratification when sulfate pools are lowest.

The possibility that S^o reduction was occurring in Wintergreen sediments was suggested by the available pool of S^o (Fig. 1) and its depletion with depth. The origin of S^o probably stems from 4 sources: 1) the oxidation of H_2S in the water column during fall turnover; 2) diffusion of H_2S from the sediment during nonstratified periods with subsequent oxidation; 3) oxidation of H_2S within the sediment by ferric

iron oxidation; and 4) the presence of large populations of photosynthetic bacteria in the overlying water column (3). A five-fold increase in the concentration of Chlorobium chlorophyll 650 immediately above the sediment-water interface was found from July through September, 1978 (Smith, R. L.; unpublished data). This was due primarily to settling of senescent green photosynthetic bacteria, which deposit S^o granules and compromised 90% of the total bacterial population at 4-5 m during the period. The same relationship between S^o and sulfate concentrations was reported for Lake Mendota sediments (17).

Although S^o is found in many environments, little is known about its reduction. Reduction of S^o has been documented in waterlogged soils (2) and in the water column of Solar Lake (10). Some sulfate reducing organisms are capable of reducing elemental sulfur to H_2S (1). In addition an organism has been reported, Desulfuromonas acetoxidans, which reduces elemental sulfur but not sulfate (18). The rate of S^o reduction in profundal surface sediments of Wintergreen Lake when amended with S° represents less than 40% of the lowest observed in situ rate of sulfate reduction. Therefore elemental sulfur reduction by sulfate reducers would not appear to be of sufficient magnitude to appreciably affect or sustain the reported in situ rate of sulfate reduction. Indeed the two activities may involve two totally different populations of organisms since in addition to Desulfuromonas photosynthetic bacteria and cyanobacteria can reduce S° in dark reactions (see 10). S° reduction, however, is difficult to accurately measure due to the insolubility of S° in aqueous phase. The surface area of exogeneous $^{35}S^\circ$ may not reflect the surface area, and hence

availability, of *in situ* S° particles. This is further complicated by the dissolution of S° with H_2S to form polysulfides. Even with these complications the observed rate is insignificant in relation to the observed rate of sulfate reduction.

A general equation for the oxidation of organic matter by sulfate reduction is given by:

$$H_2SO_4 + 2(CH_2O) \rightarrow 2CO_2 + H_2S + 2H_2O$$

where 2 moles of carbon are oxidized per mole of sulfate reduced. As an example of sulfate reduction in Wintergreen Lake profundal sediments the reported cumulative rate of sulfate reduction (Table 1) for 0-15 cm corresponds to 30.6 mmoles carbon oxidized m^{-2} day^{-1}. The average rate of methane release or production in Wintergreen Lake for 1976 and 1977 was 8.1 moles CH_4 m^{-2} from May-November (15) which is 38.5 mmoles CH_4 produced/m^2 x day. The stoichiometery of methane production is given by:

$$2(CH_2O) \rightarrow CO_2 + CH_4$$

Thus 77 mmoles organic C/m^2 x day was mineralized. Therefore the two processes together mineralized 108 mmoles C/m^2 x day or 106% of the reported particulate organic matter entering the sediments (15). The input estimates are primarily of sestonic autochthonous production and the combined autochthonous and allochthonous inputs are undoubtedly higher since the rate of sedimentation is 6 mm yr^{-1} (12).

Thus sulfate reduction rates in Wintergreen Lake profundal sediments are much higher than might be predicted on the basis of the low sulfate concentrations, and even fall within the range reported for marine sediments. However, methane production is still the predominant terminal metabolic process in these sediments. Approximately 2.5 times the amount of organic carbon on a given date was mineralized through

methane production as compared to sulfate reduction. It is interesting to note however, that relatively high rates of both processes are occurring concurrently within the same sediment interval (Klug, et al., in prep.) in light of several reports of the incompatibility of the two processes (4, 21). Such an ecosystem would lend itself to a study of the metabolic interactions of these two groups of organisms under natural conditions.

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CHAPTER III

ELECTRON DONORS UTILIZED BY SULFATE-REDUCING BACTERIA ISOLATED FROM EUTROPHIC LAKE SEDIMENTS

INTRODUCTION

The reported range of carbon substrates utilized by sulfate-reducing bacteria is limited to low molecular weight compounds (12, 16). Lactate is considered to be the preferred electron donor for sulfate reduction in both pure culture (16, 17) and in freshwater sediments (4, 5) and consequently is generally used to enrich and isolate sulfate-reducing bacteria from natural habitats (1, 3, 6, 7, 8, 11, 17, 20). However the narrow range of electron donors reportedly used by sulfate-reducing bacteria may be the result of enrichment and isolation of these organisms virtually exclusively with lactate. This is supported by the isolation of a sulfate-reducing, acetate-oxidizing strain of Desulfotomaculum (21) and a propionate-oxidizing, sulfate-reducing bacteria (10) from enrichments using acetate and propionate respectively. These organisms were previously described as lacking the capability to oxidize either acetate or propionate.

This report describes the enrichment and isolation of sulfate-reducing bacteria from freshwater eutrophic lake sediments utilizing a variety of carbon substrates and the subsequent characterization of carbon sources utilized by each isolate. The results indicate that as a group sulfate reducers isolated from these sediments could utilize an array of substrates for carbon and/or

electron donors, suggesting that a variety of substrates could serve as potential electron donors for sulfate-reducing bacteria in the sediments.

MATERIALS AND METHODS

Sediment samples: Profundal sediments from Wintergreen Lake, a shallow (z_m=6.5m) hypereutrophic lake located in Southwestern Michigan (14) were collected by gravity coring in 3 inch ID core tubes containing a row of vertical ports at 3 cm intervals, which were sealed with pressure sensitive tape during coring. Subsamples of sediment were obtained from the cores with a cut-off 5 ml syringe.

MPN estimates: Most probable number estimates (MPN) of sulfate-reducing organisms were conducted in an anaerobic glovebox (Coy Manufacturing) containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. The MPN media consisted of two parts (values in g/l): A) basal medium; K_2HPO_4, 0.58; KH_2PO_4, 0.31; $MgCl_2$-$2H_2O$, 0.46; $CaCl_2$-$2H_2O$, 0.098; $FeCl_2$-$4H_2O$, 0.5; NH_4Cl, 1.0; yeast extract, 2; trace element solution (15), 11.1 ml; resazurin, 2.2 ml of a 1% solution; Na_2SO_4, 3.1; B) organic carbon source (see Table 1 for list), 40; $NaHCO_3$, 33.6. Both parts were adjusted to pH 7.4 prior to sterilization. A was autoclaved and the headspace flushed with O_2-free N_2 while cooling. B was flushed with O_2-free N_2 and filter sterilized in the glovebox, where 190 ml A and 10 ml of B were combined and transferred to sterile prescription bottles. The 0-3 cm sediment interval from three profundal cores were combined in the glovebox and used as inocula for 10-fold serial dilutions in the bottles. Six-5 ml aliquots of each dilution were transferred to sterile tubes and stoppered with foam plugs. The tubes were enclosed in plastic bags to prevent desiccation, incubated at

ambient temperature (23-27°C) in the glovebox for several weeks, and scored for the presence of sulfate-reducing activity. Positive evidence of dissimilatory sulfate reduction was a blackening (FeS production) of the precipitate. Preliminary MPN estimates with media containing various sulfur-containing reducing agents resulted in "false positives" in the absence of sulfate. As a result chemical reducing agents were not added to the medium, however the E_h of the freshly prepared medium was below -50 mv and remained below this value if stored in the glovebox due to the presence of Fe^{++}, NH_4^+, and yeast extract in the basal medium.

Isolations: A second set of enrichment tubes with the same array of carbon sources were inoculated in triplicate with a 100-fold final dilution. The tubes were incubated 4 weeks and streaked onto media containing the same carbon substrate plus 1.5% agar. Black colonies that developed were picked and successively restreaked on the same medium until at least two consecutive transfers were obtained with a single colony morphology. Isolates were maintained on slants containing the carbon source on which they were isolated.

Carbon survey: Each isolate was tested for growth in liquid culture with a variety of sole carbon sources. The basal medium was the same as that used for the MPN estimates except the yeast extract concentration was lowered to 0.5 g/l and sodium thioglycolate, 0.5 g/l, used as a reducing agent. The media was flushed with prereduced N_2(93%)/CO_2(7%) after autoclaving. The carbon substrate concentration in part B was adjusted to 100 g/l. The complete media was dispensed while being flushed with N_2/CO_2 into screw top tubes, which were filled to the neck, and stoppered with butyl rubber septum stoppers (Bellco,

Inc.). Starter cultures of each isolate were grown with the carbon source upon which the strain was isolated and a 0.01 ml inoculum aeseptically transferred by syringe and needle to the appropriate tubes. The tubes were incubated at 25°C and scored for production of FeS.

Growth with hydrogen: Each of the above starter cultures were streaked in the glovebox onto duplicate plates containing the carbon survey basal medium plus 1.5% agar and 0.2% sodium acetate. Duplicate plates were divided and placed into one of two anaerobe jars, one of which the headspace was flushed daily with O_2-free N_2 (93%)/CO_2(7%), the other with O_2-free N_2(85%)/H_2(10%)/CO_2(5%). The jars were incubated at ambient temperature (23-27°C) for 2 weeks, after which the plates were scored for growth and the diameter of well isolated colonies measured with a dissecting microscope.

RESULTS

The MPN estimates of sulfate-reducing bacteria ranged from 3.3 x 10^6 cells g^{-1} of dry sediment when enriched with lactate to 7.1 x 10^3 cells g^{-1} of dry sediment when no carbon substrate was included in the enrichment (Table 1). These values represented 0.1-60% of the isolatable, anaerobic, heterotrophic, bacterial population. Sulfate reduction was not observed with any carbon source in the absence of sulfate.

Positive enrichments were obtained with all of the carbon substrates tested; however, growth on a number of substrates by sulfate-reducing bacteria was not obtained on solid media beyond one or two successive streakings (Table 2). Sulfate-reducing bacteria were isolated with media containing lactate, pyruvate, and acetate as carbon

Table 1. MPN estimates of sulfate-reducing bacteria in Wintergreen Lake profundal surface sediments[1].

Carbon Source		Cells/g dry wt sediment	Percent of the isolatable, anaerobic, heterotrophic, bacterial population[2]
Lactate	Range	$1.9 \times 10^5 - 3.3 \times 10^6$	3-60
	Ave	1.1×10^6	20
Succinate		1.9×10^6	34
Malate		1.9×10^6	34
Formate		1.1×10^6	20
Methanol		1.1×10^6	20
Ethanol		7.2×10^5	13
Fumarate		7.2×10^5	13
Pyruvate		4.5×10^5	8.2
Acetate		3.7×10^5	6.7
Propionate		3.3×10^5	6.0
Glutamate		1.4×10^5	2.5
Butyrate		7.2×10^4	1.3
Casamino acids		5.5×10^4	1.0
Glucose		4.5×10^3	0.1
None		7.1×10^3	0.1

[1]Inocula were sediments collected in September.

[2]As reported by Molongoski and Klug (13).

Table 2. Isolates of sulfate-reducing bacteria from Wintergreen Lake profundal sediments[1].

Carbon Source	Enrichment	First Streak	Second Streak	Isolates
Lactate	+	+	+	1F001,1F002,1F004,1F006 1F007,1F008,1F009,1F0010 1F011,1F012,1F013
Succinate	+	+	-	none
Malate	+	+	-	none
Formate	+	+	+	none
Methanol	+	+	-	none
Ethanol	+	-	-	none
Fumarate	+	+	-	none
Pyruvate	+	+	+	8F002,8F003,8F006,8F007 8F008,8F009,8F010,8F011 8F012,8F013
Acetate	+	+	+	9F001,9F002
Propionate	+	-	-	none
Glutamate	+	-	-	none
Casamino acids	+	-	-	none
Glucose	+	-	-	none
None	+	+	+	15F001,15F002,15F003, 15F004

[1]Inocula were surface sediments collected in August.

sources and with the basal medium without an added carbon source (Table 2). Eleven isolates were obtained in a medium containing lactate, 10 with pyruvate, 2 with acetate, and 4 with no added carbon source.

The results of a carbon survey testing the capability of these isolates to utilize a variety of sole carbon sources for growth are given in Table 3. Every isolate grew in lactate, pyruvate, casamino acids, or ethanol containing media, while methanol and malate supported growth of nearly all of the isolates. All substrates tested supported growth of at least one isolate, but no isolate was capable of growing in the basal medium in the absence of an added carbon substrate. Most strains of sulfate-reducing bacteria isolated with pyruvate containing media were able to utilize formate for growth while strains isolated with lactate containing media generally did not grow on formate. Organisms isolated from the MPN basal medium alone were able to grow on only seven of the substrates tested.

Hydrogen stimulated colony growth of the sulfate-reducing isolates when grown with an acetate containing medium (Table 4). Only strain 1F010 did not display a significant difference in mean colony diameter when grown in an atmosphere of H_2/CO_2 as compared to controls grown in a N_2/CO_2 atmosphere. The degree of H_2 stimulation varied among strains, with 8F003 demonstrating the greatest stimulation. The Desulfovibrio desulfuricans reference strain (ATCC 7757) was stimulated by H_2 as well, though the 95% confidence intervals of the mean colony diameter overlap slightly.

DISCUSSION

The results of the MPN enrichments (Table 1) indicate that sizeable populations of sulfate-reducing organisms are present in

Table 3. Sole carbon substrates utilized for growth by sulfate-reducing bacteria isolated from Wintergreen Lake profundal sediments.

Carbon substrate utilized for isolation	Number of isolates tested	Growth Substrate[a]														
		Lactate	Succinate	Malate	Formate	Methanol	Ethanol	Fumarate	Pyruvate	Acetate	Propionate	Glutamate	Butyrate	CAA	Alanine	None
Lactate	11	11	9	11	0	9	11	2	11	6	8	5	4	11	6	0
Pyruvate	8	8	4	8	6	6	8	3	8	4	2	5	2	8	1	0
Acetate	2	2	2	2	1	2	2	0	2	2	1	2	0	2	1	0
None	4	4	0	0	2	4	4	0	4	1	0	0	0	4	0	0
ATCC 7757[b]	-	1	0	1	0	0	1	0	1	0	0	1	0	1	1	0

[a]Entries give the number of isolates for which growth was recorded. CAA represents casamino acids.

[b]*Desulfovibrio desulfuricans*.

Table 4. H_2 utilization by sulfate-reducing bacteria isolated from Wintergreen Lake profundal sediments.

Strain	Colony Diameter (mm)[a]			
	H_2 ATM		N_2 ATM	
1F002	0.81	(0.10;6)	0.38	(0.03;6)
1F004	0.97	(0.06;6)	0.42	(0.03;6)
1F006	0.68	(0.09;6)	0.43	(0.05;7)
1F007	0.76	(0.05;6)	0.40	(0.06;6)
1F008	0.70	(0.07;6)	0.43	(0.03;6)
1F010	0.42	(0.05;6)	0.45	(0.05;6)
1F011	0.95	(0.03;6)	0.41	(0.02;6)
1F012	0.93	(0.10;6)	0.48	(0.04;6)
1F013	0.51	(0.08;6)	0.36	(0.02;6)
8F002	1.07	(0.16;6)	0.61	(0.02;6)
8F003	1.40	(0;2)	0.61	(0.04;4)
8F006	0.71	(0.16;6)	0.37	(0.04;6)
8F008	1.27	(0.19;3)	0.70	(0;1)
8F011	0.81	(0.07;6)	0.45	(0.05;6)
8F012	0.58	(0.13;6)	0.35	(0.05;6)
9F001	1.06	(0.17;6)	0.57	(0.04;6)
9F002	0.97	(0.21;6)	0.57	(0.03;6)
15F001	0.62	(0.09;6)	0.32	(0.03;6)
15F002	0.64	(0.08;6)	0.42	(0.05;6)
15F003	0.77	(0.14;6)	0.58	(0.03;5)
ATCC 7757[b]	0.61	(0.10;6)	0.45	(0.08;6)

[a]Brackets enclose 95% confidence interval estimator and n.

[b]*Desulfovibrio desulfuricans*.

Wintergreen Lake profundal surface sediments. The average estimate when lactate was utilized as a carbon substrate (1.6×10^4 cell/cm^{-3} of sediment) is within the range of 9.3×10^4 sulfate-reducing organisms/cm^{-3} of sediment reported for a coastal marine sediment (8) and the $2\text{-}10 \times 10^3$ sulfate-reducing organisms/cm^{-3} of sediment reported for freshwater surface sediments (3). The range of estimates obtained with lactate as a carbon source was nearly an order of magnitude, which was probably due to both sampling and sediment heterogeneity since populations of sulfate-reducing bacteria typically vary with sediment depth (3, 8).

Lactate is the preferred carbon source of most cultures of sulfate-reducing bacteria and therefore most frequently utilized for enrichments and isolations of these organisms. Lactate provided the highest MPN estimates of sulfate reducers present in Wintergreen Lake sediments. However a number of other carbon sources also enriched for sulfate-reducing organisms (Table 1). The electron donors utilized by sulfate reducers growing in control enrichments were probably natural sediment substrates, the yeast extract present in the medium, or the H_2 present in the glovebox atmosphere. These results do not imply that sulfate-reducing bacteria are capable of directly utilizing any given one of these substrates for either carbon or energy, for an intermediate group(s) of organisms may be involved in the initial catabolism of the substrate while the sulfate reducers are utilizing subsequent metabolites. The results do however indicate that the heterotrophic utilization of these carbon substrates in the sediments potentially involves sulfate-reducing organisms.

Many strains of sulfate-reducing bacteria require growth factors, which typically are provided as yeast extract in growth media (16, 17).

Yeast extract was included in the media employed in these studies to facilitate isolation of as many groups of sulfate-reducing bacteria as possible. Controls containing yeast extract along with impurities in agar, but no added carbon substrate (i.e. none), supported growth of several strains of sulfate-reducing organisms. Growth was relatively slow and these organisms were unable to grow when the enrichments were streaked upon media containing many of the tested carbon substrates. The yeast extract concentration was lowered to 0.05% and liquid media used to survey carbon substrates utilized by the sulfate-reducing isolates to avoid subjective determinations relative to control results.

As a group the sulfate-reducing bacterial isolates utilized all of the sole carbon substrates tested for growth (Table 3). Included among these substrates were acetate, propionate, and butyrate. Acetate-oxidizing, sulfate-reducing bacteria have been reported in only three instances (see 18, 10, 21), yet 52% of these isolates were able to grow in an acetate containing medium. These acetate-oxidizing isolates also oxidized a number of other carbon substrates, including lactate, which distinguishes them from Desulfotomaculum acetoxidans (21) and suggests that acetate-oxidizing, sulfate-reducing bacteria in natural sediments are not restricted to acetate as the sole electron donor. To the author's knowledge Desulfovibrio rebentschikii is the only sulfate-reducing bacteria described capable of oxidizing butyrate (1, see 18), though it was lost long ago, while propionate oxidation by sulfate reducers has only been recently reported (10). Thus a significant fraction of these isolates oxidize carbon substrates which are not considered to be characteristic substrates for sulfate-reducing

bacteria. Lactate, pyruvate, malate, succinate, and ethanol also supported growth of most isolates and are commonly oxidized by many strains of sulfate-reducing bacteria (16). All strains tested were capable of good growth with casamino acids as a carbon source, though this substrate is not normally included in the list of carbon substrates utilized by sulfate-reducing bacteria. This may be a characteristic common to many sulfate-reducing bacteria since a number of sulfate-reducing isolates grow with casamino acids as a substrate, including standard laboratory strains (19). The Desulfovibrio reference strain used here (ATCC 7757) also utilized casamino acids (Table 3). The ability to utilize amino acids as electron donors for sulfate reduction may be significant in Wintergreen Lake sediments which receive large inputs of proteinaceous seston (14).

Differences were evident in the carbon sources utilized by the sulfate-reducing isolates relative to the carbon substrate with which they were isolated (Table 3). This is particularly true for growth with formate or alanine by lactate isolated organisms versus pyruvate isolated organisms and succinate and malate utilization by the control isolates as compared to the other isolates. These differences illustrate the hazard of making general inferences as to the carbon substrates utilized by sulfate-reducing bacteria in natural habitats when most of the standard strains of sulfate-reducing bacteria have been isolated with a single substrate (i.e. lactate).

The wide range of carbon sources utilized by the sulfate-reducing isolates confirms the MPN results and reemphasizes the role of sulfate-reducing bacteria in the heterotrophic utilization of these carbon sources in the sediments. For example MPN estimates with

succinate, malate, formate, and methanol as carbon sources were high (Table 1) and many isolates were able to oxidize these substrates (Table 3), although attempts to isolate sulfate-reducing bacteria with media containing these carbon sources were unsuccessful. All the isolates utilized lactate, pyruvate, ethanol, and casamino acids as growth substrates, yet MPN results for pyruvate and casamino acid containing media were significantly lower than MPN results with lactate. This may have been due to other organisms successfully outcompeting the sulfate-reducing populations for pyruvate and casamino acids in the MPN enrichments. It is also possible that lactate-oxidizing sulfate reducers were present in the sediment which could not oxidize casamino acids or pyruvate, but such organisms were not isolated.

Included among the list of electron donors utilized by sulfate-reducing bacteria is molecular H_2 (2, 16), provided an organic carbon source, such as yeast extract or acetate, is present in the medium (2). The MPN enrichments (Table 1) were incubated in an atmosphere containing 10% H_2. The results indicate that H_2 was not the principle electron donor since the control estimate was low, while several carbon sources displayed MPN estimates significantly above that of the control. The sulfate-reducing bacterial isolates were able to utilize H_2 as a supplemental electron donor, however, as evidenced by a stimulation of growth. A solid medium was employed for these tests to maximize exposure to H_2, and as noted previously growth was evident from agar impurities. Thus controls incubated with a N_2 atmosphere exhibited growth as well, though significantly less. Only one strain was apparently unable to oxidize H_2 while most of the others were able

to do so to a greater extent than the Desulfovibrio reference strain. These results indicate that hydrogen can be a potential electron donor for sulfate reduction in Wintergreen lake sediments. The significance of H_2 oxidation by sulfate-reducing bacteria in eutrophic sediments is unclear however, in light of the report that H_2 contributes little to the energy metabolism of sulfate-reducing bacteria grown on lactate plus sulfate (9). If this is the case H_2 oxidation by sulfate reducers in eutrophic sediments may be a relatively minor process.

In summary these results indicate that sulfate-reducing bacteria from freshwater sediments can be enriched with an array of carbon sources, while a wide range of carbon sources were utilized for growth by sulfate-reducing bacteria isolated from these enrichments. These included several strains capable of oxidizing acetate and propionate. The substrates tested represent carbon sources available in eutrophic freshwater lake sediments. The results do not establish the natural electron donors for sulfate-reducing bacteria, however, they do establish that a number of carbon sources can potentially serve as electron donors for sulfate reduction in these sediments.

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CHAPTER IV

ELECTRON DONORS UTILIZED BY SULFATE-REDUCING BACTERIA IN SEDIMENT CONTAINING FLOW-THROUGH FLASKS

INTRODUCTION

Previous studies have indicated that significant rates of sulfate reduction occur in eutrophic lake sediments in spite of low interstitial sulfate concentrations (Chapter II). Although methane production is the predominant terminal metabolic process in these sediments, sulfate reduction can account for up to 30% of the total anaerobic carbon mineralized. A comprehensive view of the role of sulfate reduction in these sediments must include a description of the electron donors utilized for sulfate reduction. Few studies, however, have investigated natural electron donors for sulfate reduction. Even in marine sediments, where sulfate reduction is the primary terminal electron acceptor, specific electron donors and the contribution of each to total sulfate reduction have not been fully established.

The response of a microbial community as it is subjected to environmental perturbations often yields valuable information regarding the activities of that community. Therefore anaerobic microbial ecosystems have been frequently subjected to artificial manipulations such as physical changes (5, 12) or substrate additions (3, 6, 16, 19) to characterize the prevalent metabolic pathways. Such an approach has been utilized to investigate proposed electron donors for sulfate reduction in marine sediments by measuring the response of sulfate

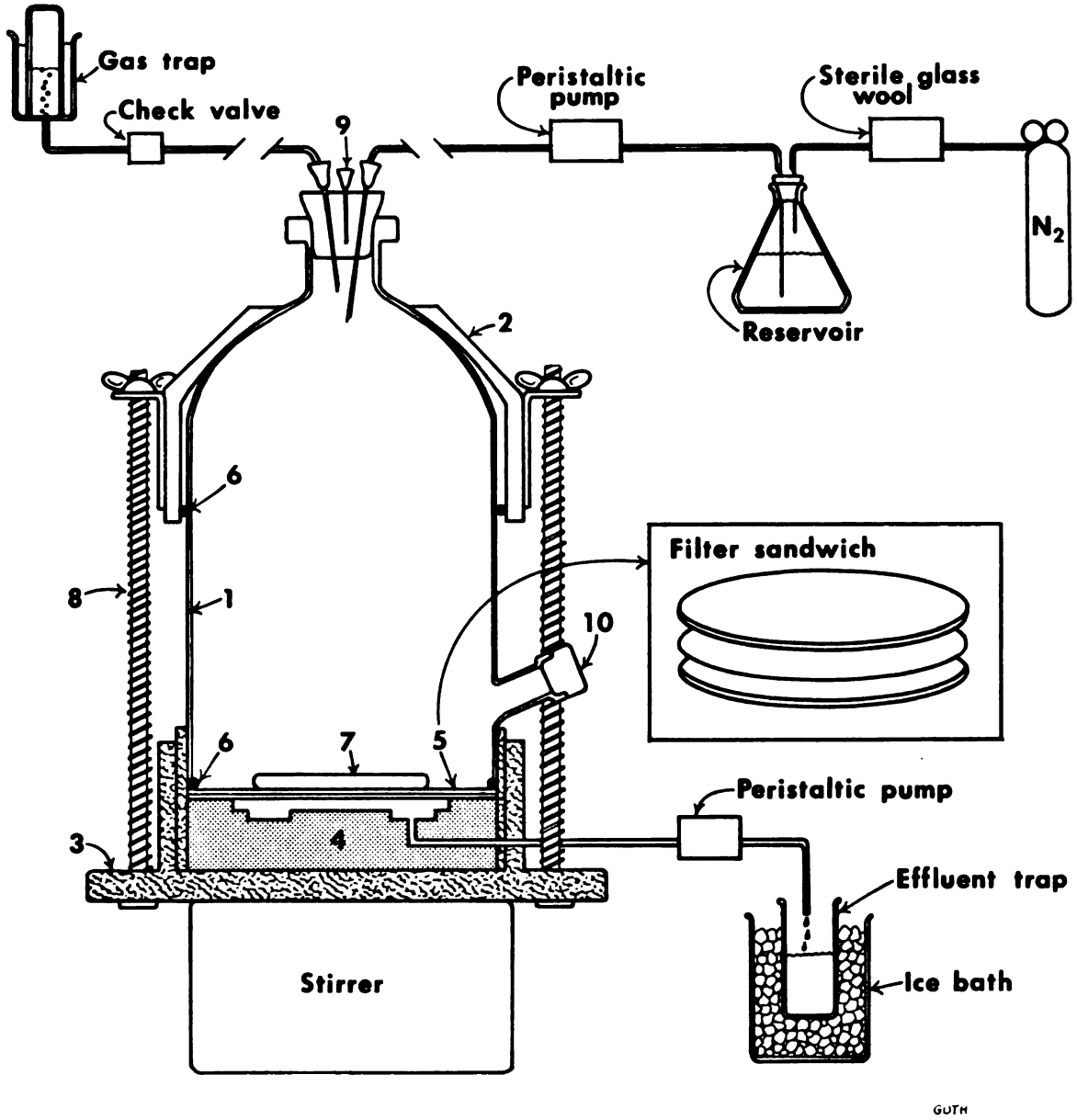
reduction to additions of lactate, acetate, pyruvate, hydrogen, or formate (1, 2, 13, 14). Metabolic activities in freshwater sediments, where sulfate concentrations are low, offer an excellent opportunity to examine the competition between sulfate-reducing organisms and other heterotrophs for electron donors. Investigation of these activities with perturbation experiments, however, must maintain the low levels of sulfate present in the sediment since the rate of sulfate reduction, and to a certain extent other sediment activities, depends directly on the sulfate concentration available.

This investigation describes the use of sediment containing flow-through flasks designed to maintain a continuous low level input of sulfate, which simulates sulfate diffusion into freshwater sediments. Changes in potential rates of sulfate reduction as a function of added carbon sources and varying sulfate concentrations were examined in this system to investigate natural electron donors for sulfate reduction in eutrophic lake sediments.

MATERIALS AND METHODS

Sediment reactors: Profundal surface sediment from Wintergreen Lake, a shallow (z_m=6.5 m) hypereutrophic lake located in Southwestern Michigan (9), were sampled with an Eckman dredge. Jars were completely filled with sediment, sealed, and stored at 10°C; experiments were initiated the day of collection. Sediment was homogenized in the jars with a paint shaker, transferred to an anaerobic glovebox, and the sediment pooled. Subsamples (700 ml) were transferred to flow-through flasks (called reactors for brevity, Fig. 1), stoppered with a butyl rubber stopper, and sealed with pressure sensitive tape. The reactors were removed from the glovebox and placed on stirring platforms in a

Figure 1. Diagram of the sediment reactor system. 1) bottomless 1 L reagent bottle with sidearm; 2) 4" to 3" PVC reducing union; 3) 4" PVC closet connector with 1/8" inner Plexiglass® sleeve; 4) milled Plexiglass® insert; 5) filter sandwich, a) outer layers of porous polyethylene sheet (35 μm pore size) and b) inner layer of Nitex (10 μm pore size); 6) butyl rubber O-ring; 7) magnetic stir bar; 8) threaded rod; 9) needle guide; 10) butyl rubber stopper with crimp seal.


Gas trap
Check valve
9
Peristaltic pump
Sterile glass wool
N_2
Reservoir
2
6
8
1
10
Filter sandwich
6
7
5
Peristaltic pump
3
4
Effluent trap
Stirrer
Ice bath
GUTH

10°C incubator. Each reactor was connected in series to a check valve (1/3 psi) and a water displacement gas trap by 3/16" O.D. Tygon tubing connected to a syringe needle, which was inserted through the reactor stopper. Headspace gas was flushed 10 minutes with prereduced N_2 through a syringe needle and exhausted via the gas trap. Input and output flow rates were maintained at 2 ml/min by a Technicon proportioning pump (Technicon Corp.); residence time of the effluent was less than one hour in the effluent lines. Effluent was collected in traps maintained in an ice bath and frozen until analysis. Analysis of interstial water obtained directly from the reactor flasks indicated that the concentration of volatile fatty acids and sulfate in the effluent were the same as those in the reactor sediment.

The reactor input solution contained (per liter): a) 10 ml of a trace element solution (15); b) Na_2SO_4, 0.53g; c) $NaHCO_3$, 0.5g; d) K_2HPO_4, 0.2g. A solution containing items a and b was adjusted to pH 7.2 in the resevoir flasks, autoclaved, and flushed with prereduced N_2 while cooling. Filter sterilized stock solutions of items c and d, pH 7.4, were added when cool. The flasks were stoppered and sealed to preclude the entrance of O_2 and connected to the reactors with sterile 0.03" I.D. Tygon microbore tubing. A constant pressure of N_2, 3 psi, was maintained in each resevoir headspace. The reactors initially received the input solution for 3 or 4 days followed by addition of a second input solution containing the appropriate sulfate concentration and carbon substrate. This was designated as T_o. When added with another substrate the sulfate concentration was 0.53 g/l, which corresponds to an input rate of 0.18 mmoles reactor $^{-1}$ day^{-1}. Carbon sources included were (mmoles/l): glucose, 15 and lactate, 15. When

H_2 was included as a substrate, the gas trap was not connected to the reactor except briefly prior to each H_2 addition to vent the excess pressure. Twice daily H_2 (50 ml, 1 atm) was added to the appropriate reactors through a syringe and needle.

Sulfate reduction: Triplicate subsamples (5.0 ml) of sediment were taken by syringe and needle through the reactor sidearms and transferred to 30 ml Wheaton serum bottles previously flushed with prereduced N_2(93%)/CO_2(7%) and sealed with Teflon lined rubber septa (Supleco, Inc.). To each sample 0.5 ml of prereduced 10 mM $Na_2{}^{35}SO_4$ (New England Nuclear, 3-6 μCi/ml) was added through a syringe and needle. The samples were blended with a Vortex mixer and incubated at 10°C for 1 hour. The reaction was terminated by the addition of 0.5 ml of prereduced 10% zinc acetate and the samples frozen until analysis. The $H_2{}^{35}S$ produced was distilled into 2% $CdCl_2$ as previously described (Chapter II), 10 ml of Aqueous Counting Scintillant (Amersham Searle) added to each trap (scintillation vials), and the radioactivity determined with a Beckman LS8000 liquid scintillation counter (Beckman Instruments). Since the sulfate-reducing activity was measured at sulfate concentrations demonstrated to be saturating for sulfate reduction at in situ sulfate concentrations (Chapter II), the rates measured here will hereafter be referred to as potential rates of sulfate reduction.

Chemical analyses: The concentration of methane in the headspace of each reactor was subsampled daily by syringe and needle. Methane was analyzed gas chromatographically as previously described (10). Effluent sulfate concentrations were determined turbidimetrically (18); alkalinity was monitored with a Hach Chemical Corporation test kit.

Volatile fatty acids were assayed using a Varian 3700 gas chromatograph equipped with a flame ionization detector. Analysis was made with a glass column (1.8 m x 2 mm ID) packed with 15% SP 1220 plus 1% H_3PO_4 on a solid support of Chromosorb W AW (100/120 mesh) at 110°C. Effluent lactate concentrations were analyzed by adding 0.1 ml of 0.4 M tetraethylammonium hydroxide to 5 ml of reactor effluent. The samples were dried and redissolved in 1 ml of an acetone benzylbromide solution, 400:1 (V/V), and incubated 2 hours at room temperature. Benzyl lactate was analyzed on a stainless steel column (2 m x 2 mm ID) packed with 10% butanediol succinate on Supelcoport (100/120 mesh) at 180°C using the chromatograph described above.

RESULTS

Two distinct rates of methane production were observed in the sediment reactors (Fig. 2); an initial rate of 0.57 mmoles CH_4 l^{-1} of sediment day^{-1} was observed for 2-3 days (day -1 to day 1) and a second, lower rate of 0.04 mmoles CH_4 l^{-1} of sediment day^{-1} observed for the duration of the experiment. The second rate corresponded with an increase in the effluent acetate concentration (Fig. 3). The effluent pH and alkalinity did not change over the same period. The final rate of methane production was inhibited by 40%, 75%, and 95% when the sulfate addition after T_o was 0.18, 1.4 (not shown), and 14 mmoles $SO_4^=$ $reactor^{-1}$ day^{-1} respectively, as compared to a control receiving no sulfate. Addition of H_2 to the reactor headspace stimulated methane production when the sulfate input after T_o was 0.18 mmole sulfate $reactor^{-1}$ day^{-1} (Fig. 2). This rate of sulfate input, 0.18 mmoles $reactor^{-1}$ day^{-1}, simulates the rate of sulfate diffusing into Wintergreen Lake profundal surface sediments (Chapter II).

Figure 2. Methane concentration in the sediment reactor flask headspace. Reactor input prior to T_o was 0.18 mmoles $SO_4^=$ $reactor^{-1}$ day^{-1}. After T_o sulfate inputs as indicated.

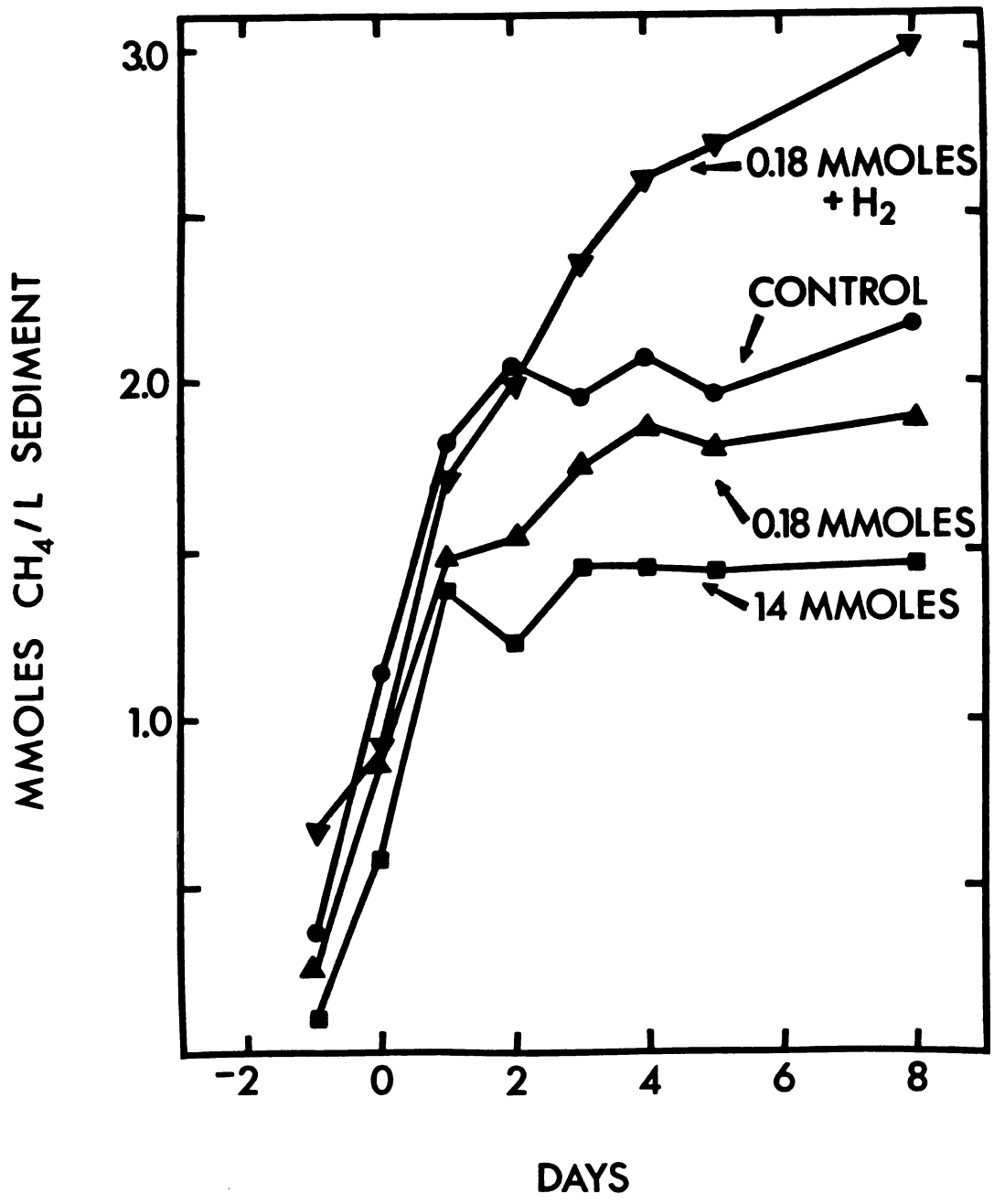
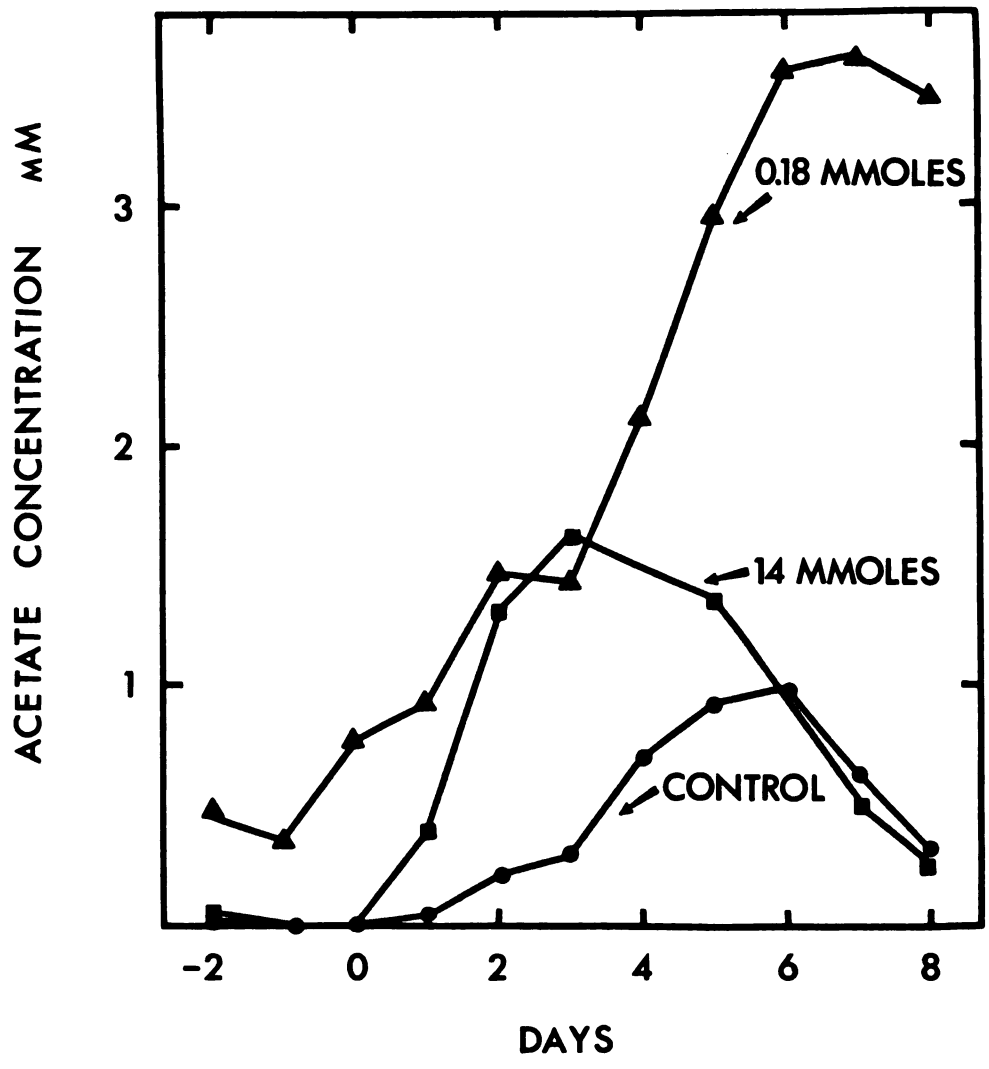

3.0
2.0
1.0
MMOLES CH_4/L SEDIMENT
0.18 MMOLES + H_2
CONTROL
0.18 MMOLES
14 MMOLES
−2
0
2
4
6
8
DAYS

Figure 3. Effluent acetate concentration from sediment reactor flasks receiving varying concentrations of sulfate. Reactor input prior to T_o was 0.18 mmoles $SO_4^=$ $reactor^{-1}$ day^{-1}. After T_o sulfate input as indicated.


ACETATE CONCENTRATION mM
3
2
1
0.18 MMOLES
14 MMOLES
CONTROL
-2
0
2
4
6
8
DAYS

Potential rates of sulfate reduction in sediments of control reactors receiving no sulfate after T_o (Fig. 4a) were reduced nearly 50% in 6 days. When the sulfate addition continued after T_o the potential rates of sulfate reduction decreased in relation to increasing additions of sulfate, up to an addition rate of 1.4 mmoles $SO_4^=$ reactor^{-1} day^{-1} (Fig. 4a). The rate of decrease was greater than that displayed by control reactors at all concentrations of amended sulfate. The effluent sulfate concentration from the reactors receiving the two higher sulfate inputs markedly increased throughout the 8-day time course (both were greater than 3 mM after day 2), indicating that the sulfate-reducing activity was saturated with respect to sulfate. An increase in the rate of sulfate reduction was observed in these latter two reactors (Fig. 4a) after day 2; the highest increase (2.5 fold) observed in the reactor receiving the highest sulfate input.

Sulfate additions increased the effluent acetate concentration over that of the control (Fig. 3). The reactor receiving the highest sulfate input, 14 mmoles $SO_4^=$ reactor^{-1} day^{-1}, displayed a significant drop in the effluent acetate concentration, after an initial increase, this decrease corresponded with an observed increase in the potential rate of sulfate reduction (Fig. 4), which subsequently dropped as the acetate concentration continued to decrease.

The addition of lactate to sediments contained in a reactor flask maintained the original rate of potential sulfate reduction when sulfate was included in the input (Fig. 5). When added alone lactate decreased the potential rate of sulfate reduction with time. Glucose and H_2 additions resulted in a drop in the potential rates of sulfate

Figure 4. Potential rates of sulfate reduction in sediment reactor flasks receiving H_2, glucose, and/or varying concentrations of sulfate. Reactor input prior to T_o was 0.18 mmoles $SO_4^=$ $reactor^{-1}$ day^{-1}. After T_o inputs as indicated. When sulfate was included input rates were the same as prior to T_o, except for A where values indicate rates of sulfate input.

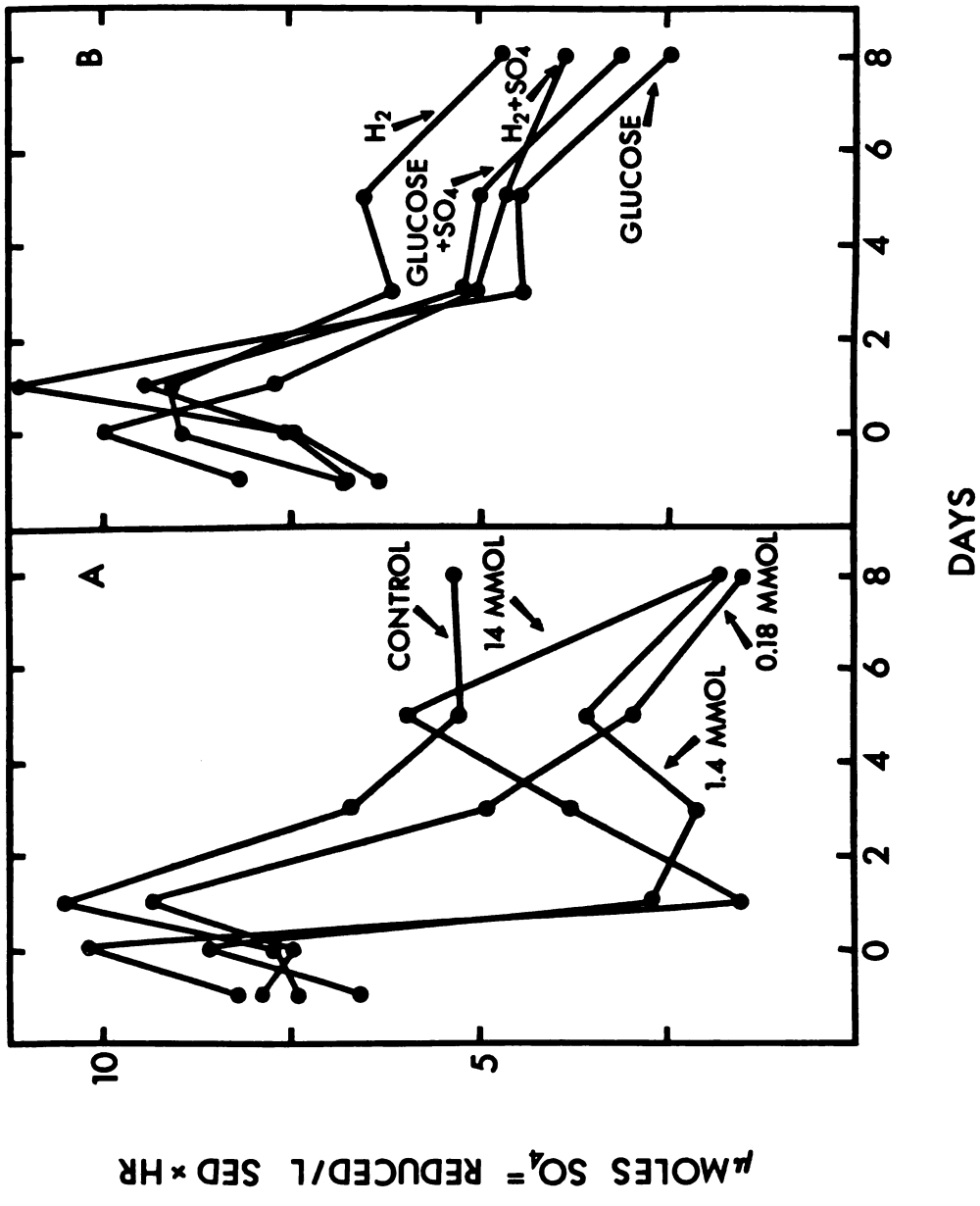
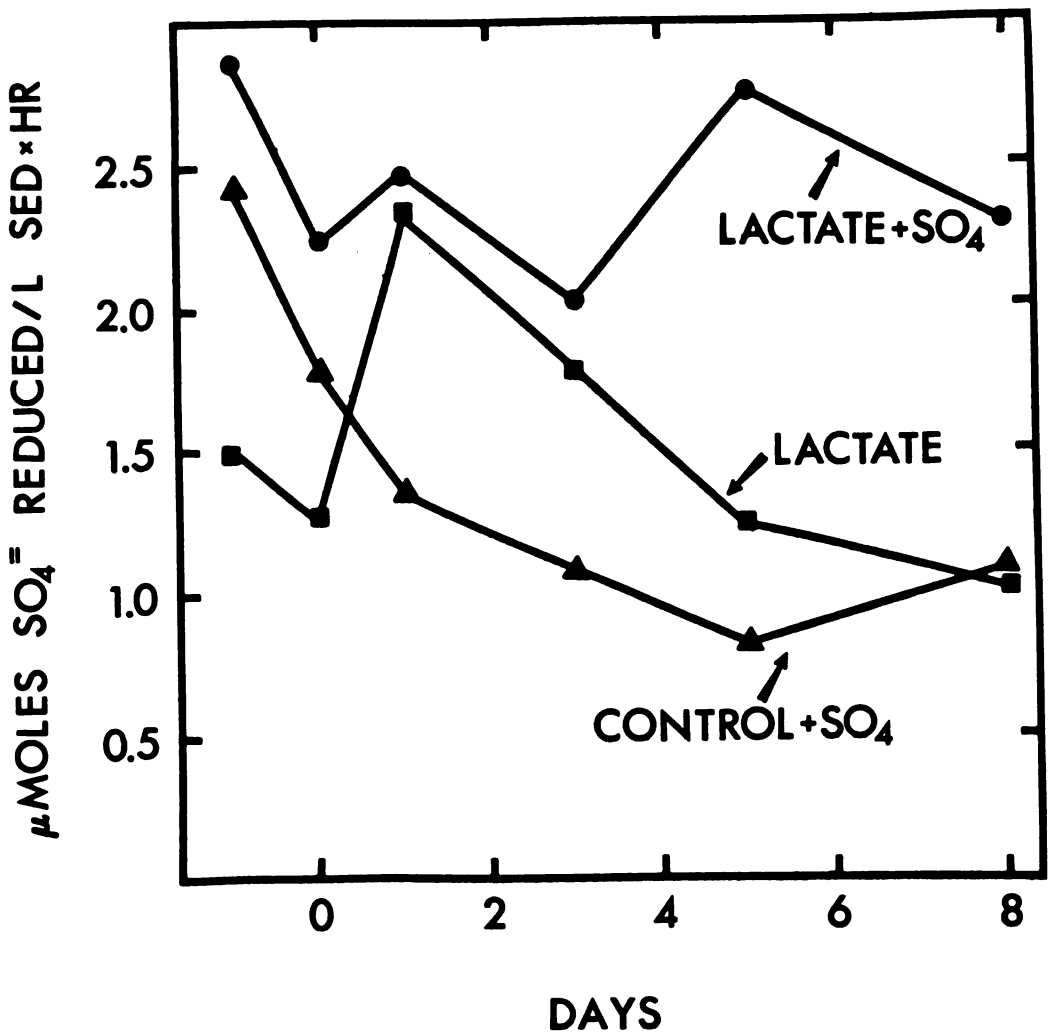

A
B
CONTROL
14 MMOL
1.4 MMOL
0.18 MMOL
H_2
GLUCOSE +SO_4
H_2+SO_4
GLUCOSE
10
5
0
2
4
6
8
DAYS
μMOLES $SO_4^=$ REDUCED/L SED × HR

Figure 5. Potential rates of sulfate reduction in sediment reactor flasks receiving lactate. Reactor input prior to T_o was 0.18 mmole $SO_4^=$ $reactor^{-1}$ day^{-1} and after as indicated.


μMOLES $SO_4^=$ REDUCED/L SED×HR
2.5
2.0
1.5
1.0
0.5
0
2
4
6
8
DAYS
LACTATE+SO_4
LACTATE
CONTROL+SO_4

reduction relative to control flasks (Fig. 4b), however the addition of either glucose or H_2 plus sulfate increased the potential sulfate reduction rate relative to controls receiving only sulfate. No sulfate was detected in effluents of reactors amended with sulfate and glucose, H_2, or lactate.

Acetate, propionate, and lactate concentrations increased in the reactor effluent when lactate was added to the reactor influent (Fig. 6). No differences were apparent in these pools relative to sulfate additions until after day 5, when an additional increase in the effluent acetate concentration, 0.9 mM, and a decrease in the effluent propionate concentration, 0.8 mM, was observed in the reactor amended with sulfate and lactate. A transient increase was observed in the effluent lactate concentration of both reactors, which was delayed 2-3 days by the addition of sulfate (Fig. 6). The ratio of the effluent concentration of acetate from reactors without added sulfate to those with added sulfate are shown in Figure 7. A value less than 1 indicates increased effluent acetate concentrations in the presence of sulfate. Additions of H_2, glucose, and lactate increased the ratio relative to control reactors.

DISCUSSION

Although continuous flow and semi-continous flow sludge digestors have long been utilized to study anaerobic metabolism in sludge, few studies have investigated anaerobic activities in freshwater sediments with this technique. The sediment reactors utilized in these studies permit low level inputs of sulfate without diluting the sediment, thus simulating sulfate diffusion into freshwater sediments. Initial sediment activities (i.e. methane production and potential rates of

Figure 6. Effluent acetate, propionate, and lactate concentrations from sediment reactor flasks receiving lactate. Reactor input prior to T_o was 0.18 mmoles $SO_4^=$ reactor^{-1} day^{-1} and after as indicated; control + SO_4 (•••••), lactate (••••••), and lactate + SO_4 (——-). Lactate input was 0.7 mmoles reactor^{-1} day^{-1}.

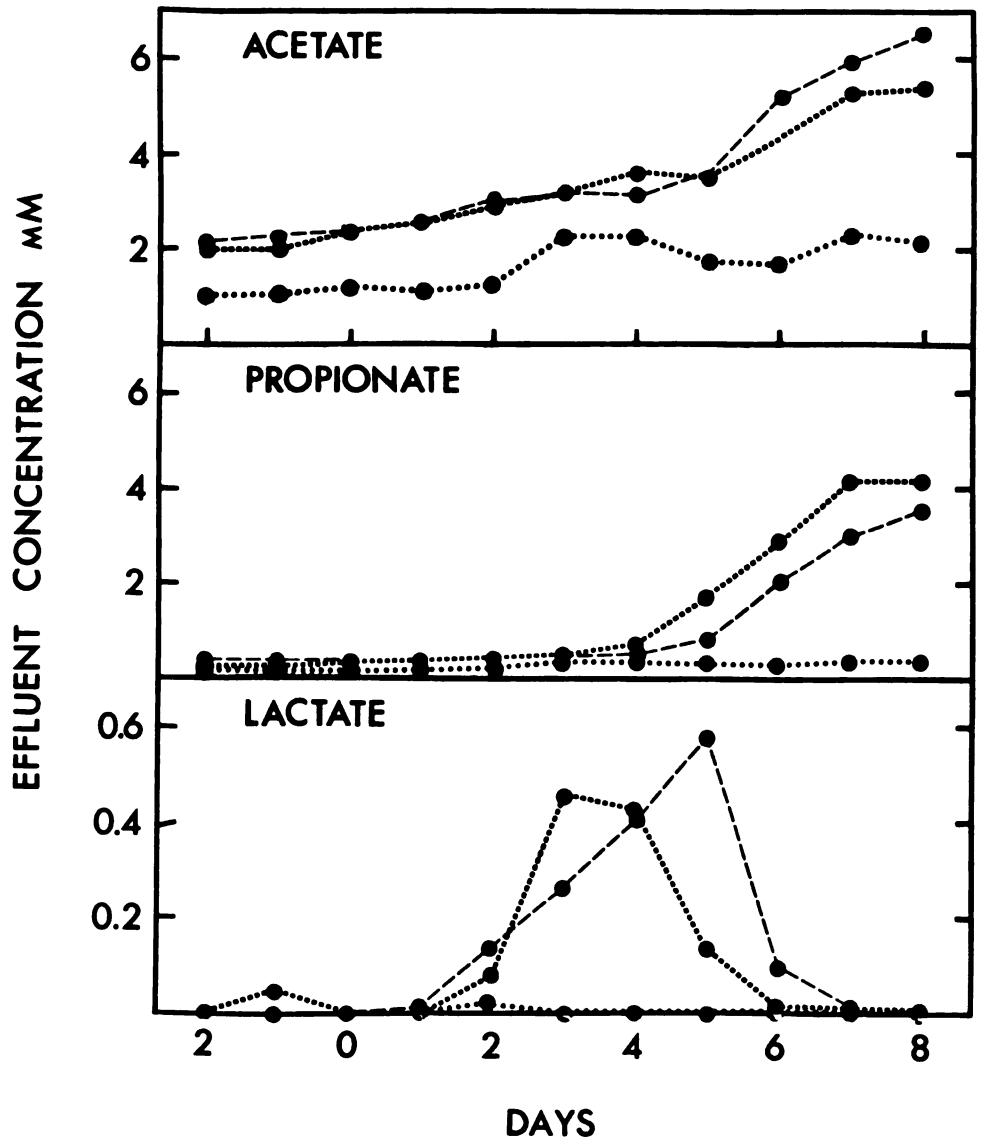
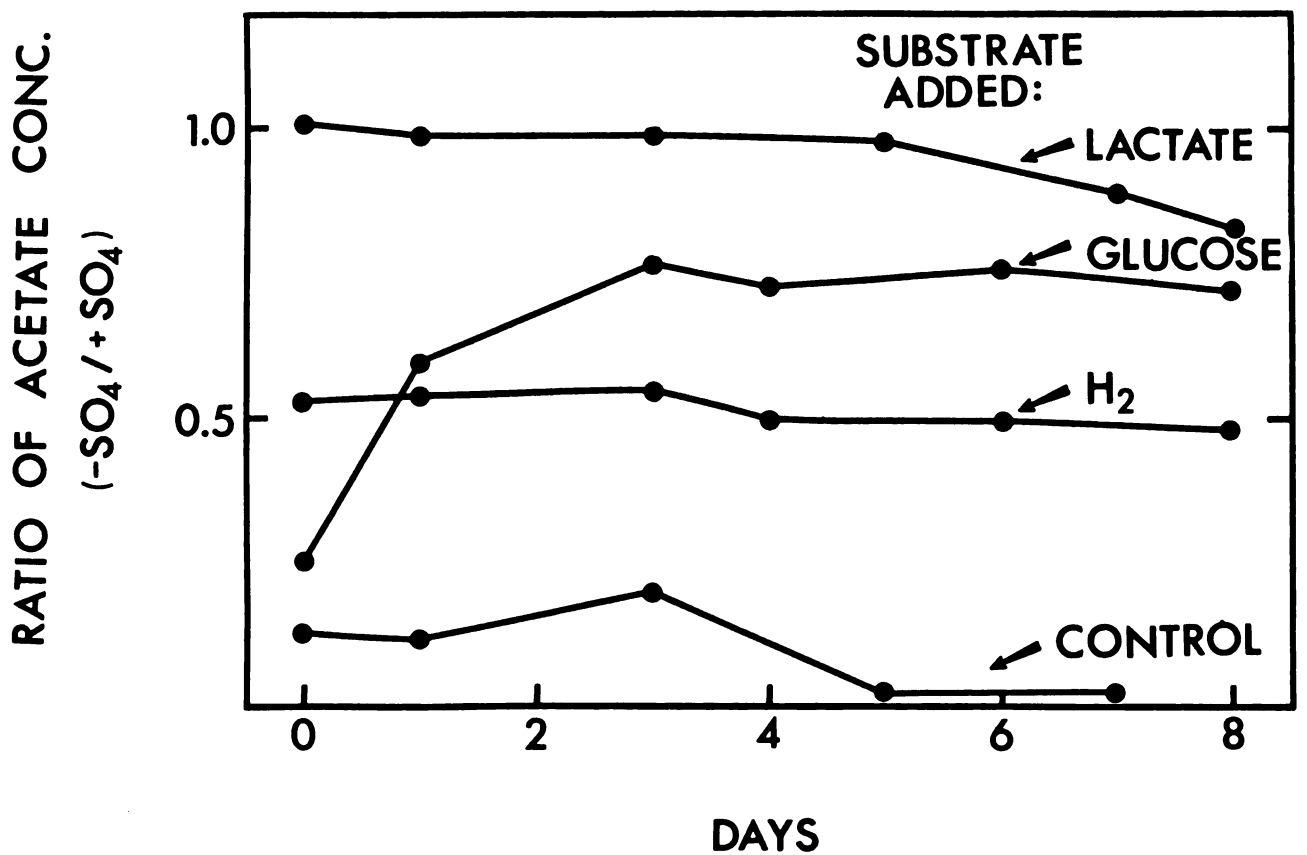

ACETATE
PROPIONATE
LACTATE
EFFLUENT CONCENTRATION mM
DAYS
6
4
2
0.6
0.4
0.2
2
0
2
4
6
8

Figure 7. Ratio of the effluent acetate concentrations from reactors receiving no sulfate relative to those receiving sulfate. Sulfate input was 0.18 mmoles $SO_4^=$ $reactor^{-1}$ day^{-1}.


RATIO OF ACETATE CONC.
(-SO4 / +SO4)
1.0
0.5
0
2
4
6
8
DAYS
SUBSTRATE ADDED:
LACTATE
GLUCOSE
H2
CONTROL

sulfate reduction) in the reactor flasks were very similar to those observed *in situ* in Wintergreen Lake profundal surface sediments (see 10, 17, Chapter II). However after 3-4 days methane production dropped to a lower rate (Fig. 2) and was accompanied by an increase in the effluent acetate concentration (Fig. 3). The mechanism responsible is unknown. The changes may be due to continual physical mixing or the effects of the artificial medium upon the anaerobic microorganisms present in the sediment. It would not appear to be due to a depletion of essential nutrients since the interstial water retention time is 12-13 days and the rate of methane production does not continually decrease, as might be expected from a dilution of an essential nutrient. In addition acetate availability would not appear to be limiting to methane production as the effluent acetate concentration increases once the lower rate of methane production was established. Hydrogen stimulated methane production while high levels of sulfate inhibited methane production, which are responses typical of sediment methane production (16, 19). Furthermore the lower rate of methane production does not appear to be due to the sulfate added prior to T_o since once sulfate is depleted in control reactors methane production remains at the lower rate. The important factor is that once the sediment had adapted to the reactor conditions (2-3 days) metabolic activities in control reactors appeared to be constant for the duration of the experiments (Figures 2, 3, 6, 7). Thus changes in sulfate-reducing activity during this time period (days 1-8) relative to controls can be inferred as responses to the given substrate added.

Sulfate reduction in sediments subsampled from reactor flasks was measured at a sulfate concentration demonstrated to be saturating for

sulfate reduction in Wintergreen Lake profundal sediments (Chapter II) and is therefore termed the potential rate of sulfate reduction. These rates were not dependent upon the determination of effluent sulfate concentrations, which were often very low (e.g. reactors amended with lactate plus sulfate) and therefore subject to error. Potential rates could also be determined for comparative purposes in control reactors which contained no measurable interstitial sulfate. These reactors did, however, contain demonstrable sulfate-reducing activity. Actual rates of sulfate reduction could not be estimated by the decrease in effluent sulfate concentration relative to the input concentration since such estimates were higher than the potential $^{35}SO_4^=$ reduction rates. This disparity must arise from nondissimilatory sulfate uptake and/or abiological sulfate adsorption. When sulfate was removed from the influent of control reactors potential rates of sulfate reduction dropped with time, probably due to decreasing electron acceptor availability.

Continual or increased sulfate inputs to sediment reactors initially resulted in decreasing rates of potential sulfate reduction with increasing additions of sulfate (Figure 4). Since sulfate was not limiting for determinations of potential rates of sulfate reduction this suggests the decrease in the potential rate was due to decreasing electron donor availability with time. Sulfate additions increased the rate of sulfate reduction in Wintergreen Lake sediments (Chapter II), which implicitly increases the utilization rate of electron donors. Similarily increasing additions of sulfate to reactors would result in increasing rates of electron donor utilization, therefore decreasing electron donor availability, and hence resulting in lower rates of

potential sulfate reduction. Since the observed effluent sulfate concentration at day 1 indicated that the initial actual rate of sulfate reduction for the reactors receiving the higher sulfate inputs was saturated with respect to sulfate, then electron donor utilization by sulfate reducers in these reactors was occurring at the maximal rate. Thus the initial decrease (days 0-1) of the potential rate of sulfate reduction in sediment subsamples from these reactors represents the maximal initial rate of decrease.

Increased effluent acetate concentrations (10-20 times) in reactors receiving sulfate relative to controls (Fig. 3, 7) suggests that the increased acetate is produced by sulfate-reducing bacteria. Electron donors for sulfate-reducing bacteria are generally viewed as low molecular weight intermediates, which are oxidized to acetate (4, 7). Addition of substrates which stimulated potential rates of sulfate reduction (e.g. H_2, and glucose) increased the effluent acetate ratio relative to sulfate as the availability of other electron donors decreased acetate production by sulfate-reducing bacteria (Fig. 7). The initial acetate increase was the same in rectors receiving either 0.18 or 14 mmoles $SO_4^=$ $reactor^{-1}$ day^{-1}. These results also indicate the limited availability of low molecular weight intermediates as electron donors for sulfate reduction, since increasing the sulfate addition, from a non-saturating to a saturating concentration for sulfate reduction, did not increase the amount of acetate produced.

Acetate-oxidizing, sulfate-reducing activity has been described in sludge digestors (8) and implicated in marine sediments (11), yet increases in acetate concentrations did not stimulate potential rates of sulfate reduction when the rate of sulfate addition (i.e. 0.18

mmoles $SO_4^=$ $reactor^{-1}$ day^{-1}) simulated the rate of sulfate diffusing into Wintergreen Lake sediments. Either acetate was not an electron donor for sulfate reduction in this case or the sulfate-reducing bacteria were oxidizing acetate at the maximal rate prior to the increase in the acetate concentration. The latter seems the most likely situation in Wintergreen Lake surface sediments in which the acetate concentration (100-200 μM (10)) is greater than the sulfate concentration (45 μM, Chapter II) and the rate of sulfate reduction limited by the sulfate concentration (see Chapter II). Acetate oxidation was evident in the reactor receiving the highest sulfate addition as the rate of potential sulfate reduction increased simultaneous with a drop in the effluent acetate concentration (Fig. 3, 4). The enrichment was evident in only 5 days suggesting the prior presence of an acetate-oxidizing population of sulfate-reducing bacteria limited only by the sulfate concentration. Studies with 2-^{14}C-acetate demonstrated that sulfate-reducing bacteria do oxidize acetate at <u>in situ</u> sulfate concentrations in Wintergreen Lake profundal sediments (Chapter V). Once the effluent acetate concentration was lowered the sulfate-reducing activity again dropped, presumably due to a limitation of electron donor availability. The reason for the drop in the effluent acetate concentration in the control reactor after day 6 is unknown.

Additions of lactate, H_2, and glucose stimulated potential rates of sulfate reduction when added with sulfate relative to controls receiving sulfate (Fig. 4, 5). The effect of additions of H_2 and lactate upon sulfate reduction was evident 3 days before the effect of the glucose addition. This indicates that either sulfate-reducing

bacteria adapted to utilize glucose as an electron donor or were utilizing other metabolic products which had been produced from the added glucose by other sediment microorganisms. The predominant pathway of carbon flow in anoxic sediments containing active populations of H_2-consuming organisms is via oxidized organic intermediates, primarily acetate (D. Lovley, personal communication). Large increases in the production of acetate and CO_2 (data not shown) were evident in reactors to which glucose was added, indicating catabolism of glucose to CO_2 and acetate. Increased acetate concentrations did not stimulate potential rates of sulfate reduction at the low sulfate input, which may account for the relatively small stimulation of potential sulfate reduction by additions of glucose. In reactors receiving lactate and sulfate the added sulfate was insufficient to oxidize all the added lactate, therefore the reactor lactate concentration increased and apparently enriched for a group of organisms which also metabolized lactate to propionate (Fig. 6). The decrease in the effluent propionate concentration in the sulfate amended reactor represents lactate oxidation to acetate by sulfate-reducing bacteria. The addition of H_2 stimulated potential rates of sulfate reduction (Fig. 4) and increased the acetate effluent ratio relative to sulfate to 0.5. Thus less acetate was produced in the reactor to which H_2 and sulfate was added than in the control reactor receiving sulfate alone, indicating that H_2 served as an electron donor for sulfate reduction which resulted in a decreased production of acetate by sulfate-reducing bacteria.

The reactor system described here appears to be well suited for studying perturbations to anoxic sediments. This is particularly true

if continual low level inputs are necessary to maintain a given sediment activity. These results indicated that included among the primary electron donors for sulfate reduction in these sediment reactors are substrates which can be oxidized to acetate, even though the availability of these substrates is limited. Acetate was also suggested to be an electron donor, while at in situ sulfate and acetate concentrations acetate oxidation by sulfate reduction may be saturated with respect to acetate. While these results are not necessarily indicative of in situ sulfate-reducing activity, the response of sediment ecosystems to experimental perturbations serves as the basis for a better understanding of the activities of natural populations of sulfate-reducing bacteria.

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CHAPTER V

ELECTRON DONORS UTILIZED BY SULFATE-REDUCING BACTERIA IN EUTROPHIC LAKE SEDIMENTS

INTRODUCTION

In marine sediments, sulfate reduction is the predominant terminal electron accepting process in carbon metabolism (10). Significant rates of sulfate reduction also occur in freshwater sediments despite low sediment sulfate concentrations (20). Although sulfate reduction is of significance to carbon and electron flow in both of these ecosystems, the key electron donors utilized by natural populations of sulfate-reducing bacteria have not been definitively delineated.

Sulfate-reducing bacteria can potentially compete with methanogenic bacteria for H_2 and acetate in both marine (1, 2, 12) and freshwater sediments (23) and have been demonstrated to oxidize the major portion of added hydrogen in marine sediments (15, 17). Additions of lactate stimulated sulfate reduction in some San Francisco Bay sediments though additions of acetate, pyruvate, and formate had no effect (16). Lactate has been implicated as the major electron donor for sulfate reduction in freshwater sediments (6) while acetate stimulated thermophilic sulfate reduction in the water column of Solar Lake, Sinai (11). However, these studies do not represent a systematic determination of electron donors for sulfate-reducing bacteria within a single system at or near <u>in situ</u> concentrations of the electron donors.

This investigation examined the effect of Na_2MoO_4, an inhibitor of sulfate-reducing bacteria, upon mineralization rates of tracer additions of ^{14}C-substrates in freshwater sediments in order to delineate natural electron donors for sulfate-reducing bacteria.

Materials and Methods

Sediment collection: Profundal surface sediments from Wintergreen Lake, a shallow (z_m = 6.5m), hypereutrophic lake located in Southwestern Michigan (13, 14) were sampled with an Eckman dredge. Jars were completely filled with sediment, sealed, stored at 10°C, and subsampled within 24 hours. Sediment was homogenized in the jars with a paint shaker and 5 ml samples transferred with a syringe to either 30 ml Wheaton serum bottles or anaerobic pressure tubes (Bellco) which were flushed with O_2-free N_2 throughout the subsampling. Samples for sulfate reduction were sealed with Teflon lined rubber septa (Supelco). All other vessels were stoppered with butyl rubber anaerobe stoppers (Bellco). For H_2 experiments the headspace gas was flushed with O_2-free H_2 through syringe needles.

Sulfate reduction: Prereduced carrier-free $Na_2{}^{35}SO_4$ (New England Nuclear), diluted with unlabelled Na_2SO_4 when appropriate, was added through a syringe and needle (0.5 ml containing 1-2 μCi) to each sample. Sediment samples were blended with a Vortex mixer and incubated at 10°C (with shaking when H_2 was present in the headspace) for appropriate periods. Bottles containing a H_2 headspace were preincubated 30 minutes prior to amendment with $Na_2{}^{35}SO_4$, as were controls which had been killed by the addition of 0.5 ml of 10% zinc acetate. Sulfate reduction was terminated by the addition of 0.5 ml of prereduced 10% zinc acetate and the samples frozen until analysis. The

$H_2{}^{35}S$ produced was trapped with a flushing train as described by Smith and Klug (20). The headspace gas in the serum bottle was flushed with O_2-free N_2 for 2 minutes to purge the system of O_2 prior to the addition of 5 ml of O_2-free 3 N HCl to the frozen sediment. Samples were flushed for 30 minutes into 2 traps containing 8 ml of 2% $CdCl_2$. Upon completion of flushing, 10 ml of Aqueous Counting Scintillant (Amersham Searle) was added to each trap (scintillation vials) and the radioactivity determined with a Beckman LS8000 liquid scintillation counter (Beckman Instruments).

Effect of molybdate upon sediment activities: Prereduced Na_2MoO_4, 0.5 ml at appropriate concentrations, was added to sediment subsamples, preincubated 30 min at 10°C, and sulfate reduction determined as described above. Due to the formation of insoluable MoS_3 at low pH (5) the $^{35}S^{=}$ produced was quantified as described by Oremland and Silverman (1979). Sediment samples were thawed and filtered through Millipore HA filters (0.45 μm). The bottles and filters were rinsed several times with 1% Na_2SO_4, the filters dried, and sediment subsamples placed in scintillation vials containing 5 ml of H_2O. The vials were shaken overnight to disperse the sediment, 10 ml of Aqueous Counting Scintillant added, and the samples dark adapted 24 hours prior to the determination of radioactivity. The counting efficiency of each sample was determined by adding an internal standard of $H_2{}^{35}SO_4$.

The effect of Na_2MoO_4 upon methane production in profundal sediments was measured by dispensing sediment and Na_2MoO_4 into anaerobic pressure tubes (Bellco) as described above. Headspace gas was flushed with 93% N_2/7% CO_2 and the samples preincubated for 30 minutes at 10°C. Methane production was determined by analyzing the

change in headspace CH_4 concentration at several time points over the course of 12 hours. Methane was analyzed on a Varian 600 D gas chromatograph as previously described (14). The effect of Na_2MoO_4 on the mineralization of 2-^{14}C-acetate was measured in a second set of subsamples prepared as above. Prereduced 2-^{14}C-acetic acid, sodium salt, (New England Nuclear, 7.4 µCi/ml, 54 Ci/mole, 0.2ml) was added through a syringe and needle and the samples incubated at 10°C for 30 minutes. Biological activity was stopped by quick-freezing in a dry ice-ethanol bath. $^{14}CH_4$ and $^{14}CO_2$ in the headspace gas were determined with a Varian 3700 gas chromatograph (Varian Instruments) equipped with a thermal conductivity detector connected in series with a gas proportional counter. The GC analysis was with a stainless steel column (1.8 m x 2 mm ID) packed with Porapak N at 40°C. The distribution of $^{14}CO_2$ between the aqueous and gaseous phases was determined by adding 0.1 ml of a $Na_2H^{14}CO_3$ (New England Nuclear, 1 µCi/ml) solution to the tubes and following equilibration, the radioactivity of the headspace gas determined a second time. Results are presented as a ratio, termed the respiratory index (RI) by Winfrey and Zeikus (37), where RI = $^{14}CO_2$/ ($^{14}CO_2$ + $^{14}CH_4$).

Mineralization of ^{14}C-substrates: A 0.5 ml aliquot of a prereduced solution of one of four different ^{14}C substrates was added by syringe and needle to subsamples of sediment. The ^{14}C-substrates added were: U-^{14}C-lactic acid, sodium salt, (New England Nuclear, 2 µCi/ml, 139 Ci/mol); 1-^{14}C-propionic acid, sodium salt (New England Nuclear, 0.4 µCi/ml, 10 Ci/mol); U-^{14}C-glucose (New England Nuclear, 2 µCi/ml, 183 Ci/mole); and U-^{14}C-amino acid mixture (New England Nuclear, 0.3 µCi/ml, containing 15 individual L-amino acids). Controls

were killed by the addition of 0.5 ml of prereduced 50% glutaraldehyde and preincubated for 30 minutes prior to the addition of ^{14}C-substrate. A second set received 0.5 ml of a prereduced solution of Na_2MoO_4 of the appropriate concentration and also preincubated 30 minutes. Sediment samples were blended with a Vortex mixer, incubated at 10°C for appropriate lengths of time, and the biological activity terminated by quick-freezing in a dry ice-acetone bath. The samples were kept frozen at -10°C until analysis. Incubation intervals and/or the position of the ^{14}C label (i.e. 1-^{14}C-propionic acid) were chosen to insure minimal $^{14}CH_4$ production. Frozen samples from the longest incubation periods were placed in a boiling water bath for 10 min, then allowed to cool. Headspace gas from these samples was analyzed for $^{14}CH_4$ with the gas chromatograph-gas proportional counter system described above.

$^{14}CO_2$ was trapped in the flushing train as previously described except that a third trap was added, 1N KOH substituted for $CdCl_2$, and the volume increased to 18 ml in each trap. Upon completion of flushing a 1 ml aliquot of each trap was added to 1 ml of a saturated $BaCl_2$ solution contained in scintillation vials, followed by the addition of 5 ml of 0.4 N tris buffer, pH 1.3, and 8 ml of Aqueous Counting Scintillant (Amersham Searle). The scintillation vials were dark adapted for several hours and the radioactivity determined as described. Counting efficiency of the $BaCO_3$-gel suspension was 84%.

RESULTS

Effect of Na_2MoO_4 upon sediment processes: Na_2MoO_4 completely inhibited sulfate reduction during a 30 min. incubation in Wintergreen Lake profundal sediments at all concentrations of Na_2MoO_4 tested (Table 1). Total methane production was inhibited 51% by 200 mM

Table 1. Effect of Na_2MoO_4 upon methane production and sulfate reduction in Wintergreen Lake profundal sediments.[1]

Addition[2]	Methane Production[3]	%Inhibition	Sulfate Reduction[4]	%Inhibition
Control	35 (3)	0	420 (12)	0
0.2 mM $MoO_4^=$	30 (10)	14	0	100
2 mM $MoO_4^=$	32 (3)	9	0	100
20 mM $MoO_4^=$	28 (2)	20	0	100
200 mM $MoO_4^=$	17 (2)	51	N.D.[5]	-

[1] Sediments collected September, 1980.

[2] Final concentration of Na_2MoO_4 . Control was amended with O_2-free H_2O.

[3] μmoles CH_4 produced/l sed x hr. Brackets enclose standard error.

[4] μCi of $^{35}S^=$ produced/l sed x hr. Brackets enclose standard error.

[5] Not determined.

Na_2MoO_4 (Table 1), however at lower concentrations (<20mM) methane production was only slightly inhibited (10%). Production of $^{14}CH_4$ from 2-^{14}C-acetate was essentially unaffected by Na_2MoO_4 concentrations below 20mM (Table 2), while nearly complete inhibition (98%) was noted after addition of 200 mM Na_2MoO_4. Mineralization rates of U-^{14}C-glucose, as measured by $^{14}CO_2$ production, were unaffected by a final concentration of 20 mM Na_2MoO_4 (Figure 1). No $^{14}CH_4$ was detected during the time course of the experiment in either the presence or the absence of the inhibitor.

Mineralization of ^{14}C-substrates: The principle mineralization product of 2-^{14}C-acetate in Wintergreen Lake sediments was $^{14}CH_4$, as evidenced by an RI value of 0.2 (Table 2). In the presence of 0.2 mM Na_2MoO_4 the RI value for acetate mineralization was 0.05. This four fold decrease is due to a decrease in $^{14}CO_2$ production relative to $^{14}CH_4$ production. At higher concentrations of Na_2MoO_4 $^{14}CO_2$ production remained relatively constant while $^{14}CH_4$ production was inhibited, resulting in an increasing RI value for acetate mineralization in the sediments as the Na_2MoO_4 concentration increased. The mineralization rate of 1-^{14}C-propionate was linear for 20 minutes in the presence ($r^2 = 0.83$) or absence ($r^2 = 0.96$) of 20 mM Na_2MoO_4 (Figure 2). No $^{14}CH_4$ was detected in either set of samples within this time period. The mineralization rate was 38% lower in the presence of Na_2MoO_4. Table 3 summarizes the results of similar experiments for sediments amended with 4 different ^{14}C-substrates. Na_2MoO_4 significantly inhibited the mineralization rates of lactate, propionate, and a mixture of amino acids, but not the mineralization of glucose. In each case mineralization rates were linear without an apparent time lag.

Table 2. Effect of Na_2MoO_4 upon mineralization of 2-^{14}C-acetate in Wintergreen Lake profundal sediments.[1]

Addition[2]	TOTAL GAS PRODUCED[3]		RI Value[4]
	$^{14}CO_2$	$^{14}CH_4$	
Control	48 (4)	196 (20)	0.20
0.2mM $MoO_4^=$	9 (1)	160 (7)	0.05
2mM $MoO_4^=$	22 (2)	229 (25)	0.09
20mM $MoO_4^=$	15 (2)	105 (7)	0.13
200mM $MoO_4^=$	14 (5)	4 (4)	0.78

1) Sediments collected September, 1980.

2) Final concentration of Na_2MoO_4. Control was amended with O_2 free H_2O.

3) Data reported as nCi. Brackets enclose standard errors.

4) RI = $^{14}CO_2/(^{14}CO_2 + ^{14}CH_4)$.

Figure 1. Effect of Na_2MoO_4 upon the mineralization of U-^{14}C-glucose at 10°C. Controls were pretreated with glutaraldehyde. Data points are a mean of triplicates. Circles with $MoO_4^=$; squares without $MoO_4^=$. Error bars represent $\pm$ one standard error.

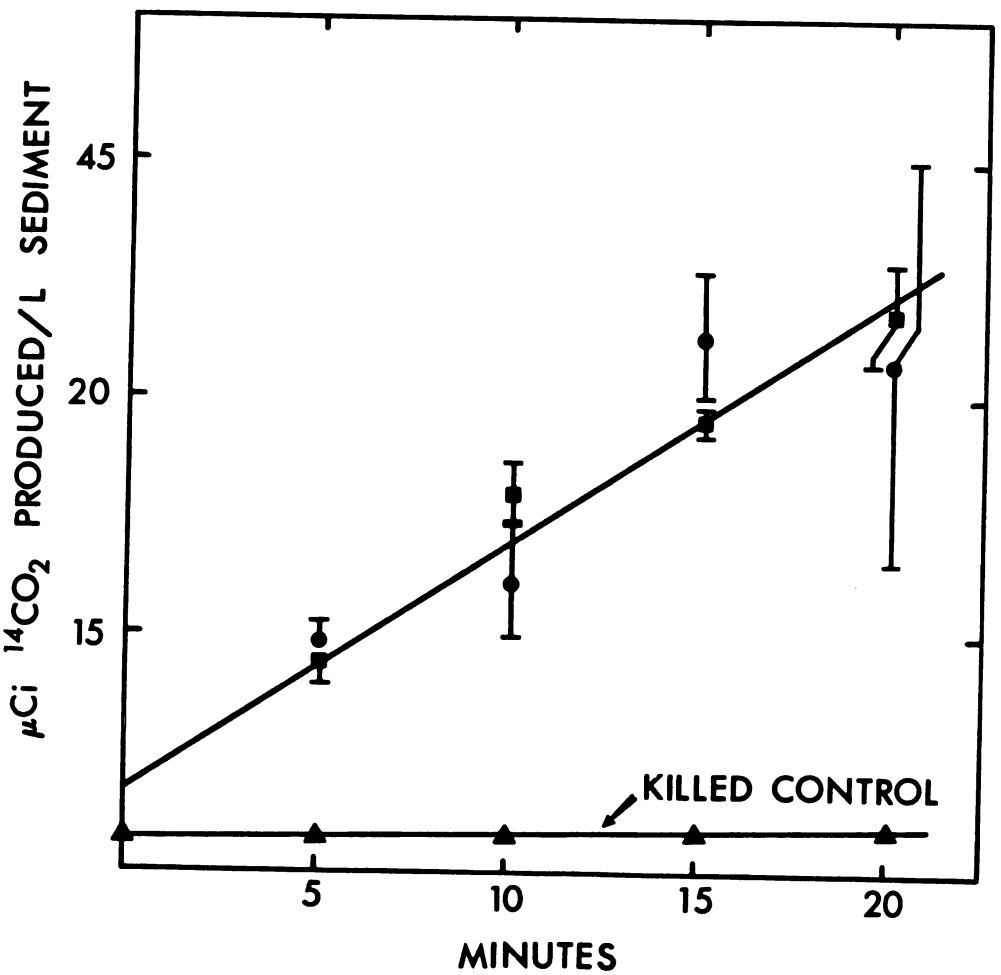
μCi $^{14}CO_2$ PRODUCED/L SEDIMENT
45
20
15
KILLED CONTROL
5
10
15
20
MINUTES

Figure 2. Effect of Na_2MoO_4 upon the mineralization of $1-^{14}C$-propionate at 10°C. Controls were pretreated with glutaraldehyde. Data points are a mean of triplicates. Error bars represent $\pm$ one standard error.

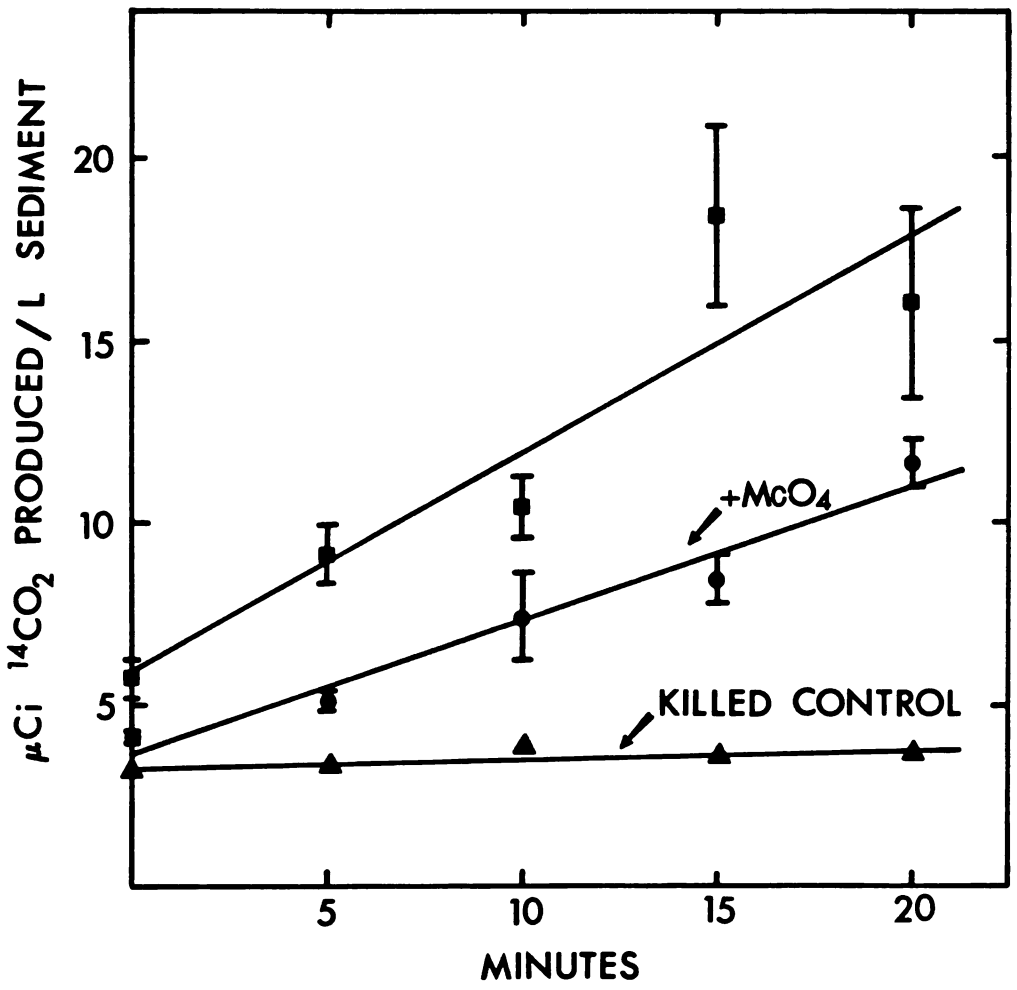
μCi $^{14}CO_2$ PRODUCED / L SEDIMENT
20
15
10
5
+MnO_4
KILLED CONTROL
5
10
15
20
MINUTES

Table 3. Effect of Na_2MoO_4 upon mineralization rates of ^{14}C-substrates in Wintergreen Lake profundal sediments.[1]

Substrate	Na_2MoO_4 concentration (mM)	% Inhibition[2]
lactate	20	47.0
	1	58.3
propionate	20	52.8
	1	51.7
L-amino acid mixture	20	84.6
glucose	20	5.7

[1]Sediments collected in October 1979, July, August, and September 1980.

[2]n = 2 for 20mM Na_2MoO_4; n = 1 for 1 mM Na_2MoO_4.

The inhibition of both lactate and propionate mineralization were unaffected over the range of Na_2MoO_4 concentrations of 1-20 mM.

Effect of H_2 on sulfate reduction: Sulfate reduction in Wintergreen Lake profundal sediments was stimulated by the addition of H_2 to the reacting flask headspace (Table 4). A stimulation was evident in sediments amended with both carrier-free $^{35}SO_4^=$ and 1 mM $^{35}SO_4^=$ (final concentration). The reduction rate was linear for 10 minutes at in situ $SO_4^=$ concentrations and the entire time course (20 minutes) at 1 mM $SO_4^=$ concentration (data not shown). Turnover times reflect differences in the sediment sulfate pool.

DISCUSSION

Molybdate is well established as an inhibitor of sulfate-reducing bacteria (8, 19, 22). It is stereochemically similar to sulfate and has been demonstrated to inhibit ATP-sulfurylase, the first enzyme in the sulfate-reducing pathway (18). Since inhibition is specific for biochemical processes involving sulfate, molybdate appears to be well suited for studies of sulfate-reducing bacteria in natural habitats when conducted on a short-term basis (<< 1 generation time). In such a situation the effect of molybdate upon the total metabolism of dissimilatory sulfate-reducing bacteria would be far greater than corresponding effects upon assimilatory sulfate-reducing bacteria. Taylor and Oremland (22) have demonstrated that organisms reducing sulfate were much more sensitive to molybdate than were other physiological types of bacteria.

Few studies have employed the selective inhibition by molybdate to investigate the role of sulfate-reducing bacteria in natural habitats. The inhibitor was used to investigate the interaction of

Table 4. Effect of H_2 upon sulfate reduction in Wintergreen Lake profundal sediments.[1]

Experiment	Sulfate concentration[2]	Headspace gas	T_t[3] (hr)	% Stimulation
1	*in situ*	N_2	1.0	-
		H_2	0.4	250
	1mM	N_2	326	-
		H_2	204	160
2	*in situ*	N_2	4.8	-
		H_2	1.7	282

[1]Sediments collected 4/80 for experiment 1; 7/80 for experiment 2.

[2]Final sediment sulfate concentration.

[3]Turnover time of the $^{35}SO_4^{=}$ pool, based upon initial rates of sulfate reduction.

sulfate-reducing bacteria and methanogenic bacteria in marine sediments (22). Huisingh and Matrone (7) demonstrated that molybdate inhibited sulfate reduction in sheep fed Na_2SO_4 but stimulated sulfide production from methionine (9). The concentration of molybdate necessary to effect inhibition in natural habitats is dependent upon the sulfate concentration, since inhibition is competitive in nature. It may also be dependent upon the sulfide concentration as complexes of $MoO_2S_2^{-2}$ and MoS_4^{-2} are formed (25). Sulfate reduction in marine sediments is completely inhibited by 20 mM molybdate (16). However, in freshwater sediments, where the sulfate concentration is much lower, sulfate reduction was completely inhibited by 0.2 mM molybdate (Table 1).

High concentrations (>20 mM) of Na_2MoO_4 inhibited both total methane production and $^{14}CH_4$ production from 2-^{14}C-acetate in profundal sediments. A comparison of total methane production (Table 1) and production from acetate (Table 2) indicates that methane production from acetate was inhibited to a greater extent by Na_2MoO_4 than was methane production from H_2 and CO_2. Both sources of methane production were relatively unaffected at lower concentrations of Na_2MoO_4 (i.e. <20mM). Heterotrophic metabolic processes not involving immediate precursors for sulfate reduction or methane production appeared to be unaffected by Na_2MoO_4 as rates of glucose mineralization were unaltered in the presence of 20 mM Na_2MoO_4 (Figure 1).

The RI index for 2-^{14}C-acetate mineralization indicates that 20% of the total mineralization of the methyl group of acetate was oxidation to CO_2. Na_2MoO_4 inhibited 69% of this oxidation, indicating that 14% of the acetate mineralization in the absence of Na_2MoO_4 could be attributed to sulfate reduction, while 6% of the acetate was mineralized by some other group of organisms in the sediment.

The mineralization rates of acetate, lactate, propionate, and an amino acid mixture in the presence and absence of Na_2MoO_4 strongly imply that sulfate-reducing bacteria are directly involved in the mineralization of these substrates in Wintergreen Lake profundal sediments (Table 2 and 3). The actual mineralization rates of glucose, lactate, and free amino acids can't be extrapolated from these data since although low concentrations of ^{14}C-substrates were added these exogenous additions are considered to have altered the natural concentration. Additions of ^{14}C-propionate or ^{14}C-acetate did not significantly alter the natural sediment concentrations (see 14). Although 52% of the total propionate mineralization could be attributed to sulfate reduction compared to 14% of total acetate mineralization, conversely these data do not indicate the relative contribution of acetate and propionate to sulfate reduction since the sediment propionate concentration is 10-fold lower than the sediment acetate concentration (D. Lovley, personal communication). The inhibition of mineralization by Na_2MoO_4 at natural substrate concentrations represents a minimum estimate of mineralization by sulfate-reducing bacteria since inhibition of sulfate reduction can increase a given substrate's availability to other sediment microorganisms.

Hydrogen can also serve as an electron donor for sulfate-reducing bacteria (3). Cocultures of Desulfovibrio can participate in interspecies H_2 transfer as well by oxidizing H_2 and reducing sulfate (1, 4). Oremland and Taylor (17) determined that sulfate-reducing bacteria were primarily responsible for H_2 consumption in marine sediments, while a 3-fold stimulation of sulfate reduction by hydrogen addition was reported in salt marsh sediments (2). H_2 stimulated

sulfate reduction 2.5-2.8 fold in Wintergreen Lake profundal sediments, indicating potential hydrogen oxidation by sulfate reduction in freshwater sediments. However methanogenic bacteria in these sediments are the primary H_2 consuming organisms (21). This is supported by a lack of an increase in total methane production in the presence of Na_2MoO_4 (Table 1), indicating that hydrogen oxidation by sulfate-reducing bacteria at in situ hydrogen concentrations is relatively minor.

Few studies have determined natural electron donors for sulfate reduction in anoxic sediments. Most evidence has been determined indirectly with lactate, acetate, and hydrogen being commonly implicated as electron donors (17, 23). The data presented here indicate that lactate, acetate, free amino acids, and propionate potentially provide electrons for sulfate reduction in the freshwater sediments examined. Each of these carbon substrates can be oxidized directly in the absence of H_2 by one or more of several sulfate-reducing isolates from Wintergreen Lake profundal sediments (Chapter III). Thus it appears that an array of substrates serve as natural electron donors for sulfate reduction in these sediments and that sulfate reducers are of potential significance to the in situ mineralization of lactate, propionate and free amino acids, though mineralization of any given substrate may represent only a small fraction of the total electron flow through sulfate reduction.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

This study has demonstrated that high rates of sulfate reduction occur in the sediments of eutrophic lakes despite low interstitial sulfate concentrations. Although profiles of organic sulfur and H_2S concentrations suggest otherwise dissimilatory sulfate reduction appears to be the major mechanism for H_2S production. The highest rates of sulfate reduction occur in surface sediments and appear to be limited by the rate of sulfate diffusing into the sediments from the overlying water column. These two processes serve to maintain the low interstitial sulfate concentration, which has a rapid turnover time. The significance of sulfate reduction is emphasized by total sulfate reduction in a sediment depth profile, which can account for 30% of the mineralization of the particulate organic matter entering the sediments.

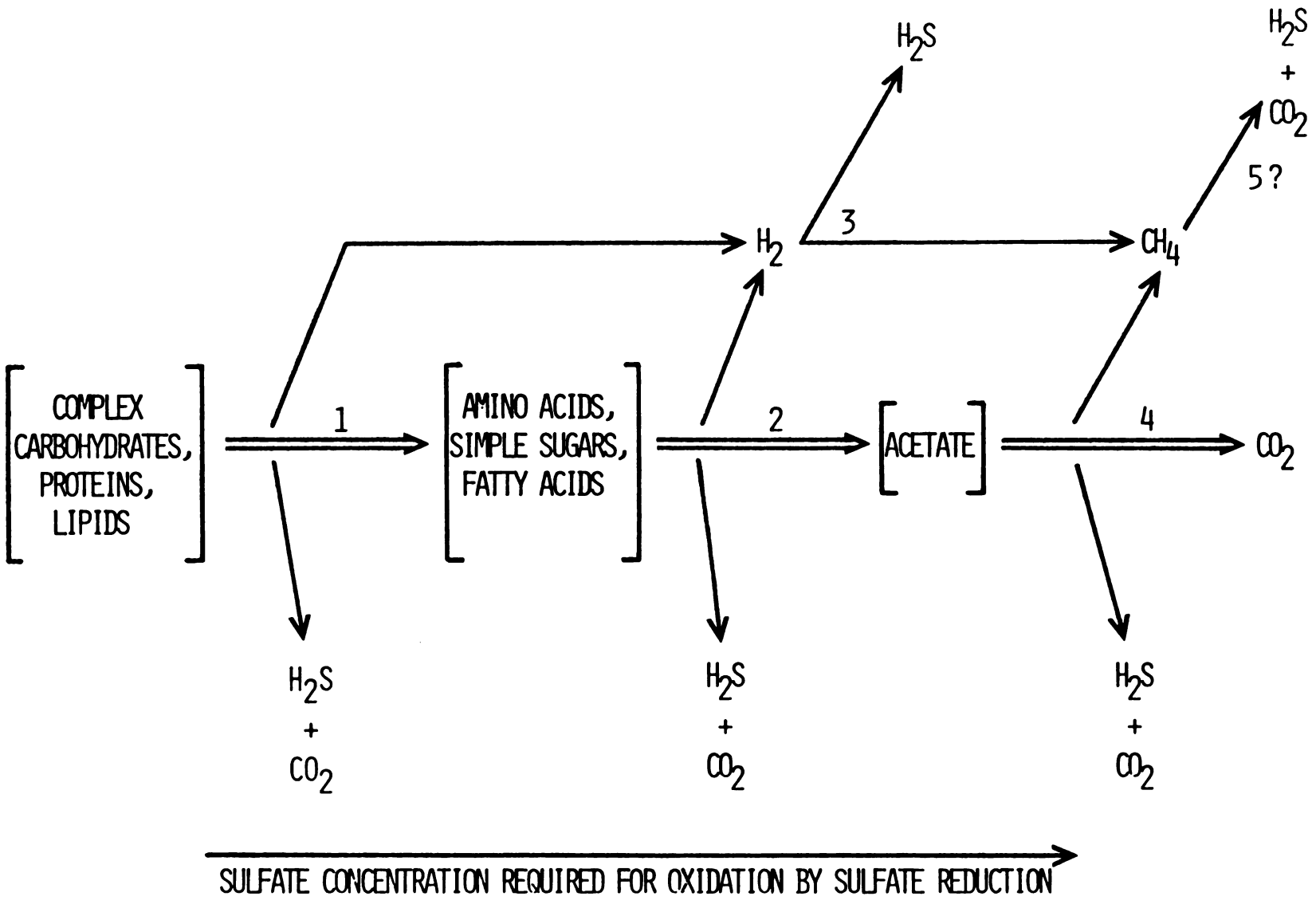
Sulfate-reducing bacteria isolated from these sediments can utilize a wide array of substrates for carbon and/or electron donors; substrates that are typically found in eutrophic sediments. The range of substrates supporting growth by isolates and MPN enrichments indicates that electron donors utilized for sulfate reduction in natural habitats has been previously underestimated. Sulfate-reducing bacteria oxidize intermediate molecular weight compounds to acetate in sediment contained in reactor flasks and are stimulated by additions of

lactate, H_2, and glucose. Mineralization experiments in the presence and absence of Na_2MoO_4, an inhibitor of sulfate reduction, also indicates that sulfate reducers utilize an array of substrates as electron donors and are of potential significance to the in situ mineralization of lactate, propionate and free amino acids. Since the acetate concentration is greater than the sulfate concentration in these sediments the oxidation of acetate by sulfate reduction appears to be saturated with respect to acetate.

Based upon these results a scheme conceptualizing sulfate reduction in anoxic sediments as it relates to carbon flow is given in Figure 1. The pathway of carbon flow (double-lined arrows) is the same whether CO_2 or $SO_4^=$ is the principle electron acceptor. The figure demonstrates that sulfate-reducing bacteria can be involved with each major step of oxidative carbon metabolism (steps 1-4), the magnitude of the involvement is dependent primarily upon the sulfate concentration available. Ample evidence indicates that when the sulfate concentration is sufficient sulfate reduction mediates carbon flow. Marine ecosystems are the classic example of carbon metabolism dominated by sulfate reduction when sulfate concentrations are not limiting.

The nature of decompositional processes predicates that the energy available to sulfate-reducing bacteria from each subsequent step of anaerobic mineralization is less than previous steps. Thus based upon thermodynamic considerations succesively increasing concentrations of sulfate are required to favor oxidation by sulfate reduction for steps 1 through 5. The energy available at standard conditions strongly favors those sulfate reducers that can utilize steps 1, 2, or 3 for

Figure 1. A generalized scheme of the role of sulfate reduction in anaerobic carbon metabolism in anoxic sediments. (Modified from (2)).


COMPLEX CARBOHYDRATES, PROTEINS, LIPIDS
1
AMINO ACIDS, SIMPLE SUGARS, FATTY ACIDS
2
ACETATE
4
CO2
H2S + CO2
H2S + CO2
H2S + CO2
H2
3
H2S
CH4
5?
H2S + CO2
SULFATE CONCENTRATION REQUIRED FOR OXIDATION BY SULFATE REDUCTION

electron donors (except for fatty acid oxidation). However based upon in situ sediment concentrations the energy available to sulfate reducers from H_2 oxidation is considerably less (Table 1), making the reaction thermodynamically competitive with only acetate and propionate oxidation.

Thermodynamic considerations are not the sole factors determining electron donors utilized by sulfate-reducing bacteria. Electron donor availability and competition with other organisms are determinative factors as well. This study has demonstrated that amino acids and propionate can serve as electron donors for sulfate reduction in eutrophic freshwater sediments, in addition to lactate, acetate, and H_2. Amino acid oxidation and propionate oxidation by sulfate-reducing bacteria have not been previously demonstrated in natural habitats. Unique among the organisms isolated is the ability of a large percentage to oxidize acetate, lactate, and amino acids. Since the sediment concentration of lactate and free amino acids is very low, a model is proposed in which sulfate-reducing bacteria oxidize available higher molecular weight intermediates (i.e. lactate and amino acids) and simultaneously oxidize the more energy deficient substrates (i.e. H_2, acetate, and in some cases propionate) despite the low sediment sulfate concentrations. If the sulfate-reducing bacteria that were isolated from Wintergreen Lake sediments are representative of freshwater sediment sulfate reducers then individual organisms would be responsible for a multitude of oxidative processes; a novel concept concerning sediment sulfate reduction. Implicit in this scheme is the complete oxidation of the higher molecular weight intermediates to CO_2 by sulfate reduction. The exact contribution of the higher

Table 1. Free energy released by electron donor/sulfate reduction couples demonstrated in Wintergreen Lake profundal sediments.

Electron Donor	Product	$\Delta G^{o'}$	$\Delta G'$
2 Alanine	2 acetate + NH_4^+	-32.7[a]	-38.3[b]
2 Lactate	2 acetate	-38.3	-37.0
4/3 Propionate	4/3 acetate	-12.0	-12.8
4 H_2	4 H_2O	-36.3	-12.6
Acetate	CO_2	-11.3	-10.4

[a]kcal/mole $SO_4^=$ reduced (6).

[b]kcal/mole $SO_4^=$ reduced. Based upon Wintergreen Lake in situ concentrations of: sulfate, 50μM (Chapter II); sulfide, 200μM (Chapter II); acetate 130μM (2): propionate, 10μM (2); dissolved inorganic carbon, 12.5 mM (1); NH_3, 2mM (4); H_2, 3.7 x 10^{-5} atm. (5); and the presupposed concentrations of 1μM for lactate and alanine.

molecular weight electron donors to total sulfate reduction, however, remains to be determined when more sensitive techniques have been developed to measure the concentrations and turnover times of these intermediates.

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