METHANOGENESIS IN MARINE SEDIMENTS OF THE MISSISSIPPI RIVER DELTA

A Dissertation

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ABSTRACT

The highest most probable numbers (MPNs) of methanogens (1.18 X 10^6 cells per 100 g sediment, dry weight) and sulfate-reducers (0.31 X 10^6 cells per 100 g sediment, dry weight) in unstable, gas-rich, Mississippi River delta sediments occurred together in samples taken 0.2 m below the sediment-water interface. The presence of sulfate-reducers was directly related to dissolved sulfate in the interstitial water. Dissolved methane in the sediments was inversely related to the presence of sulfate-reducing bacteria. Methanogens and sulfate-reducers were recovered from maximum depths of 29.3 and 75.0 m, respectively. Two horizons of dissolved sulfate, with peaks of sulfate-reducer MPNs, indicated the presence of two sulfate-reducing zones in Site 1 sediments at depths of 0-2, and 10-12 m below the sediment-water interface. The extensive depth distributions of methanogens and sulfate-reducers, plus the occurrence of two sulfate-reducing zones, were the result of sediment mixing during undersea landslides that frequently occurred in the delta. *In vitro* methanogenesis was stimulated to a high degree by the addition of methylamine, dimethylamine, trimethylamine, methanol, and CO_2 plus H_2 (48.5-64.7 mol% conversion), and to a lesser degree by methanethiol, methylhydrazine, choline, and pectin. Although $^{14}CH_4$ was produced from ^{14}C-2-acetate, the addition of unlabeled acetate had no effect on *in vitro* methanogenesis. Methanogenesis was sensitive to 2-bromoethanesulfonate, chloroform, nitrate, air, and formaldehyde, but not to added β-fluoroacetate, sulfide, or sulfate. The optimum temperature for methanogenesis in enrichments and in unadulterated sediments

was 35-40° C and 45° C, respectively. *In vitro* methanogenesis in 0.2 m-deep sediments at 20° C (the *in situ* temperature) was 0.24 nmol CH_4/g sediment, dry weight/day. Actively methanogenic broth enrichments containing methanol and methylamine were dominated by irregularly coccoid cells that closely resembled *Methanococcus mazei* in morphology. Seventy-five strains of desulfovibrios closely resembling *Desulfovibrio salexigens*, and five strains of *Desulfotomaculum* sp. were isolated from 0.2 m-deep delta sediments. The *Desulfotomaculum* isolates did not closely resemble any described species and may constitute a new taxon. The production of methane from choline, in addition to the isolation of desulfovibrios capable of producing trimethylamine from choline, implicated trimethylamine as a possible methane precursor in the delta sediments.

INTRODUCTION

The final processes of carbon mineralization in anaerobic sediments are chiefly governed by two groups of strictly anaerobic microorganisms: sulfate-reducers and methanogens. The biogenesis of methane gas is the terminal reaction of carbon mineralization and occurs to some degree in virtually all anoxic sediments, both freshwater and marine, as well as in a variety of other anaerobic environments such as the gastrointestinal tract of mammals, sewage sludge and waste digestors, wetwood rot of certain trees, and in the decomposing algal-bacterial mats of thermal springs (80). The process in which methane is formed as a metabolic waste product is dependent upon strict anaerobic conditions, and only occurs if the redox potential of the surrounding medium is poised at or below -150 to -200 mV. Although the actual formation of methane is accomplished by a unique and morphologically diverse group of chemoautotrophic and chemoheterotrophic bacteria, the overall process of the degradation of organic material to methane involves a wide variety of bacteria. The methanogens are capable only of converting a limited number of small molecular weight compounds (CO_2, formate, acetate, methanol, N-methylamines, methanethiol, and dimethylsulfide) to methane, and are often dependent upon heterotrophic anaerobes and facultative anaerobes for the degradation of large molecular weight biopolymers, such as cellulose, which contribute to the organic nutrient load in natural environments.

Much of our knowledge about microbial methanogenesis has resulted from the studies of the microbiology of the rumen, sludge

digestion, and nutrient cycling in freshwater (especially Lake Mendota) sediments. Very little is known about methanogenesis in a marine sediment. The bulk of our knowledge concerning marine methane production and distribution has come from oceanographic and sediment chemistry studies of a few marine environments. Only recently has some attention been given to the study of methanogens and their involvement in the diagenetic processes that are characteristic of marine sediments.

The purpose of the present study was basically three-fold. First, the distribution of methanogens and sulfate-reducers in a series of core samples from Mississippi River delta sediments was compared to certain physical and chemical characteristics of the sediments. Second, studies on the conversion of known methane precursors to methane were undertaken in order to predict possible *in situ* substrate utilization. Changes in pH and redox potential, as well as the effects of certain inhibitory compounds on methanogenesis, were measured in sediment microcosm experiments. Third, attempts were made to isolate and partially characterize methanogens and sulfate-reducers from the Mississippi River delta sediments in order to better understand the physiology of the strict anaerobes that dominate anaerobic carbon cycling in this environment.

LITERATURE REVIEW

Distribution of Methane in Anaerobic Sediments

The presence of methane gas in anaerobic sediments has been known for many years. Hutchinson (26) reported that as early as 1830

Dalton found that methane, in addition to N_2 and CO_2, was a major constituent of gases evolved in an English lake. The methane was responsible for the buoyancy of "floating islands" in the lake. Koyama (33) utilized labeled substrates to follow methanogenesis in lake sediments and paddy soils, and found that methane was derived mostly from acetate and CO_2 in the sediment. He also studied the effects of high pressure and elevated salt concentrations (to simulate a marine environment) on methanogenesis in a freshwater sediment. These experiments showed that high hydrostatic pressure (450 atm) had no measurable effect on methanogenesis. However, high concentrations of sea salt severely inhibited methanogenesis. Koyama (33) also attempted to estimate the total worldwide production of methane from paddy soils, lakes, forest soils, and other sources, but neglected to include marine sources (probably owing to his experiments on methanogenesis inhibition by sea salts) and probably underestimated the total methane budget.

Bell (6) studied the evolution of various gases from closed flooded soil systems during the decomposition of glucose, peptone, and cellulose. He found that production of gases from these added substrates followed a predictable pattern based upon the redox potential (E_h) of the flooded soil. Soon after flooding, the E_h of the system stabilized at approximately +200 mV, the production of CO_2 and H_2 was detected, and nitrate was rapidly depleted. After further incubation, the E_h dropped and stabilized at approximately -250 mV. At this lower E_h, methane was the predominant gaseous end product. Chen, *et al.* (17) reported similar results after adding

$^{15}NO_3^-$ to Lake Mendota sediments. They found that nitrate had only a transitory existence in anoxic lake sediments, and that most of the gas evolved *in situ* was methane.

Macgregor and Keeney (36) studied *in vitro* methanogenesis in lake sediments and found that *de novo* methanogenesis was greater in sediments from "hardwater" lakes than from "softwater" lakes. They speculated that this was due to the presence of higher levels of $CaCO_3$ in the hardwater lakes. They also noted that the addition of nitrate and sulfate to sediments inhibited methanogenesis. They suggested that these additions would raise the E_h of the sediment to a more positive value than is tolerated by methanogens, but this response could also be due to a diversion of the electron flow to other electron acceptors that are more thermodynamically favorable than CO_2.

The studies discussed above serve to indicate that methanogenesis occurs in a variety of anaerobic sediment systems, and is the last oxidation/reduction reaction to occur, following the reductions of O_2, nitrate, and sulfate. They also showed the possible involvement of CO_2 and acetate as methane precursors. In addition to these studies on freshwater methanogenesis, other workers investigated the distribution of methane and other gases in marine sediments and water columns. The following studies are important in that they are indicative of the wide distribution of methanogenic activity in marine systems.

Lamontagne, *et al*. (34) measured the concentration of methane in the water columns of several marine habitats worldwide. They found that, in general, dissolved methane in tropical open ocean areas was greater in surface waters than at great depth, but was four orders

of magnitude greater in deep, naturally anoxic regions such as the Cariaco Trench (off the coast of Venezuela), the Black Sea, and Lake Nitinat (an anoxic fjord in British Columbia, Canada). The methane levels were also high in certain shallow, nearshore and bay environments (three orders of magnitude greater than open water). These levels were highly dependent upon temperature, local pollution, and tidal mixing, and were therefore, highly variable. Open ocean water was slightly supersaturated with methane, while estuarine systems were highly supersaturated.

The survey by Lamontagne, *et al.* (34) of dissolved methane in various marine systems did not address the question of how or where the methane was produced. With the exception of extremely deep and anoxic waters (such as the Cariaco Trench), most ocean waters remain oxidized throughout the water column, probably due to mixing and the diffusion of O_2, and hence, are not likely to be the actual source of methane production. Several groups have focussed their attention on the occurrence of dissolved methane in marine sediments, and are reviewed below.

Reeburgh (53) measured summer and winter depth distributions of methane and other gases in Chesapeake Bay sediments. Methane was not detected in the overlying water, or in the upper 10-12 cm of sediment core samples, but reached levels of up to 150 ml of methane per liter of interstitial water at a sediment depth of 60 cm. Based upon the solubility of methane in seawater, Reeburgh (53) believed that the concentration of methane deep in the sediment was a function of the rate of ebullition of methane from the sediment. He attributed

the low level of methane in the upper 25 cm of sediment either to oxidation, or to a low rate of production.

High levels of dissolved methane have been found in the organic-rich marine sediments of Long Island Sound by Martens and Berner (39). They compared dissolved methane and sulfate in sediment cores taken from water depths of 1.5-4.0 m, and found that high methane concentrations did not occur unless the level of sulfate approached zero. They showed that *de novo* methanogenesis in a large sealed jar containing Long Island Sound sediment, occurred only after a total depletion of added sulfate had occurred. A clear line of demarcation was shown in the Long Island Sound sediments, with sulfate-rich and low-methane sediment occupying the upper strata, and methane-rich, sulfate-depleted regions below. Based on these observations, Martens and Berner (39) reasoned that methanogenesis and sulfate-reduction are mutually exclusive processes in a marine environment. Subsequently, Martens and Berner (40) again examined sulfate and methane concentrations with depth in core samples from Long Island Sound. They postulated that the low levels of dissolved methane in the upper sulfate-rich strata were attributable to both the diffusion of methane upward from the lower strata, and to the anaerobic oxidation of methane by sulfate-reducing bacteria.

To measure the *in situ* rate of methanogenesis in a marine sediment, Oremland (43) placed cylindrical, Plexiglas incubation chambers into a shallow-water tropical marine sediment in the Florida Keys. The chambers that blocked sunlight (hence, stopped photosynthesis and O_2 evolution inside the chamber) showed the greatest methane production. While the addition of H_2 plus CO_2 to laboratory sediment

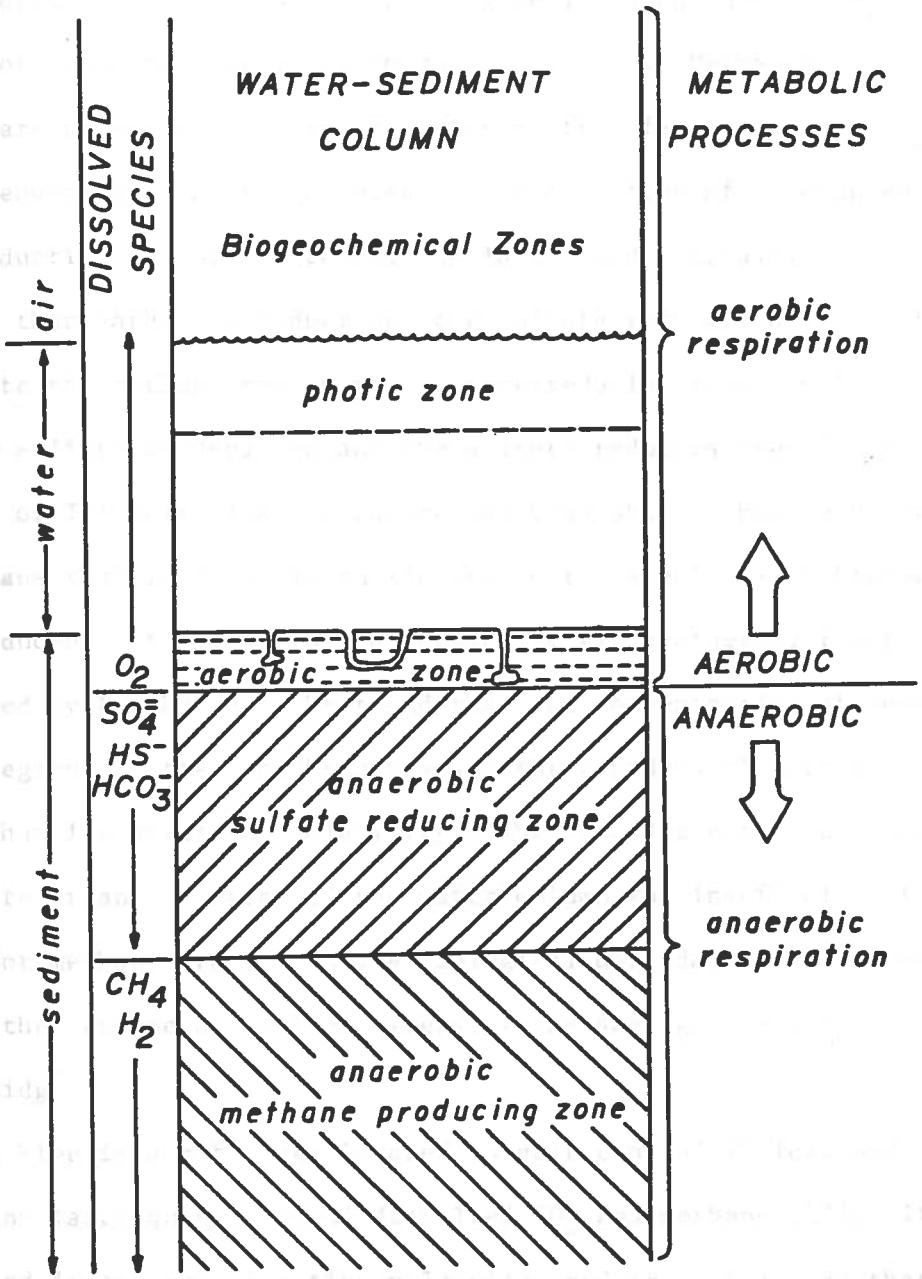
incubations stimulated methanogenesis above endogenous levels, the addition of acetate and formate did not. Thus, methane production in this tropical sediment (i) was limited by photosynthetic O_2 production in the uppermost layer of sediment; (ii) was apparently not limited by the concentration of either acetate or formate in the sediment; or (iii) proceeded via a different pathway (i.e., by the oxidation of H_2 and reduction of CO_2). A high rate of methanogenesis occurred in the upper 5 cm of the "dark" chamber. Oremland (43) reconciled this observation with previous reports of dissolved sulfate inhibition of methanogenesis in the upper strata by suggesting that sulfate-reduction was rapid in this tropical sediment, becoming quickly depleted within the chamber.

The works of Martens and Berner (39, 40), Reeburgh (53), and Oremland (43) show that the two most important anaerobic respiring populations of bacteria, sulfate-reducers and methanogens, appear to exist in stratified layers in stable, anoxic marine sediments. These assumptions are based not on actual cell counts, but are deduced from interstitial water chemistry. Invariably, the sediments nearest the sediment-water interface contained high concentrations of dissolved sulfate (approximately 10 mM) and low levels of dissolved methane (approximately 0.1 mM). Below a depth of about 20-25 cm, depending upon the source of the sediment, the organic load, pollution, and tidal mixing, sulfate was depleted (below the limits of detection) and dissolved methane levels were much higher, approaching 150 ml per liter of interstitial water. This has been interpreted to mean sulfate-reduction and methanogenesis are mutually exclusive,

but this explanation may not be complete. Claypool and Kaplan (18) point out that there is an ecological succession in organic-rich marine sediments. Three distinct biogeochemical environments, each possessing a dominant form of respiratory metabolism, are seen. The three zones are: (i) the upper, aerobic zone, where O_2 is the major terminal electron acceptor (TEA); (ii) the anaerobic sulfate-reducing zone, where sulfate is the major TEA; and (iii) the anaerobic methane-producing (or carbonate-reducing) zone, where CO_2 or HCO_3^- is the major TEA. Each zone is characterized by a dominant respiratory metabolite, and each is a successively less efficient mode of respiration. In each zone, the dominant microbial population exploits its environment and depletes its favored TEA, but at the same time creates a new environment that favors the growth of the dominant microbial population beneath it. The transition with depth from aerobic to sulfate-reducing to methane-producing sediment strata are "geochemical consequences of species-induced changes, i.e., the depletion and exhaustion of the required respiratory metabolite and the generation of new products" (18).

Claypool and Kaplan (18) provide an idealized cross-section of an organic-rich, reducing marine sediment which is shown in Figure 1, in adapted form. Claypool and Kaplan (18) also addressed the question of why low levels of methane are found in the sulfate-reducing zone. They suggested that H_2S produced in the sulfate-reducing zone may be toxic to methanogens, but this explanation is not consistent with the fact that the levels of free sulfide are actually quite low in marine sediments, and that methanogens are obligate

Figure 1. Idealized cross-section of an organic-rich, reducing, marine sediment, adapted from Claypool and Kaplan (18). This illustration depicts the various metabolic processes that occur in marine sediments, and distinguishes the zones dominated by aerobic respiration from those dominated by anaerobic respiration.


WATER-SEDIMENT COLUMN
METABOLIC PROCESSES
Biogeochemical Zones
DISSOLVED SPECIES
air
water
sediment
aerobic respiration
photic zone
aerobic zone
AEROBIC
ANAEROBIC
O_2
$SO_4^=$
HS^-
HCO_3^-
anaerobic sulfate reducing zone
anaerobic respiration
CH_4
H_2
anaerobic methane producing zone

sulfide requirers. Claypool and Kaplan (18) offered an alternative explanation that seems more plausible. Both methanogens and sulfate-reducers compete for H_2 in the sediment. Some sulfate-reducers possess a hydrogenase electron transport system, and can utilize H_2 as a source of reducing power, but do not require it. Methanogens, however, are dependent upon interspecies H_2 transfer and require H_2 for CO_2 reduction. Therefore, because the oxidation of H_2 coupled to the reduction of sulfate to sulfide is thermodynamically more favorable than carbonate reduction, the sulfate-reducers are able to out-compete the methanogens in the sulfate-reducing zone. Only after the sulfate is depleted and the sulfate-reducers are limited by a lack of TEA molecules are the methanogens able to become dominant.

Methane that is found in anoxic sediments is not always biologically produced. It can be formed chemically via geothermal reactions, as reported by Brooks (9). He found that the concentration of methane in some regions of the Caribbean Sea was three orders of magnitude greater than its predicted solubility. The data suggested that the particulate organic content of the water column was insufficient to account for methane production via biological degradative processes, and that the methane arose from extensive gas seepage along the Jamaica Ridge.

Lake Kivu is a rift lake located in east-central Africa, and it contains large quantities of dissolved CO_2 and methane (20). It is situated in an area of active volcanism, and it is believed that part of the evolved methane in the water column is due to volcanic venting (20). Deuser, *et al.* (20) have shown, however, that most of

the evolved methane is formed from the reduction of abiogenic CO_2 by methanogenic bacteria. Both the H_2 and CO_2 utilized in this oxidation/reduction system are of volcanic origin.

Sediment Instability and Methane Distribution in the Mississippi River Delta

A great deal of effort has been devoted to studying stable, organic-rich sediments, i.e., those that remain stratified after deposition and are mixed only minimally by such physical processes as gas ebullition, tidal influences, or through the movement of macrofauna and meiofauna within the sediment (bioturbation). A large group of researchers has recently begun an extensive study of the geology, hydrology, and geomorphology of the Mississippi River delta. Prior and Coleman (51) used side-scan sonar imaging, combined with seismic and fathometer data, to reveal the presence of various types of underwater landslides in the delta. They surveyed sediment movement on the upper delta front on slopes of 0.2-1.5 degrees in water depths ranging from 5-100 m. Features such as collapse depressions, mud slides, slumps, mud-flow gullies, and overlapping mudflow lobes indicated sediment movement rates of up to 1.5 km per year. The modern Mississippi River delta is the youngest (formed within the last 600 years) of the delta lobes and is composed of three main distributary channels (51). The deltaic sedimentation rates vary, being greatest (over 1 meter/year) near the mouths of the distributaries, and consists largely of water and coarse-grained particles. The interdistributary bays (including the study area of the present

work) contain fine-grained clay sediments, large amounts of organic material, and exhibit slower rates of deposition than at the distributary mouths. Some regions in the interdistributary bays possess sediments composed of up to 15% methane (by volume). Areas that are particularly high in methane can be mapped seismically due to their poor reflection of 3.5 kHz acoustic signals (51). Collapse depressions are particularly common in the interdistributary bays, and probably result from volumetric changes in the sediment after massive venting of methane gas and pore water, followed by an immediate surface depression of 1-3 m. The resulting volume of instability in the sea floor reaches depths of 5-15 m. Other sediment failures found to be common in the interdistributary bays are bottleneck slides and elongate retrogressive slides (51). Bottleneck slides resemble collapse depressions except that the downslope end is left open, allowing discharge of the debris over the surrounding intact slope. The bottleneck slides range in length from 150-600 m and are caused by methane gas-induced sediment instability. The high methane gas and pore water pressure causes a sudden failure of the sediment, leading to a spreading failure which causes the low strength sediment to take on the consistency of a liquid. The sediment then flows out of the cavity. The retrogressive slippage continues until a stable scarp is attained (51). Elongate retrogressive slides are usually long, sinuous channels that typically form on 0.1 degree slopes, and measure 500-1500 m long. They are formed in a manner similar to bottleneck slides, but also involve the development of chutes that transport large quantities of debris downslope.

Recent advances in digitally acquired, undistorted side-scan sonar images of the delta slope have enabled Prior, *et al.* (52) to accurately map out sediment movements, yielding the first mosaics of the delta. Previous side-scan sonar work had provided useful information about bottom morphology and feature distribution, but the images suffered from two inherent scale distortions: (i) lateral distortion; and (ii) compressional distortion caused by variation in ship speed. New images with the improved system will allow re-mapping of the delta slopes with accurate analysis of temporal changes in the sediments.

In addition to the accurate mapping and classification of underwater sediments in the delta by Prior and his co-workers (51, 52), Whelan (69) studied the interstitial water chemistry of south Louisiana coastal marsh sediments. He found higher methane concentrations in northern, low salinity sediments than in those of the open marsh in the south. A significant fraction of the methane in the sediments exists as gas bubbles trapped within the sediment. In general, where sulfate-reducing activity was greatest, or where the concentration of dissolved sulfate was lowest, the level of dissolved methane was significantly higher. Due to the relatively shallow water column over these sediments (low hydrostatic pressure), and the high degree of sediment unconsolidation, methane escaped freely from these sediments (69).

Examination of the geochemistry of delta sediments by Whelan, *et al.* (70) showed that high concentrations of methane corresponded to zones of low sediment shear strength, and were found to exist in zones of depleted sulfate. They recovered core samples from as deep

as 70 m below the sediment-water interface and were able to demonstrate classical anaerobic geochemical gradients in *undisturbed* (unmixed) sediments. However, they also observed core samples that showed the coexistence of relatively high methane and sulfate levels. This suggested that the processes regulating sulfate and carbonate reduction in these sediments were complicated by convective mixing of bottom seawater and sedimentary material, and was associated with sediment movement (70). An influx of sulfate (from the overlying sulfate-rich seawater) most likely caused methanogenesis to cease temporarily until the sulfate was diminished to 0.13-0.17 $^{o}/oo$. The pore water chemistry from the delta sediments indicated that the aerobic zone was negligible in size, and that sulfate was rapidly reduced with depth. The methane-producing zone, beneath, was extensive. A model was suggested to describe how the physical and geochemical properties of the delta sediment related to sediment movement, as follows. Rapid sedimentation of fine-grained, organic-rich material followed the classical succession of anaerobic respiration gradients: sulfate-reduction was dominant in the upper zone where no methane was produced. Carbonate reduction occurred below this zone, with the concentration of methane increasing to the point of bubble formation. The resultant drop in sediment shear strength caused the formation of a failure zone deep in the sediments. With bottom pressure fluctuations due to wave-induced stress during storms, methane bubbles migrated upward, thus conveying lowered shear strength in the upper layers of sediment. This was followed by the eventual failure of the sediment and movement downslope (70).

The studies reviewed thus far generally agreed that the diminished levels of methane observed in the sulfate-reducing zone was a result of the thermodynamic favorability of the electron flow from H_2 to sulfate rather than to CO_2. A growing body of new evidence began to accumulate that postulated that the apparent mutual exclusion of methanogenesis and sulfate-reduction was the result of anaerobic oxidation of methane to CO_2 by sulfate-reducing bacteria. The first indication of this possibility was reported by Davis and Yarbrough (19), who showed that the sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, was capable of a slow oxidation of methane in the presence of lactate as the major carbon source. Barnes and Goldberg (5) measured methane in core samples taken from the Santa Barbara Basin in 590 m of water and suggested that methane diffused upward into the sulfate-reducing zone and was metabolized by sulfate-reducers (most probably, *Desulfovibrio*) in the sulfate-reducing zone. Their argument is supported by the thermodynamically favorable reaction for the oxidation of methane, shown below:

$$CH_4 + SO_4^{2-} + 2H^+ \rightarrow H_2S + CO_2 + 2H_2O$$

$$\text{Standard Free Energy, } \Delta G^o = -22.8 \text{ kcal/mol } SO_4^{2-}$$

They also indicated that the *actual* free energy, taking into account the *in situ* concentrations of the various dissolved species, was -3 kcal/mol SO_4^{2-}, and is still energetically feasible. Barnes and Goldberg (5) concluded that the primary sink for methane generated in anoxic marine sediments is sulfate-reduction, and not aerobic oxidation by methylotrophs.

In a similar study, Reeburgh (54) measured methane and total carbonates in the water column and sediments of the Cariaco Trench. The water at a depth of 300 m contained sulfide and was anoxic, therefore, no aerobic zone existed in the upper sediment layer. The source of dissolved methane in the water column was the sediments, and the oxidation of methane in the water column could not account for the total methane turnover. Reeburgh (54) calculated the methane flux to the sulfate-reducing, methane-consuming zone in the sediment to be in the range of 5-15 μmol cm^{-2} yr^{-1}, and corresponded to methane consumption rates of 0.5-1.5 mmol l^{-1} yr^{-1}, or about 10^6 times greater than the rate of oxidation in the water column. Sulfate-reducers were probably responsible for the co-metabolism of methane while utilizing lactate as the major carbon and electron source. In a follow-up study to that of Reeburgh (54), Reeburgh and Heggie (55) compiled the data from numerous studies that reported the methane distributions in freshwater and marine environments. They offered schematic summaries of depth profiles of dissolved methane in these environments and suggested that the absence of a gradient-free, low-methane, surface zone in freshwater systems (which is typical of marine sediments) was due to a 10^3-fold greater sulfate concentration in marine sediments, hence, a much more active sulfate-reducing zone and a greater propensity for consuming methane.

Syntrophic Interactions Between Sulfate-Reducers and Methanogens

In a series of investigations by Cappenberg (11, 12, 13) and

Cappenberg and Prins (15), the first real evidence of a syntrophic relationship between sulfate-reducers and methanogens was revealed. Cappenberg (11) first studied the stratified populations of sulfate-reducers and methanogens in the freshwater sediments of Lake Vechten, the Netherlands. Most probable number (MPN) counts of both types of bacteria showed that the sulfate-reducers were most abundant at depths of 0-2 cm below the sediment-water interface, while the maximum counts of methanogens occurred 3-6 cm below the surface of the sediment. The concentration of sulfide was approximately 1000 times greater in the sulfate-reducing zone, which suggested that the lower MPNs of methanogens in the sulfate-reducing zone may have been due to the sensitivity of methanogens to higher levels of H_2S. In contrast to marine systems, the concentration of sulfate in the Lake Vechten sediment was the limiting factor for the growth of sulfate-reducers. Carbonate- and formate-reducing methanogens were most abundant at a depth of 3 cm, while acetate- and methanol-fermenting methanogens were found deeper, at 5 cm. Additional substrate-utilization studies showed that methanogenesis was most stimulated in the lake sediment by the addition of acetate and lactate, and to a lesser degree by formate. Added sulfate severely diminished methanogenesis.

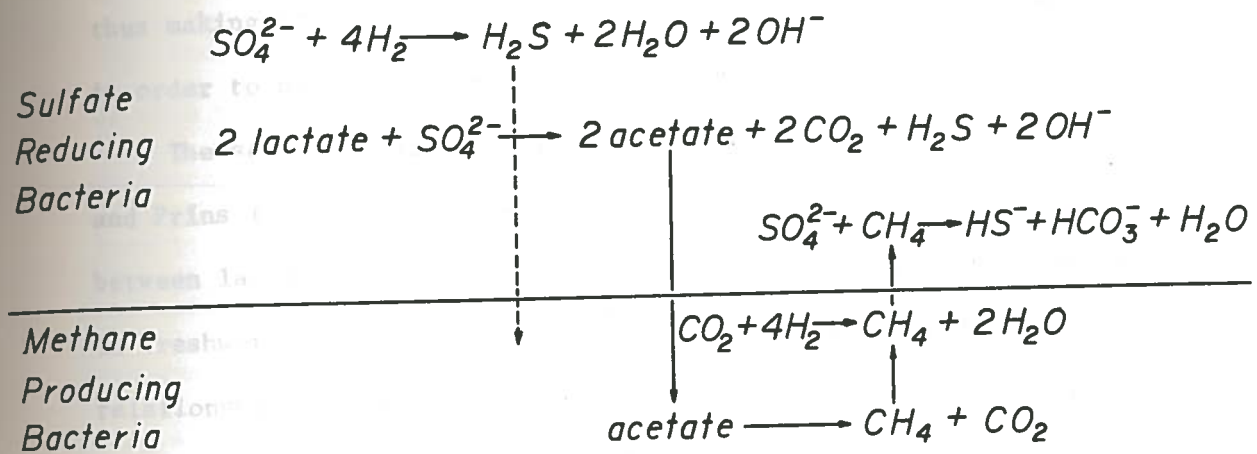
Cappenberg (12) pursued further investigations of Lake Vechten sediments with the use of inhibition studies employing β-fluoroacetate, fluorolactate, and CCl_4. All three compounds are analogues of substrates utilized either by methanogens or sulfate-reducers. Inhibition of methanogenesis by the addition of CCl_4 caused an accumulation of

acetate in the sediment. The addition of β-fluoroacetate reduced methanogenesis by 75%, indicating that acetate was the major methane precursor in the sediment. When fluorolactate was added, sulfate-reduction was totally inhibited and no gas accumulated, indicating that lactate was the major energy source for sulfate-reducers, and that H_2 was probably not a major electron donor. It also indicated that anaerobic methane oxidation did not play an important role in sulfate-reduction, although this may be important in marine systems (12).

The results of Cappenberg's studies (11, 12) gave a clear indication of the possibility of a syntrophic relationship between sulfate-reducers and methanogens in Lake Vechten sediments. Sulfate is reduced to sulfide by the oxidation of lactate, yielding acetate as a metabolic waste product. Methanogens flourish in the highly reduced sediment provided by the H_2S, and utilize acetate as a major methane precursor. A scheme was devised to illustrate some energy-yielding reactions of sulfate-reducers and methanogens, based on the ecological and inhibition studies (11, 12) and is shown in Figure 2 in adapted form.

The logical extension of the inhibition experiments of Cappenberg (12) would be to use radiolabeled compounds to unequivocally demonstrate syntrophy between sulfate-reducers and methanogens. By using ^{14}C-U-acetate and ^{14}C-U-lactate, Cappenberg and Prins (15) showed that approximately 70% of the methane in Lake Vechten sediments was derived from acetate (data which were consistent with earlier observations), and that a syntrophic relationship was evident between the two groups of bacteria. $^{14}CH_4$ was formed from ^{14}C-U-lactate,

Figure 2. Energy-yielding reactions and syntrophic interrelations between sulfate-reducing bacteria and methanogenic bacteria in anoxic, freshwater sediments, from Cappenberg (12). This figure illustrates the stratified populations of sulfate-reducers and methanogens seen in Lake Vechten, and the various reactions that occur as a result of the dominant types of anaerobic respiration that are characteristic of freshwater sediments.


$SO_4^{2-} + 4H_2 \rightarrow H_2S + 2H_2O + 2OH^-$
Sulfate Reducing Bacteria
2 lactate + SO_4^{2-} → 2 acetate + $2CO_2$ + H_2S + $2OH^-$
$SO_4^{2-} + CH_4 \rightarrow HS^- + HCO_3^- + H_2O$
Methane Producing Bacteria
$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$
acetate → CH_4 + CO_2

a substrate which cannot be utilized by pure cultures of methanogens.

Further proof of the interrelationship between sulfate-reducers and methanogens was shown by Cappenberg (14) with the use of lactate-limited, mixed continuous culture of *Desulfovibrio desulfuricans* and *Methanobacterium* sp. isolated from Lake Vechten. The decrease in the acetate concentration in the second growth vessel corresponded to a stoichiometric increase in methane production. The methanogen was extremely sensitive to sulfide produced by the sulfate-reducers, thus making it necessary to keep the lactate concentration very low in order to prevent washing out of the methanogen.

The series of studies by Cappenberg (11, 12, 13) and Cappenberg and Prins (15) leave little doubt that a syntrophic relationship exists between lactate-oxidizing sulfate-reducers and aceticlastic methanogens in freshwater sediments. There is, however, little evidence of this relationship in marine sediments. Warford, *et al.* (66) give evidence that the major methane precursor in the marine sediments of the Santa Barbara Basin is CO_2, and that methanogenesis and sulfate-reduction occur substantially in the same regions of the sediments. *In vitro* incubation of the marine sediments showed methanogenesis in all samples down to a depth of 340 cm, and that methanogenesis decreased with depth probably as a result of diminished levels of methane precursors in the deeper sediments. The use of ^{14}C-2-acetate and $NaH^{14}CO_3$ radiolabels showed that 70-85% of the $^{14}CH_4$ generated in the marine sediments was derived from $^{14}CO_2$, and only 2-11% was derived from ^{14}C-2-acetate. Addition of ^{14}C-U-lactate to the sediments initially caused a large release of $^{14}CO_2$ due to the conversion

of lactate to acetate and CO_2, but little of the acetate was converted to methane. During prolonged incubations, $^{14}CH_4$ increased at the expense of $^{14}CO_2$. Since bicarbonate was the preferred methane precursor in these sediments, as sulfate was depleted, the production of methane from CO_2 outstripped CO_2-production by sulfate-reducers that oxidized lactate. Warford, *et al.* (66) concluded that marine methanogenic sediments may be very different from those of freshwater systems. Although the data indicated that acetate was converted to CO_2, and that the major methane precursor was CO_2, the possibility of some type of syntrophic relationship between sulfate-reducers and methanogens in the Santa Barbara Basin was not ruled out. Their results supported the hypothesis of Claypool and Kaplan (18), whose isotopic data ($\delta^{13}C$) indicated that methane was derived chiefly from carbonates in marine environments.

Kosiur and Warford (32) investigated the question of why low concentrations of methane were found in the near-surface, sulfate-reducing zone of the Santa Barbara Basin. They found that *in vitro* methanogenesis in sediment samples was not inhibited by the initial interstitial water sulfate concentration of 28.3 mM. Active methanogenesis and anaerobic methane oxidation both occurred in the sulfate-reducing zone. The rate of methanogenesis decreased with depth within the sulfate-reducing zone, probably due to diminished availability of methane precursors formed by sulfate-reducers.

Additional evidence of the occurrence of anaerobic methane oxidation in marine and freshwater environments was provided by Zehnder and Brock (78). Radiotracer studies on sediment samples from Izembek

Bay, Alaska (a marine sediment), and Lake Mendota, showed that methane production was followed by anaerobic oxidation. 2-bromoethanesulfonate (an analogue of coenzyme M, mercaptoethanesulfonate), a potent inhibitor of methanogenesis, strongly inhibited anaerobic methane oxidation, indicating that methanogens may be involved in the oxidation. The addition of sulfate inhibited methane oxidation, but the inhibition was thought to be a result of the reduction of sulfate to sulfide. Free sulfide slowed methanogenesis, but was easily reversed with the addition of ferrous iron which precipitated and detoxified the sulfide. Fallon, *et al.* (22) showed that anaerobic methane oxidation during summer stratification of Lake Mendota amounted to 45% of the total methane production, and that 54% of the organic load in the lake was released as methane.

Marine Methanogens and Sulfate-Reducers

Although much effort has been devoted to the study of methanogens isolated from freshwater systems, very little is known about marine methanogens. *Methanococcus vannielii*, one of the first methanogens isolated from a marine environment, was studied by Stadtman and Barker (59) and Jones, *et al.* (27). The organism was isolated from tidal mud flats in San Francisco Bay, yet it is not an obligate halophile (27). The organism was routinely cultured in formate-containing media of low osmotic strength. In fact, the organism is not salt tolerant, and fails to grow in media containing 3% NaCl (27). Ultrastructural analysis of *Methanococcus vannielii* (27) showed that it lacked a peptidoglycan layer in the cell wall. This is consistent

with the extreme sensitivity of the organism to mechanical stress (27).

Two new marine methanogens were isolated by Romesser, *et al.* (56), *Methanogenium cariaci* from sediments in the Cariaco Trench, and *Methanogenium marisnigri* from the Black Sea. Both species are irregularly-shaped cocci, utilize CO_2 plus H_2 or formate (but not acetate or methanol), and require NaCl for growth. The cell walls of these isolates morphologically resemble that of *Methanococcus vannielii*, and also lack peptidoglycan. Both methanogens have an optimum growth temperature of 20-25^{o} C and require either acetate (*M. cariaci*) or trypticase (*M. marisnigri*) for growth. These two organisms are the first obligately halophilic methanogens isolated from a marine environment.

Jones and Paynter (28) studied methanogenesis in salt marsh and estuarine sediments of Sapelo Island, GA, and in South Carolina. In short *Spartina* marsh sediments, methanogens were most numerous at a sediment depth of 5-7 cm and decreased with depth. In tall *Spartina* marsh sediment, methanogens were most numerous at 0-2 cm. Methanogenesis was stimulated *in vitro* by the addition of CO_2 plus H_2, and formate. Jones and Paynter (29) have recently characterized a new marine methanogen isolated from salt marsh sediment. *Methanogenium paludismaritimae* produced methane from formate and $CO_2 + H_2$, and required Na^{+} and Mg^{++} for growth. Like other members of the genus, *M. paludismaritimae* lacked peptidoglycan in the cell wall. However, it differed markedly in the guanosine plus cytosine (G + C) content of its DNA (34 mol%), when compared to *M. cariaci* (52 mol%) and *M. marisnigri* (61 mol%).

Several strains of the sulfate-reducing bacterium *Desulfovibrio* are capable of growth in elevated concentrations of NaCl, but only one recognized species, *Dv. salexigens*, is an obligate halophile (50). There are no reports of any known obligately halophilic members of the spore-forming genus, *Desulfotomaculum*. Postgate and Campbell (50) have shown that *Dv. salexigens* is unable to tolerate low osmotic tensions typical of freshwater environments, and that it requires Cl^- for growth. The organism has been isolated from seawater, marine and estuarine sediments, and pickling brines. It is also easily recognized by its high resistance to the antibiotic, Hibitane, when compared to other species of the genus *Desulfovibrio*.

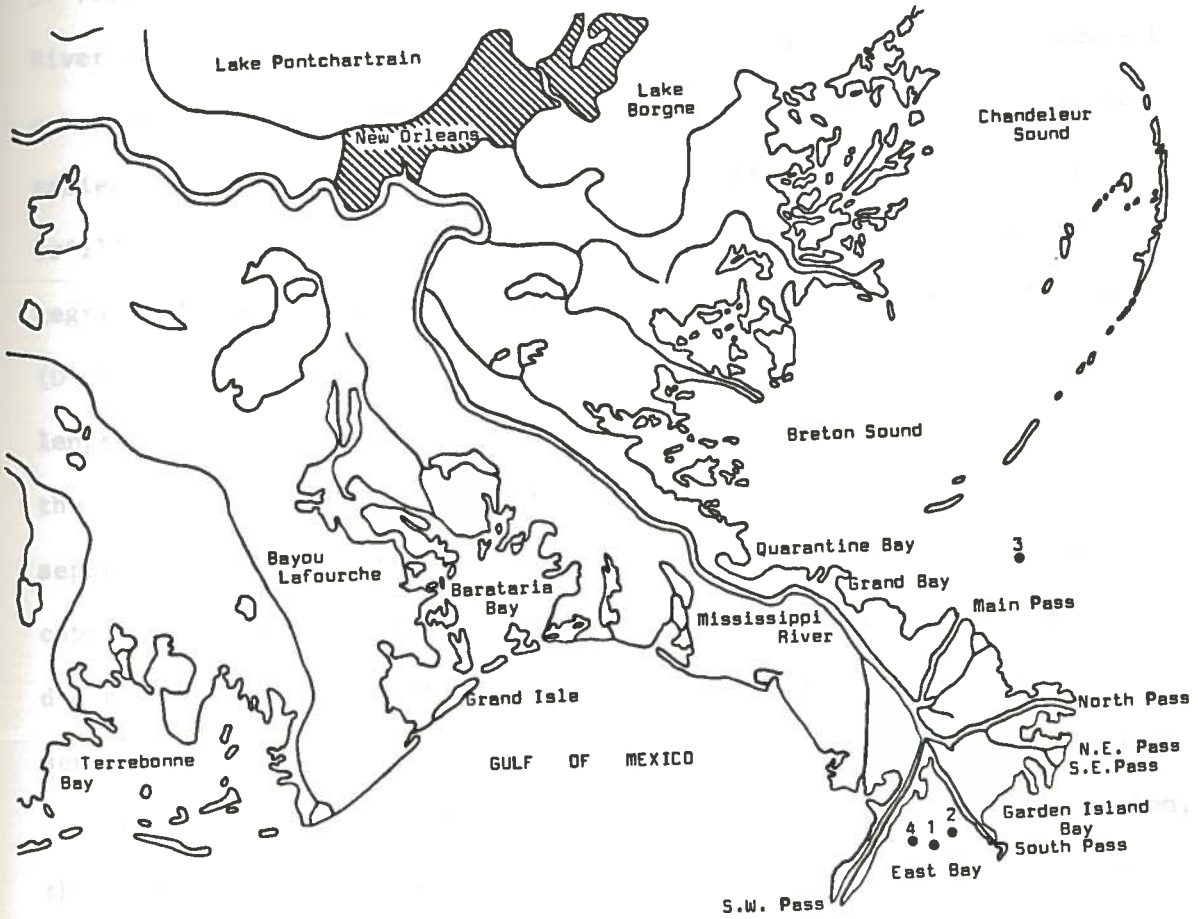
MATERIALS AND METHODS

Distribution of Methanogens and Sulfate-Reducers, and Interstitial Water Chemistry in Sediment Core Samples

Study Area. Core samples to a depth of 30.0 m were taken from Mississippi River delta sediments in the Gulf of Mexico near 28° 57' N latitude, 89° 151' W longitude. The samples were collected on 25-26 November 1978 at Site 1 near the river mouth in East Bay. East Bay is an interdistributary bay situated between South Pass and Southwest Pass (Figure 3). The water depth at the sampling site was 14 m. Previous studies employing high-resolution seismic profiles, side-scan sonar tracks, and hydrographic surveys have shown that underwater landslides have occurred near this sampling site (70).

Sampling. The sediment samples were collected from on board a shallow-water jack-up rig (mobile drilling platform) equipped with

Figure 3. Map of the Mississippi River delta showing the study area and sampling sites. Three sets of samples were collected in East Bay, an interdistributary bay situated between South Pass and Southwest Pass, about 135 km downriver from New Orleans. Site 1 was located approximately 15 km offshore. The sampling excursions were initiated from Venice, Louisiana, a drilling supply center located about 20 km upriver from the Main Pass distributary. The sampling sites in East Bay were reached via South Pass.


Lake Pontchartrain
New Orleans
Lake Borgne
Chandeleur Sound
Breton Sound
Quarantine Bay
3
Grand Bay
Main Pass
Bayou Lafourche
Barataria Bay
Mississippi River
North Pass
Grand Isle
N.E. Pass
S.E.Pass
Terrebonne Bay
GULF OF MEXICO
Garden Island Bay
4 1 2
South Pass
East Bay
S.W. Pass

wire-line sampling equipment. At Site 1, sixteen sediment cores were taken at approximately 1.5 m intervals with a 0.6 m-long, 5.6 cm (I. D.), stainless steel core barrel attached to the wire-line sampler (70). The wire-line sampler penetrated the sediment inside of a 6.4 cm (O. D.) drill pipe (70). Additional delta sediment samples that were used in this study were taken from cores reaching depths of 70.7 and 212.1 m at Sites 2 and 3, respectively, in the Mississippi River delta. These two cores were originally collected for geophysical analyses but were unopened and stored for approximately six months at ambient temperatures (25-30^{o} C). Between core sample collections, drilling "mud" was injected into the bore hole to maintain the integrity of the boring. The sediment samples were obtained in 5.5 cm (O. D.), air-tight plastic resin liners, pre-cut to 12.2 cm in length, that fit inside the core barrel. After sample collection, the sediment-containing liners were extruded from the core barrel, separated into 12.2 cm sections, and immediately sealed with rubber caps. The brief exposure of the ends of each core sample to air did not cause significant oxidation of the sediment due to the presence of FeS (8, 73, 82). All core samples were refrigerated (4^{o} C) for approximately 48 h prior to use in the laboratory. As a precaution, the ends of each core sample were cut off and discarded prior to the removal of sediment material for experiments. The discarded sediment material still retained the dark gray color of reduced sediment (oxidized sediment turns reddish-brown). Samples taken at Site 2 (70.7 m-deep core) were collected and stored in a manner similar to that of Site 1 samples. Samples taken at Site 3 (212.1 m-deep core) were collected as described above, but were wrapped in aluminum

foil and sealed in paraffin and cardboard cylinders. No special precautions were taken to ensure the anaerobic storage of these samples, and as a result, some visible oxidation of the outer 1-2 mm surface of each core sample was noted. Prior to the use of these core samples, the outer oxidized layer was removed, and only dark, reduced sediment was used for bacterial enrichments.

One hundred-ml aliquots of Site 1 sediment to be assayed for dissolved methane were removed from the liners immediately after collection, and were sealed in metal, 460 ml cans which contained 260 ml of He-purged, methane-free distilled water, and 1 ml of 1% benzalkonium chloride as a preservative (70). A second aliquot of each sample, to be used for analyses of sulfate, total dissolved inorganic carbon (ΣCO_2), dissolved organic carbon (DOC), salinity, and pH, was sealed in a tightly wrapped plastic bag for transport back to the laboratory (70). None of the interstitial water chemistry analyses required the use of anaerobic material, so no precautions were taken to exclude oxygen from these samples. However, to prevent bacteriological changes in the sediment material, the samples were refrigerated (4^{o} C) during transport.

Approximately 15 l of surface sediment was obtained with an Ekman dredge and stored on board at 4^{o} C in heavy duty plastic containers. After returning to the laboratory, the anoxic surface sediment was transferred to 10 l glass containers and stored under O_2-free N_2 at 4^{o} C before use.

In November, 1977, a 150 X 7 cm plastic drop core was used to collect the top 0.77 m of sediment at Site 4 in the East Bay area.

After returning to the laboratory, 12 subsamples at approximately 6 cm intervals were removed by drilling through the side of the plastic drop core and aseptically transferring portions of sediment to glass containers under N_2.

Bacterial Counts. Population densities of methanogenic and sulfate-reducing bacteria were estimated by the five-tube most probable number (MPN) technique (2). The medium used for the methanogen MPN counts was a modification of an enrichment medium for marine methanogens suggested by J. A. Romesser (personal communication). The carbonate-buffered methanogen MPN enrichment medium consisted of: 10% Trypticase Peptone (BBL, Cockeysville, MD), 10 ml; 10% Yeast Extract (Difco, Detroit, MI), 10 ml; 10% ammonium acetate, 5 ml; 10% sodium formate, 10 ml; trace minerals (80), 10 ml; vitamins (R. S. Wolfe, personal communication), 10 ml; 2-mercaptoethanesulfonic acid, ammonium salt (HS-CoM), 1 ml of a 1 mg/ml solution; 10% Na_2CO_3, 10 ml; 5% $NaHCO_3$, 40 ml; 0.1% resazurin (redox indicator), 1 ml; distilled water, 150 ml; artificial seawater (Seven Seas Marine Mix, Utility Chemical Co., Patterson, NJ, 40 g/l distilled water), 750 ml; and 1.25% cysteine·HCl-$Na_2S·9H_2O$ as a reducing agent (3), 32 ml. The headspace gas was a mixture of H_2 + CO_2 (80%-20%, vol/vol). The methanogen medium was prepared anaerobically using the Hungate technique (25). All gases used in media preparation were rendered free of O_2 by passage over reduced copper at 350° C. The medium was anaerobically pipetted into previously-flushed, 27 ml anaerobic culture tubes (Bellco Glass, Inc., Vineland, NJ) using a pipet bulb with a gassing line plugged into the expulsion side arm.

The tubes were sealed with gray, butyl rubber, slotted serum bottle septa and aluminum seals, and then autoclaved. After inoculation of the tubes as described below, the pressure of the gas phase was increased to 2 atm of H_2 + CO_2 (80%-20%, vol/vol) according to the method of Balch and Wolfe (3).

The medium used for the enumeration of sulfate-reducing bacteria was Bacto Sulfate API agar (Difco, Detroit, MI) supplemented with 20 g/l NaCl. This medium yielded the highest MPN counts of the various media tested (10, 49). The sulfate-reducers MPN medium was dispensed as 15 ml agar deeps into 115 X 16 mm test tubes, capped with slip-on caps, and autoclaved. Black, H_2S-producing colonies that formed within the agar deeps were considered as sulfate-reducers.

Weighed portions of wet sediments were ten-fold serially diluted anaerobically in sterile methanogen MPN medium using 0.5 ml glass disposable syringes (Glaspak, Becton-Dickinson) that were previously out-gassed with O_2-free N_2 (3). Sediment dilutions of 10^{-1} and 10^{-3} were anaerobically inoculated into the methanogen MPN medium and molten (45° C) sulfate-reducers MPN medium in compliance with the five-tube MPN procedure (2). All tubes were incubated in the dark at 30° C for three weeks. To determine sediment dry weights, weighed portions from each sample were dried at 75° C to constant weight.

Gas Chromatography. Methane production in MPN tubes, substrate utilization tubes, and other routine methane assays (see below) was measured by flame ionization gas chromatography using a Perkin-Elmer 3920B gas chromatograph. The column was a 1.83 m (6 ft) copper tube, 3.175 mm (1/8 in.) O. D., filled with 80-100 mesh Porapak QS.

A 0.5 ml gas-tight syringe (Pressure-Lok Series A-2, Precision Sampling, Baton Rouge, LA) was used to deliver 0.02-0.5 ml samples of the headspace gases to the gas chromatograph. Measurement of dissolved methane in delta sediments was accomplished by stripping the methane from the interstitial water with He (70) and injecting 0.05 ml into a gas chromatograph with a Valco gas sampling switching valve (Model V-6-HPa, Valco Instruments Co., Houston, TX). All methane assays in this study were quantitated by linear regression analyses.

Interstitial Water Chemistry. Dissolved sulfate was determined turbidimetrically by precipitation with barium chloride (2). Dissolved organic carbon (DOC) was determined by the combustion-infrared method (2). Dissolved inorganic carbon (ΣCO_2) was determined by acidifying an aliquot of interstitial water and measuring the evolved CO_2 by infrared spectroscopy with a CO_2 analyzer (2). The pH of the interstitial water was determined using a microelectrode. Salinity was measured as chloride ion by titration with silver nitrate (2).

In Vitro Utilization of Methane Precursors by Delta Sediments

Added Substrate Utilization. Ten-ml aliquots of a slurry of 0.2 m-deep sediment were dispensed anaerobically into 27 ml anaerobic culture tubes under N_2 such that each tube contained 2.8 g of sediment (dry weight). The tubes were sealed with black, butyl rubber, anaerobe stoppers (Bellco Glass, Inc., Vineland, NJ). Unless otherwise specified, the diluent (pH 7.0) used to make the sediment slurries contained, per 1 of distilled water, NaCl, 30 g; KCl, 0.7 g; $MgCl_2 \cdot 6H_2O$,

5.5 g; $MgSO_4 \cdot 7H_2O$, 6.9 g; NH_4Cl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; and K_2HPO_4, 0.3 g (J. A. Romesser, personal communication). The following potential methane precursors were added separately to triplicate sets of tubes in 100 µmol quantities: sodium acetate, sodium bicarbonate, sodium formate, dimethylsulfide, methanol, methylamine, dimethylamine, trimethylamine, methanethiol, methylhydrazine, and methionine. The following potentially stimulatory compounds were added separately in triplicate to other sets of tubes: sodium lactate, sodium propionate, sodium pyruvate, glucose, cellobiose, choline·Cl (all 100 µmol), cellulose (0.03 g/tube), pectin (0.014 g/tube), and polypectate (0.014 g/tube).

In addition to the N_2 atmosphere in all tubes (at 1 atm pressure), certain tubes containing sediment slurry received 2.25 ml of H_2 (100 µmol), or $H_2 + CO_2$ (80%-20%, vol/vol). Other sets of control tubes received N_2 alone. Sets of killed control tubes were either autoclaved or received 0.1 ml of 37% formaldehyde. All tubes were incubated at 30° C and shaken periodically with a vortex mixer to ensure that evolved methane was released into the headspace. Methanogenesis was monitored on a daily basis by injecting 0.02-0.5 ml samples of headspace gas into a gas chromatograph.

Methanogenesis from Endogenous Methane Precursors. To measure the production of methane from endogenous methane precursors, slurries of delta sediment (prepared as above without added substrates), and tubes containing portions of unadulterated (undiluted) sediments were incubated in serum bottles under N_2 at the *in situ* temperature of 20° C. Methanogenesis was monitored on a daily basis as described above.

Methanogenesis in the Presence of Inhibitors. To test the effects of specific inhibitors of methanogenesis, slurries of Site 1 sediment (prepared as above without added substrates) were supplemented with one of the following compounds. Chloroform (12) was added (at concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5%) to sediment samples taken from depths of 0.2, 3.1, 7.2, 17.8, and 30.0 m. β-fluoroacetate (12) was added (at concentrations of 0, 0.01, 0.02, 0.05, and 0.1%) to a second series of samples taken from the same depths. Other similar sets of sediment slurries were supplemented with: (i) 2.5 mM bromoethanesulfonate, BrES (an analogue of coenzyme M, mercaptoethanesulfonate) (77); and (ii) KNO_3, at concentrations of 5, 10, 50 and 100 mM. Tubes supplemented with BrES or KNO_3 were also given 2.25 ml of $H_2 + CO_2$ (80%-20%, vol/vol) as a source of carbon and energy. All tubes with added inhibitors were incubated at 30° C for 16 days (chloroform and β-fluoroacetate tubes) or 45 days (BrES and KNO_3 tubes) and monitored for methane production by gas chromatography.

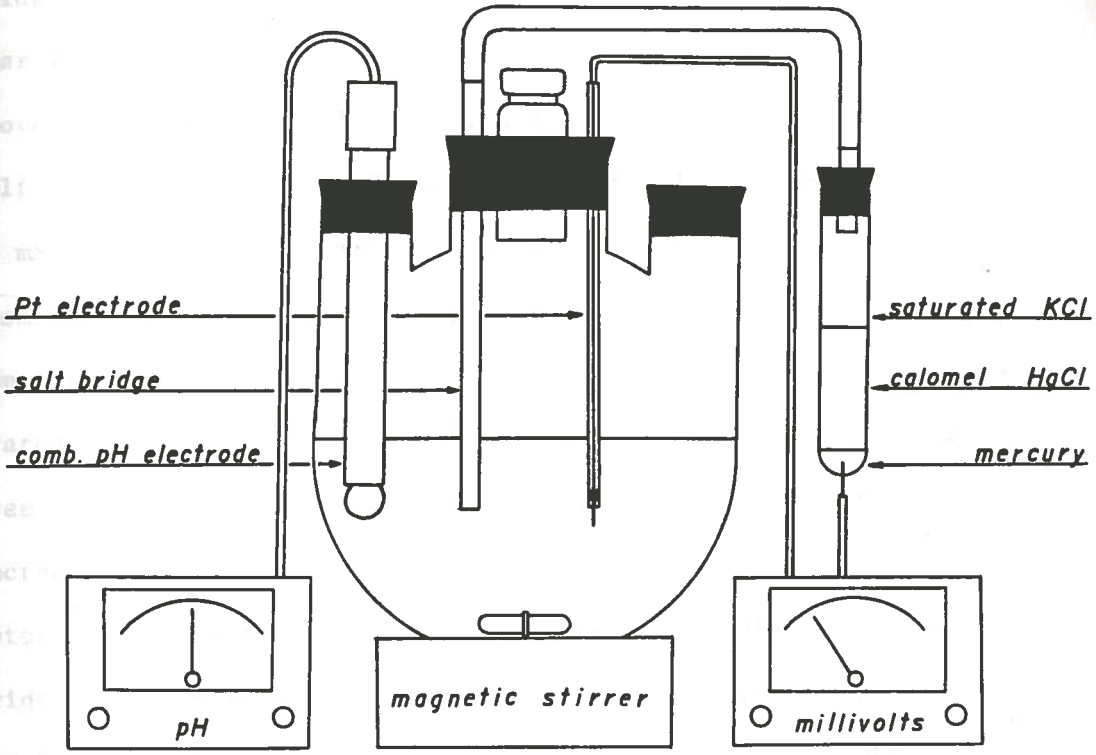
Methanogenesis in the Presence of Added Sulfide, Sulfate, and NaCl. To test the effects of added sulfide, sulfate, and NaCl on methanogenesis, separate triplicate sets of sediment slurries were supplemented with: (i) $Na_2S \cdot 9H_2O$ at concentrations of 0.1, 0.5, 1.0, 5.0, 10, 25, 50, and 100 mM; (ii) $MgSO_4 \cdot 7H_2O$ at concentrations of 0.1, 0.5, 1.0, 5.0, 10, 25, 50, and 100 mM; (iii) NaCl at concentrations of 50, 100, 500, 1000, 3000, and 5000 mM. Half of the tubes that received added sulfide, sulfate, or NaCl contained N_2 in the headspace. The other half was given N_2 plus 2.25 ml of $H_2 + CO_2$ (80%-20%, vol/vol).

All tubes were incubated at 30° C for 45 days and monitored for methane production by gas chromatography.

Effect of Temperature on Methanogenesis. In order to find the optimum temperature for methanogenesis in 0.2 m-deep delta sediments, three sets of sediment samples were prepared. One set of serum bottles contained unadulterated sediment without being diluted to a slurry, and was sealed under a N_2 atmosphere. A second set of serum bottles contained slurries of sediment diluted as described above, and was sealed under N_2. The last set of serum bottles contained sediment slurries under N_2 supplemented with 2.25 ml of $H_2 + CO_2$ (80%-20%, vol/vol). The sediment samples were incubated for 45 days at temperatures of 20, 30, 35, 40, 45, and 50° C. Methane production was measured by gas chromatography.

Measurement of Changes in Redox Potential (E_h) and pH During Methanogenesis. To monitor changes in E_h and pH during methanogenesis in 0.2 m-deep sediments, it was necessary to construct large-volume reaction vessels to contain the sediment slurries. The design of the reaction vessels was that of Patrick, *et al.* (45), and is shown in Figure 4. Each reaction vessel consisted of a one-liter, three-neck, flat-bottomed flask equipped with probes for monitoring pH and E_h, and a serum bottle septum to allow the withdrawal of gas samples from the headspace. The pH of the sediment slurry was measured with a Beckman Zeromatic IV pH meter and combination electrode. The redox potential was measured with a second pH meter (set to read millivolts) with two platinum electrodes, a salt bridge, and a calomel half cell (45). The Pt electrodes were constructed by gluing

Figure 4. Diagram of the reaction vessels used to monitor changes in redox potential (E_h) and pH during methanogenesis by Site 1, 0.2 m-deep, near-surface sediments. The design of the reaction vessels was that of Patrick, *et al.* (45), and allowed the measurement of chemical changes in sediment slurries while being mixed and maintained in a sealed, anaerobic microcosm at a controlled temperature (30^{o} C).


Pt electrode
salt bridge
comb. pH electrode
saturated KCl
calomel HgCl
mercury
pH
magnetic stirrer
millivolts

a 2 cm piece of Pt wire with epoxy cement into the end of a 3 mm O. D. glass tube, as shown in Figure 4. Electrical contact between the Pt wire (which extended 1 cm beyond the end of the glass tube) and the 10 gauge copper wire inside the glass tube was made using a small amount of mercury. The Pt electrode was connected to the electrode jack on the pH meter via 24 gauge bell wire. The salt bridge consisted of a 6 mm O. D. glass tube filled with 4% noble agar in a saturated solution of KCl. The solution of saturated KCl above the agar was continuous with the upper portion of a calomel half cell (see Fig. 4). The calomel half cell was contained in a 16 mm glass test tube with a 2 cm piece of glued Pt wire protruding from the bottom. Mercury (about 2 ml) was used to make electrical contact between the Pt wire and the slurry of calomel (HgCl in saturated KCl) occupying the lower portion of the calomel half cell (see Fig. 4). The Pt wire at the bottom of the half cell was connected to the reference electrode jack on the pH meter. The redox potential probes were standardized by immersing the Pt and salt bridge (reference) electrodes in a saturated solution of quinhydrone (pH 7.00) which has a known redox potential of +284 mV (16).

A 50% slurry of 0.2 m-deep Site 1 sediment was made by mixing 200 g (wet weight) of sediment with 200 ml of anaerobic diluent (prepared as above) and was anaerobically pipetted into the reaction vessel. The headspace gas was O_2-free N_2. The reaction vessels each contained 400 g of slurry plus a magnetic stirring bar. The entire apparatus (with pH meters and magnetic stirrers) was housed in an incubator maintained at 30^{o} C. Methane production, pH, and

E_h were monitored for 27 days.

Methanogenesis from ^{14}C-acetate. Ten-ml aliquots of 50% delta sediment slurries (prepared as above) were dispensed into 60 ml anaerobic serum bottles under a N_2 atmosphere. The sediment samples used in this experiment were collected from East Bay and were taken from depths of 0.075, 0.75, 1.5, 2.9, 3.6, 11.2, and 29.0 m. Previous enrichments with unlabeled compounds had shown that these samples were capable of active methanogenesis. ^{14}C-1-acetate, 14.95 µCi/ml, and ^{14}C-2-acetate, 10 µCi/ml, were obtained from New England Nuclear. Both ^{14}C-acetate solutions were diluted with an anaerobic diluent containing 0.05% $Na_2S \cdot 9H_2O$ plus cysteine·HCl to maintain reducing conditions and to remove any traces of O_2. Each sediment slurry received 200 µl (663,800 dpm) of anaerobically diluted radiolabel and was incubated in the dark for 10 days at 30° C.

At the end of the incubation period, 0.1 ml of 6N NaOH was added to each sediment slurry to stop all biological activity. The slurries (now pH 12) were shaken for 30 min to remove all CO_2 and $^{14}CO_2$ from the headspace, thus ensuring that the only radiolabel left in the headspace was $^{14}CH_4$ (which has an extremely low solubility in aqueous solution). The evolved $^{14}CH_4$ was counted by liquid scintillation according to the method of Zehnder, *et al.* (79). A 15 ml portion of headspace gas was removed from each slurry with an airtight glass syringe and injected into a modified liquid scintillation vial containing 5 ml of Aquasol counting cocktail. The liquid scintillation vials were modified by removing the cork-aluminum seals from the screw caps and replacing them with butyl rubber septum

seals. A hole was drilled into each cap to allow the puncturing of the vial cap to introduce labeled headspace gas. The liquid scintillation vials were capable of holding unlabeled methane gas under 2 atm pressure without leaking. The vials were shaken for 30 min to bring the $^{14}CH_4$ into solution in Aquasol. Experiments with unlabeled methane showed that the solubility of methane at concentrations matching those employed in this experiment was 71.6% at room temperature. The samples were counted in a Beckman LSC-200 liquid scintillation counter (maximum counting error, 5%) and corrected for background (40 cpm), counting efficiency (82.9%), and solubility of methane in Aquasol (71.6%).

Attempts to Isolate Delta Methanogens

Methanogenic Enrichments. Numerous successful methanogenic enrichments were made with the six basic anaerobic media listed in Tables 1-3. All methanogen enrichment media were prepared under strict anaerobic conditions using the Hungate technique (25). All six media were able to support actively methanogenic, mixed population enrichment cultures, and generally produced significant conversion of methane precursors (greater than 50%) in 2-3 days when incubated at 30-37° C. The successful enrichments were restricted to broth cultures in sealed serum bottles (60 and 115 ml, Wheaton Scientific) or anaerobe tubes (27 ml, Bellco Glass, Inc.). The media were inoculated separately with sediment material (approximately 1 g) from four different sampling sites (Sites 1-4), and from several depths ranging from near the surface to 30 m below the sediment-water

Tables 1-3. Methanogen growth media used for broth enrichments and attempted isolation by extinction dilution, Hungate roll tubes (25), and anaerobic streak plates (21). All chemicals used in these media were of reagent grade. Except where noted, the media were prepared and dispensed under O_2-free N_2.

Table 1

Mah, et al. (38) Enrichment Medium	
NH_4Cl	1.0 g
K_2HPO_4	0.4 g
$MgCl_2 \cdot 6H_2O$	0.1 g
$CaCO_3$	100 g
Ca acetate	20 g
resazurin	0.0001 %
artificial seawater	1 l

Modified Romesser, et al. (56) Medium	
10% methylamine	10 ml
10% trypticase	10 ml
10% yeast extract	10 ml
10% ammonium acetate	5 ml
10% sodium formate	10 ml
trace minerals (80)	10 ml
vitamins (3)	10 ml
HS-CoM (1 mg/ml solution)	1 ml
methanol	5 ml
10% Na_2CO_3	10 ml
5% $NaHCO_3$	40 ml
resazurin	0.0001 %
distilled water	150 ml
artificial seawater (40 g/l)	750 ml
1.25% cysteine-sulfide (3)	32 ml
H_2 + CO_2 (80%-20%, vol/vol)	gas phase

Table 2

Modified LPBM Plus 50% Sea Salts (80)	
Na formate	1 %
methanol	1 %
KH_2PO_4	0.15 g
K_2HPO_4	0.29 g
NH_4Cl	0.18 g
$MgCl_2 \cdot 6H_2O$	0.04 g
$Na_2S \cdot 9H_2O$	0.5 g
trace minerals	1.8 ml
yeast extract	0.1 %
HS-CoM (1 mg/ml solution)	0.2 %
resazurin	0.0001 %
distilled water	100 ml
artificial seawater	100 ml

Methanol-Methylamine (MM) Medium	
artificial seawater	300 ml
distilled water	70 ml
yeast extract	0.1 g
10% Na formate	4 ml
10% Na acetate	4 ml
minerals (80)	4 ml
vitamins (3)	4 ml
HS-CoM (1 mg/ml solution)	0.4 ml
5% $NaHCO_3$	16 ml
resazurin	0.0001 %
10% methylamine	4 ml
methanol	2 ml
1.25% cysteine-sulfide (3)	16 ml

Table 3

Low Yeast Extract Medium, Mah, et al. (38)	
yeast extract	0.02 %
trypticase	0.2 %
NH_4Cl	0.1 %
K_2HPO_4	0.04 %
$MgCl_2$	0.01 %
resazurin	0.0001 %
cysteine·HCl	0.05 %
$NaHCO_3$	0.05 %
$Na_2S·9H_2O$	0.01 %
methanol	1.0 %
Na acetate	0.1 M
artificial seawater	1 l

High Yeast Extract Medium, Mah, et al. (38)	
yeast extract	1.0 %
trypticase	0.8 %
NH_4Cl	0.1 %
$NaHCO_3$	0.15 %
cysteine·HCl	0.05 %
$Na_2S·9H_2O$	0.01 %
resazurin	0.0001 %
methanol	1.0 %
Na acetate	0.1 M
artificial seawater	1 l

interface. The sediment samples which contained viable methanogens caused nearly uniform methane generation in all six types of media.

The extinction dilution technique was used in an attempt to isolate methanogens in broth media using all six types listed in Tables 1-3. In this procedure, active methanogenic enrichments were ten-fold serially diluted into fresh media of the same type by means of N_2-flushed, glass, 2.5 ml syringes (41). Dilutions of up to 10^{-12} were incubated at 35° C for six to eight weeks, and periodically assayed for methane production by gas chromatography.

Attempts to Isolate Methanogens on Solid Media. Two basic types of agar-containing, solid media were used in an attempt to obtain pure cultures of methanogens from delta sediment samples: (i) the roll tube technique (25); and (ii) the anaerobic streak plate (21). Roll tubes containing all six methanogen growth media were inoculated with material from active methanogenic enrichments according to the method of Hungate (25) and incubated at 35° C. Anaerobic streak plates were poured in an anaerobe hood (Germfree Laboratories, Inc., Miami, FL) under an atmosphere of 90% N_2 and 10% H_2. They were then inoculated with material from active methanogenic enrichments and incubated at 35° C inside the anaerobe hood, or were sealed in a He-flushed anaerobe jar and incubated inside the anaerobe hood. The latter method was essentially the same as the "ultra-low O_2" chamber method of Edwards and McBride (21). Anaerobic conditions were maintained inside the anaerobe hood by the circulation of H_2 gas over a palladium catalyst. The catalyst was periodically removed (every 2-3 weeks) and rejuvenated by heating for 1 h at 160° C. Because the H_2

reacted with O_2 that diffused into the anaerobe hood, it was necessary to periodically replenish the H_2 in the hood in order to maintain a 10% H_2 atmosphere. The quality of the anaerobic atmosphere within the hood was monitored by keeping solutions of redox indicators (methylene blue and resazurin) open to the anaerobic atmosphere inside the hood.

Numerous colonies were picked from the roll tubes and anaerobic streak plates and were inoculated into fresh broth media. These enrichments were then further incubated at 35° C for six to eight weeks and monitored for methane production by gas chromatography. The anaerobic streak plates were routinely examined under long wave ultraviolet light in order to identify fluorescent methanogen colonies, according to the method of Edwards and McBride (21).

Isolation and Characterization of Delta Sulfate-Reducers

Sulfate-Reducer Enrichments. Successful enrichments of sulfate-reducing bacteria were obtained with Postgate's enrichment medium (49). The medium contained, per liter of distilled water, KH_2PO_4, 0.5 g; NH_4Cl, 1.0 g; $CaSO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; Na lactate, 3.5 g; yeast extract, 1.0 g; ascorbic acid, 1.0 g; Na thioglycollate, 1.0 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; and NaCl, 30.0 g. The distilled water that was used in the enrichment medium was boiled and cooled to drive off most of the dissolved O_2. The medium was filter sterilized (Millipore, 0.45 µm pore size), sparged aseptically with O_2-free N_2 for 15 min, and anaerobically dispensed into sterile anaerobe tubes. Tubes of Postgate's enrichment medium (49) were inoculated with

approximately 1 g (wet weight) of Site 1 delta surface sediment and incubated for 1 week at 35° C. Positive sulfate-reducing enrichments turned black due to the precipitation of sulfide as FeS.

Isolation of Sulfate-Reducing Bacteria. Numerous enrichments were set up according to a modified procedure of Postgate (49) and were incubated at 35° and 55° C. Ninety-six hour old positive enrichments were sampled and ten-fold serially diluted into fresh Postgate's enrichment medium by means of N_2-flushed, glass, 2.5 ml syringes. One-ml aliquots were removed from dilutions of 10^{-7}, 10^{-8}, and 10^{-9}, and were inoculated into fresh media. This extinction dilution technique was used to isolate 80 strains of sulfate-reducing bacteria. Purity of the isolates was checked by phase contrast microscopy and by occasionally streaking plates of Brewer's Thioglycollate Anaerobe Medium to detect non-sulfate-reducing contaminants.

Identification of Sulfate-Reducer Isolates. The taxonomic criteria of Postgate and Campbell (50) were used to identify sulfate-reducing bacteria isolated from the Site 1 surface sediments. All 80 strains were tested for their ability to grow in Medium C of Butlin, *et al.* (10) supplemented with the following compounds: lactate, pyruvate, malate, acetate, and formate, according to the method of Postgate (49). The sulfate-free medium of Postgate (47) was used to test the growth of the isolates on pyruvate and choline in the absence of sulfate. The ability of the isolates to grow in the presence of Hibitane (chlorhexidine diacetate) was tested by supplementing Postgate's medium (49) with 0.25, 1.0, 2.5, 10, 25, and 1000 µg/ml of the antibiotic. Production of desulfoviridin, a pigment produced

by members of the genus *Desulfovibrio*, was tested by adding 0.1 ml of 3N NaOH to a sample of each isolate in order to release the pigment into solution. The observation of a bright red color under longwave ultraviolet light was considered as a positive test for desulfoviridin production (48). All isolates were tested for their optimum growth temperature and minimum NaCl requirement in Postgate's medium (49).

Photo- and Electron-Microscopy of Cells. Photomicrographs of representative strains of the sulfate-reducing isolates, and of the methanogenic enrichment cultures, were taken by either phase contrast or Nomarski interference microscopy. Cells for electron micrographic examination were negatively stained with 1% phosphotungstate and viewed in a JEL 100CX transmission electron microscope.

RESULTS

Depth Distribution of Methanogens and Sulfate-Reducers

Figure 5 shows the depth distribution profiles of methanogens and sulfate-reducers in Site 1 Mississippi River delta sediments. In this series of core samples, the methanogen population was greatest (1.18×10^6 cells per 100 g sediment, dry weight) in the uppermost sample, taken 0.2 m below the sediment-water interface. The MPN of methanogens decreased rapidly with depth. At Site 1, viable methanogens were not detected at a depth of 8.7 m, or below. The MPNs of methanogens in sediment core samples from Site 2 (70.7 m-deep boring) and Site 3 (212.1 m-deep boring) were also highest in the samples collected near the sediment-water interface (Table 4). The core samples from Sites 2 and 3 also showed that methanogen populations decreased with depth.

Figure 5. Depth (in meters below the sediment-water interface) distribution profiles of methanogens (○—○); sulfate-reducers (□—□); dissolved methane (●—●); and dissolved sulfate (△—△), in Site 1 delta sediments. Most probable number (MPN) estimates of bacterial population densities were the average of two separate determinations performed on sediment dilutions of 10^{-1} and 10^{-3}, and are given as cells per 100 grams sediment, dry weight. The data points with cross-hatching indicate "less than" values, and reflect the limits of detection of these bacterial populations rather than true, finite values. Dissolved sulfate is shown as a mM concentration in the interstitial water that was squeezed from delta sediment samples. The dissolved methane is shown as mmol of methane per liter of whole, wet sediment.

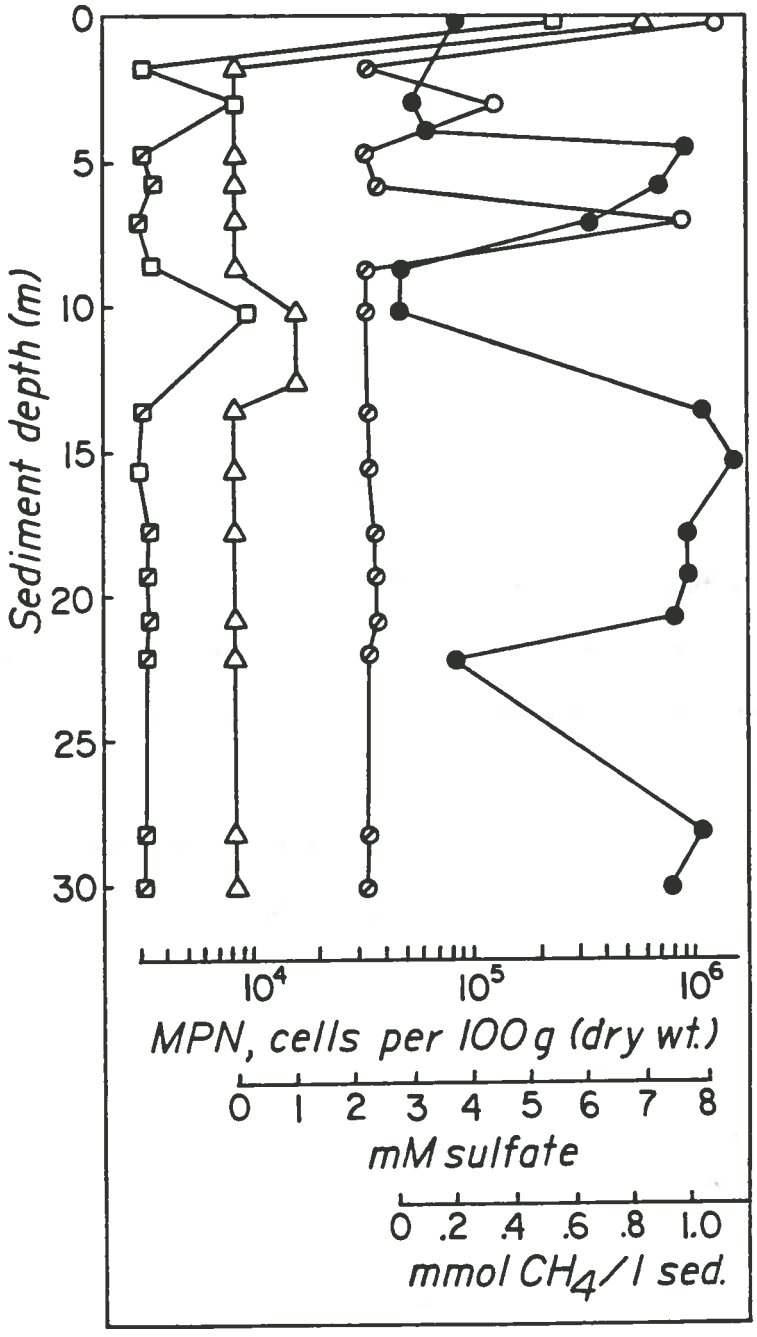

0
5
10
15
20
25
30
Sediment depth (m)
10⁴
10⁵
10⁶
MPN, cells per 100 g (dry wt.)
0 1 2 3 4 5 6 7 8
mM sulfate
0 .2 .4 .6 .8 1.0
mmol CH4/l sed.

Table 4. Most probable number (MPN) estimates of methanogen and sulfate-reducer population densities in delta sediments from Site 2 and Site 3. Depth is shown in meters below the sediment-water interface. MPN estimates were an average from two separate determinations of sediment dilutions of 10^{-1} and 10^{-3}, and are given as cells (X 10^{-4}) per 100 grams sediment, dry weight.

	Depth (m)	Cells ($\times 10^{-4}$)/100g Sediment, Dry Wt. Methanogens	Sulfate-Reducers
Site 2	1.5	>6300	61
	3.7	>2400	240
	11.3	950	210
	29.3	6.5	140
	45.1	<2.1	170
	70.7	<2.7	<2.7
Site 3	2.9	1.6	9.8
	18.4	<0.005	2.9
	75.0	<0.004	0.09
	89.0	<0.004	<0.004
	101.3	<0.004	<0.004
	124.1	<0.004	<0.004
	131.7	<0.004	<0.004
	142.1	<0.004	<0.004
	157.0	<0.004	<0.004
	187.8	<0.004	<0.004
	212.1	<0.004	<0.004

The depth distribution of sulfate-reducers in the Site 1 and Site 3 sediments followed a pattern that was similar to that of the methanogens. The highest MPN densities of sulfate-reducers occurred in the uppermost samples near the sediment-water interface. At Site 1, sulfate-reducers were detected in samples to a depth of 15.7 m, but were absent below a depth of 17.8 m. The depth distribution of viable sulfate-reducers was extensive in Site 2 and Site 3 sediments, reaching depths of 45.1 and 75.0 m, respectively. At Site 2, the highest MPN of sulfate-reducers occurred at a depth of 3.7 m, and was well removed from the sediment-water interface.

At Site 4, the upper 0.77 m of delta sediment was examined. Table 5 shows the results of MPN estimates of methanogens and sulfate-reducers in delta sediment samples ranging in depth from 0.050-0.770 m below the sediment-water interface. Neither population showed a recognizable relationship with depth, however, the MPNs of methanogens and sulfate-reducers in the uppermost sample (0.050 m-deep) were both small when compared to those at greater depths (0.355-0.420 m). In general, the MPNs of methanogens in the Site 4 samples were greater than those of sulfate-reducers. In 11 of 12 samples, methanogens outnumbered sulfate-reducers by ratios ranging from 2:1 to over 300:1.

Interstitial Water Chemistry of Site 1 (30.0 m-deep) Sediments

The depth profiles of dissolved methane and dissolved sulfate in Site 1 sediments are shown in Figure 5. Dissolved methane maxima occurred at depths of approximately 5, 14-20, and 29-30 m. Very low concentrations of dissolved methane were observed at the surface (0.2-4.0 m), at 9-10 m, and at 22 m. The large population of methanogens

Table 5. Most probable number (MPN) estimates of methanogen and sulfate-reducer populations in delta sediments from Site 4 that were collected in a 1.5 m-long plastic drop core. Depth is shown in meters below the sediment-water interface. MPN estimates were averages from two separate determinations of sediment dilutions of 10^{-1} and 10^{-3}, and are given as cells (X 10^{-4}) per 100 grams sediment, dry weight. The ratio of methanogens to sulfate-reducers is indicated in the far right column.

Depth (m)	Cells ($\times 10^{-4}$)/100 g Sediment, Dry Weight: Methanogens	Sulfate-Reducers	Ratio
0.050	7.3	<3.1	>2
0.077	960	<2.6	>370
0.100	480	53	9.1
0.165	1200	9.8	123.8
0.225	<5.7	82	0.1
0.290	110	51	2.2
0.355	>3100	30	>100
0.420	2600	100	25.6
0.485	210	<6.9	>30
0.545	23	<4.5	>5
0.600	940	18	51.2
0.770	>4200	16	>250

in the 0.2 m sample, therefore, appeared to coincide with a greatly diminished level of dissolved methane.

The greatest concentration of dissolved sulfate occurred in the uppermost sample at a depth of 0.2 m (Fig. 5). Dissolved sulfate was rapidly depleted below a depth of 2.0 m, and was not detected below this depth, except for a second horizon of dissolved sulfate at 10-12 m. The dissolved sulfate maxima at depths of 0.2 m and 10-12 m both coincided with high MPNs of sulfate-reducing bacteria. In addition, the three major peaks of sulfate-reducers (at 0.2, 3.0, and 10.0 m) coincided with significant decreases in dissolved methane at those depths. Conversely, samples that contained low MPNs of sulfate-reducers exhibited high levels of dissolved methane.

Figure 6 shows depth profiles of ΣCO_2, DOC, total carbon, pH, percent water, and salinity in Site 1 delta samples. The ΣCO_2 concentration decreased rapidly with depth, dropping by about one-half from the 0.2 m sample (55 mg C/l) to the 2.0 m sample (22 mg C/l). A second peak of ΣCO_2 (50 mg C/l) occurred at a depth of 9.0 m. The concentration of DOC appeared to fluctuate between 40 and 60 mg C/l, and showed no apparent relationship with depth. Similarly, pH (7.5-8.0) and salinity (30.5-35.5 g Cl^-/l) fluctuated and did not appear to be related to depth. Percent water was greatest (57%) in the uppermost, 0.2 m-deep sample.

In Vitro Methanogenesis from Added Substrates

The results of the separate additions of 19 potential methane precursors are summarized in Table 6. The following known methane precursors were rapidly converted (3-7 days) to methane by Site 1

Figure 6. Site 1 depth (in meters below the sediment-water interface) profiles of dissolved inorganic carbon, ΣCO_2 (□—□); dissolved organic carbon, DOC (△—△); total carbon (○—○); pH (■—■); percent water (▲—▲); and salinity (●—●). ΣCO_2, DOC, and total carbon are shown as mg C per liter of interstitial water. pH is given in units measured at 25° C. Percent water is shown as grams of water per 100 g wet sediment before drying to constant weight at 75° C. Salinity is shown as chloride ion concentration in grams per liter of interstitial water.

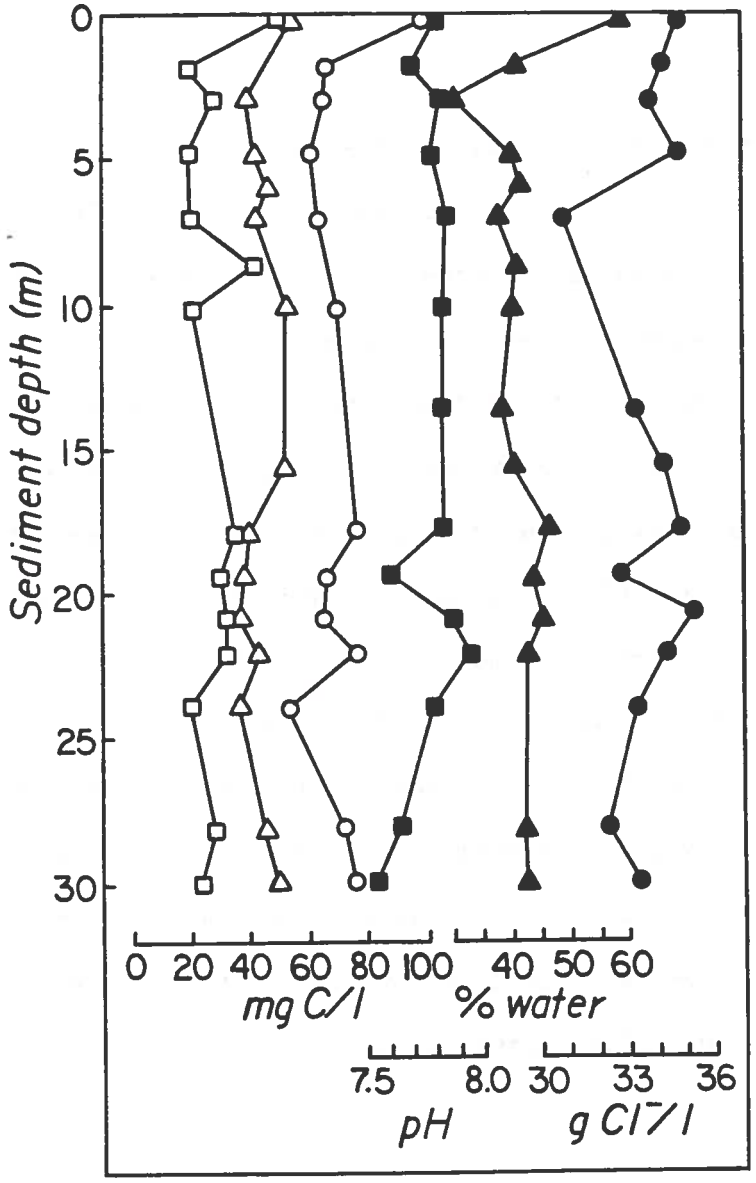

Sediment depth (m)
0
5
10
15
20
25
30
0 20 40 60 80 100
mg C/l
40 50 60
% water
7.5 8.0
pH
30 33 36
g Cl⁻/l

Table 6. *In vitro* methanogenesis resulting from the addition of separate 100 µmol quantities of potential methane precursors and potentially stimulatory compounds. Sediment slurries (50%) of Site 1, 0.2 m-deep, near-surface material were dispensed into triplicate sets of anaerobe tubes and incubated for 16 days at 30° C. *In vitro* methanogenesis is given as the average µmol methane produced per gram sediment, dry weight, in three separate determinations, plus or minus one standard deviation ($\pm \sigma^2$). Percent conversion is shown as mol% of added carbons converted to methane. The incubation period culminating in the maximum production of methane in each triplicate set of tubes is given in days. Each value was corrected for methanogenesis from endogenous methane precursors in triplicate control tubes that received no added substrate. Tubes with $CO_2 + H_2$ received 2.25 ml of a gas mixture of 20%-80%, vol/vol. Tubes with cellulose each received 0.03 g of the substrate.

In Vitro Methanogenesis (30°C), 100 µmol Added Substrate

Added Substrate	µmol CH_4/g	% Conversion	Days
methylamine	19.27±3.25	64.7	4
dimethylamine	39.44±2.75	66.3	4
trimethylamine	52.59±4.86	58.9	3
methanol	19.39±3.08	65.2	4
CO_2 & H_2	9.70 ± 0.59	48.5	7
methanethiol	1.45 ± 0.13	4.9	7
methylhydrazine	1.68 ± 0.87	5.6	16
hydrogen	0.97± 0.11	-	7
dimethylsulfide	0.17 ± 0.13	0.6	8
formate	0.09 ± 0.01	0.3	16
acetate	0.08 ± 0.06	0.1	16
bicarbonate	0.00 ± 0.04	0.0	16
lactate	0.18 ± 0.06	0.2	16
propionate	0.06 ± 0.05	0.1	16
pyruvate	0.39 ± 0.15	0.4	16
methionine	1.03 ± 0.30	1.2	16
glucose	2.21 ± 0.40	1.2	5
cellobiose	0.67 ± 0.21	0.2	7
cellulose	0.27 ± 0.00	0.0	16

delta sediments from the 0.2 m-deep sample: methylamine, dimethylamine, trimethylamine, methanol, and $CO_2 + H_2$. The mol% conversion of added carbons with these five substrates ranged from 48.5-66.3%. The remaining 14 substrates, in general, caused much lower productions of methane. Among these were two compounds which are known methane precursors, formate and acetate. Both substrates stimulated only minimal production of methane above that in controls without added substrates. The addition of methanethiol (CH_3SH) or methylhydrazine (CH_3NHNH_2) resulted in the stimulation of methanogenesis (4.9 and 5.6 mol% conversion, respectively). Methane production from methylhydrazine has not been previously reported.

Figures 7 to 11 show the *in vitro* methanogenesis (and methane consumption) in Site 1, 0.2 m-deep sediment from added methane precursors and potentially stimulatory compounds in 45 day incubations at 30^{o} C. The rapid conversion of methanol and N-methyl compounds to methane is shown in Figure 7. Methane production from these compounds peaked in 3-4 days, and exhibited a slow, steady consumption until the end of the 45 day incubation period. The production of methane from cellulose is shown in Figure 8. A lag period of approximately 15 days was followed by a significant increase in methane production from day 22 to 45. Methanogenesis from methanethiol, methionine, methylhydrazine (Fig. 8), and from carbon monoxide (Fig. 9), each showed a similar extensive lag period (7-8 days) prior to the onset of substrate turnover. Methanogenesis from glucose (Fig. 9), and from pyruvate and dimethylsulfide (Fig. 10), was rapid, but only a small percentage of the added carbons was converted to methane.

Figure 7. *In vitro* methanogenesis with time from the addition of separate 100 µmol quantities of trimethylamine (●—●); dimethylamine (■—■); methylamine (○—○); and methanol (□—□). Sediment slurries (50%) of Site 1, 0.2 m-deep, near-surface material were dispensed into triplicate sets of anaerobe tubes and monitored for methane production for 45 days. *In vitro* methanogenesis is given as µmol methane produced (at 30° C) per gram sediment, dry weight, from the tube in each set yielding the maximum methane production. Each value was corrected for methanogenesis from endogenous methane precursors in triplicate control tubes that received no added substrates.

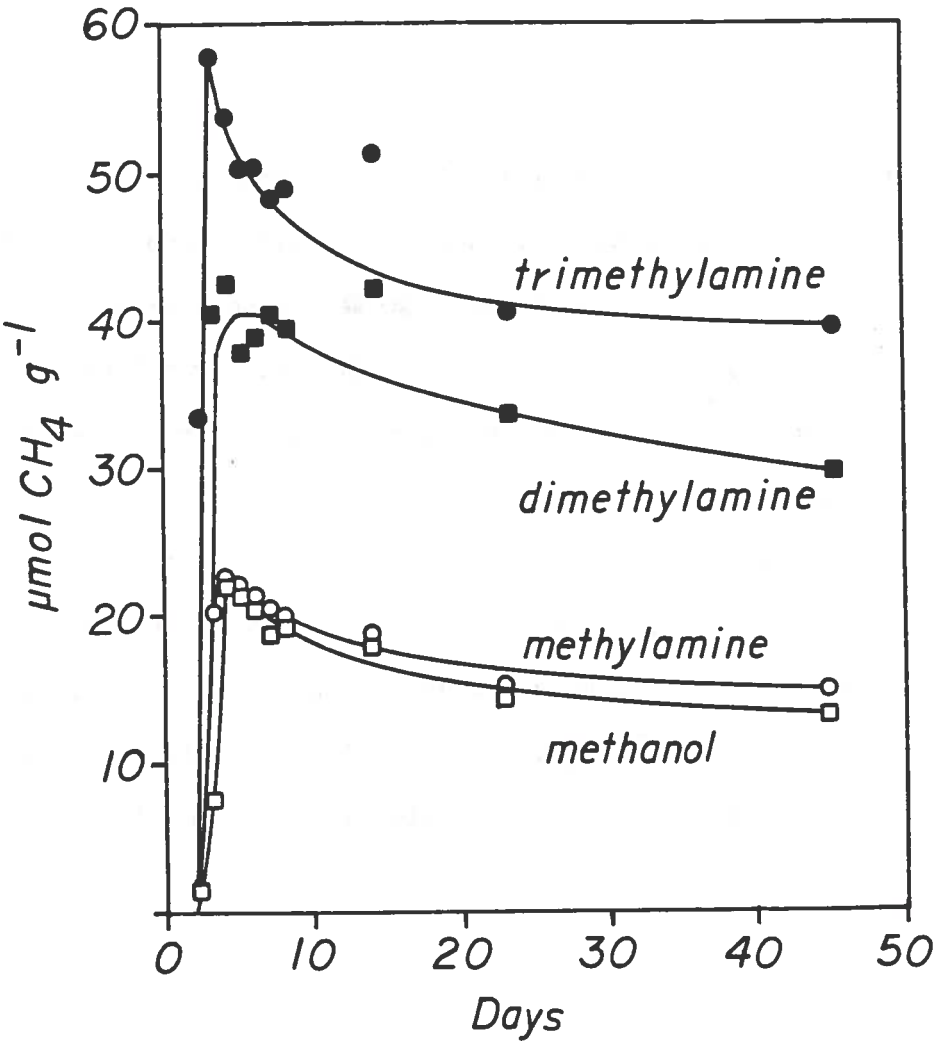

60
50
40
30
20
10
µmol CH_4 g^{-1}
trimethylamine
dimethylamine
methylamine
methanol
0
10
20
30
40
50
Days

Figure 8. *In vitro* methanogenesis with time from the addition of separate 100 µmol quantities of methanethiol (○—○); methionine (□—□); and methylhydrazine (■—■), and from the addition of 0.03 g cellulose (△—△) per tube. Sediment slurries (50%) of Site 1, 0.2 m-deep, near-surface material were dispensed into triplicate sets of anaerobe tubes and monitored for methane production for 45 days. *In vitro* methanogenesis is given as µmol methane produced (at 30° C) per gram sediment, dry weight, from the tube in each set yielding the greatest methane production. Each value was corrected for methanogenesis from endogenous precursors in triplicate control tubes that received no added substrates.

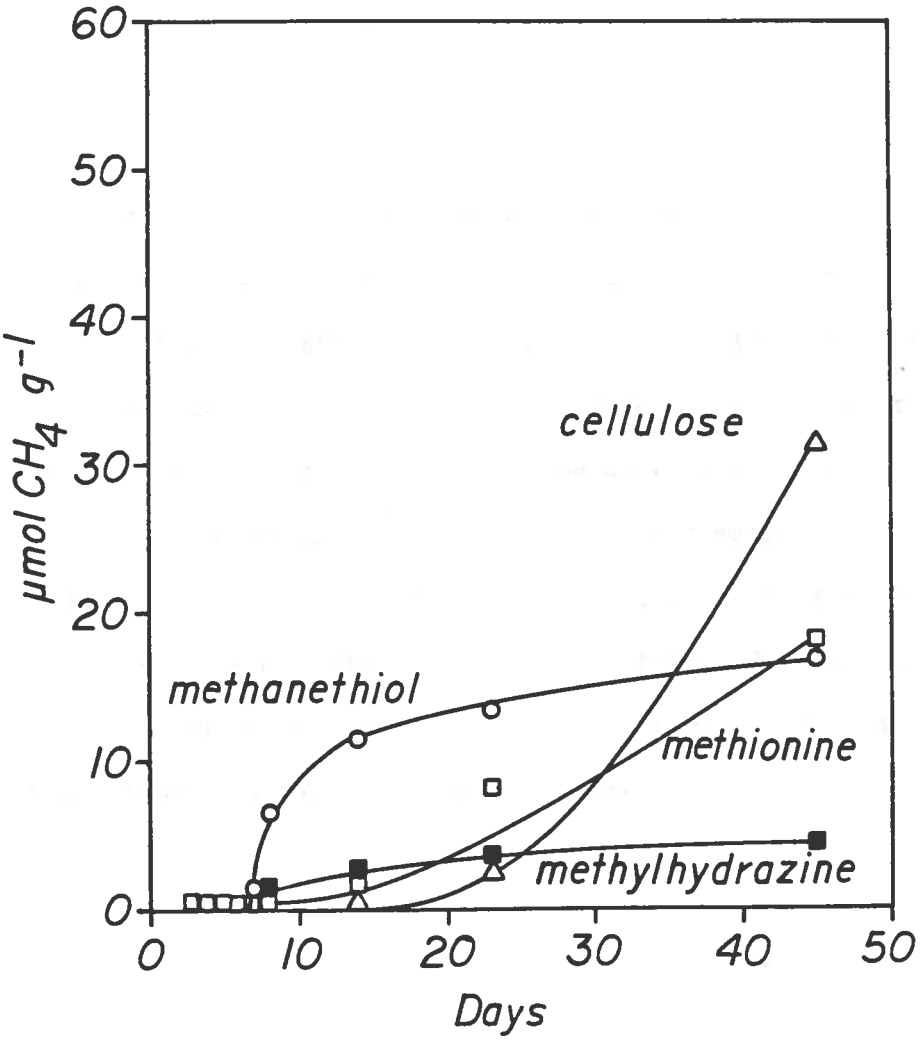

60
50
40
30
20
10
0
µmol CH_4 g^{-1}
cellulose
methanethiol
methionine
methylhydrazine
0
10
20
30
40
50
Days

Figure 9. *In vitro* methanogenesis with time from the addition of separate 100 µmol quantities of glucose (□—□); carbon monoxide (○—○); and cellobiose (△—△). Sediment slurries (50%) of Site 1, 0.2 m-deep, near-surface material were dispensed into triplicate sets of anaerobe tubes and monitored for methane production for 45 days. *In vitro* methanogenesis is given as µmol methane produced (at 30° C) per gram sediment, dry weight, from the tube in each set yielding the maximum methane production. Each value was corrected for methanogenesis from endogenous precursors in triplicate control tubes that received no added substrates.

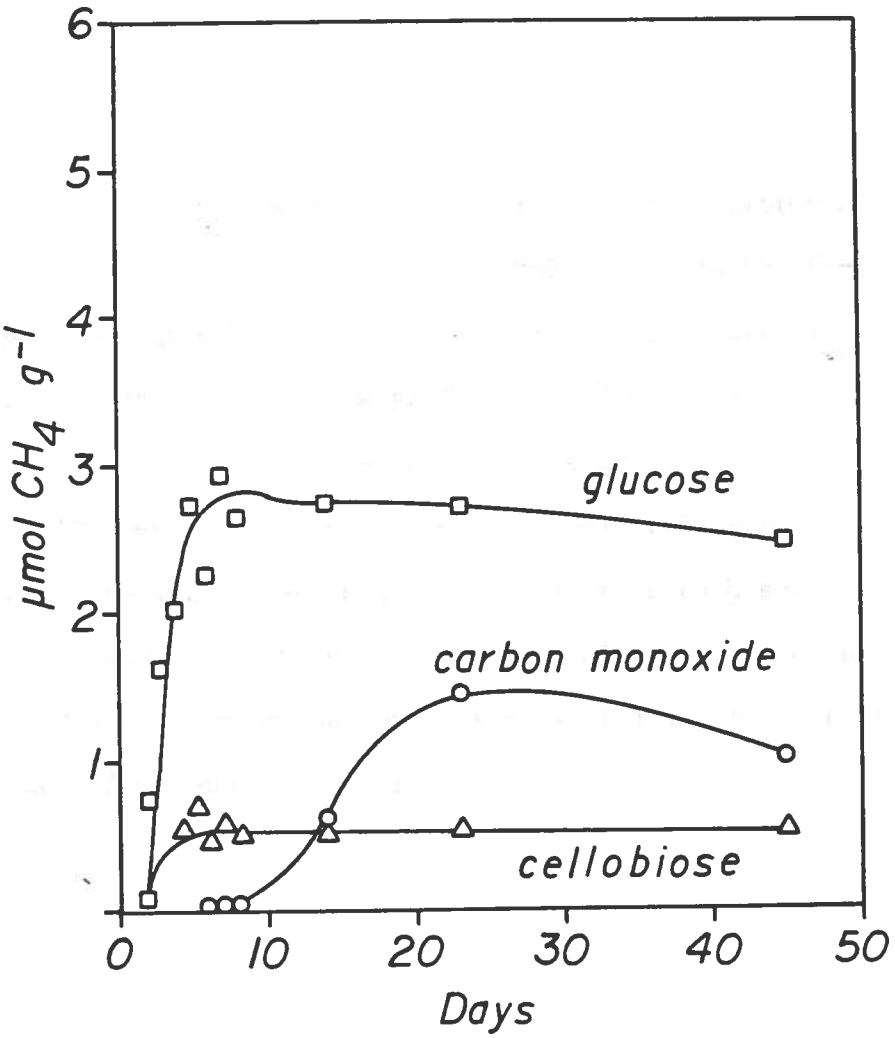

6
5
4
3
2
1
μmol CH_4 g^{-1}
glucose
carbon monoxide
cellobiose
0
10
20
30
40
50
Days

Figure 10. *In vitro* methanogenesis with time from the addition of separate 100 µmol quantities of pyruvate (○—○); propionate (□—□); and dimethylsulfide (△—△). Sediment slurries (50%) of Site 1, 0.2 m-deep, near-surface material were dispensed into triplicate sets of anaerobe tubes and monitored for methane production for 45 days. *In vitro* methanogenesis is given as µmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production. Each value was corrected for methanogenesis from endogenous precursors in triplicate control tubes that received no added substrates.

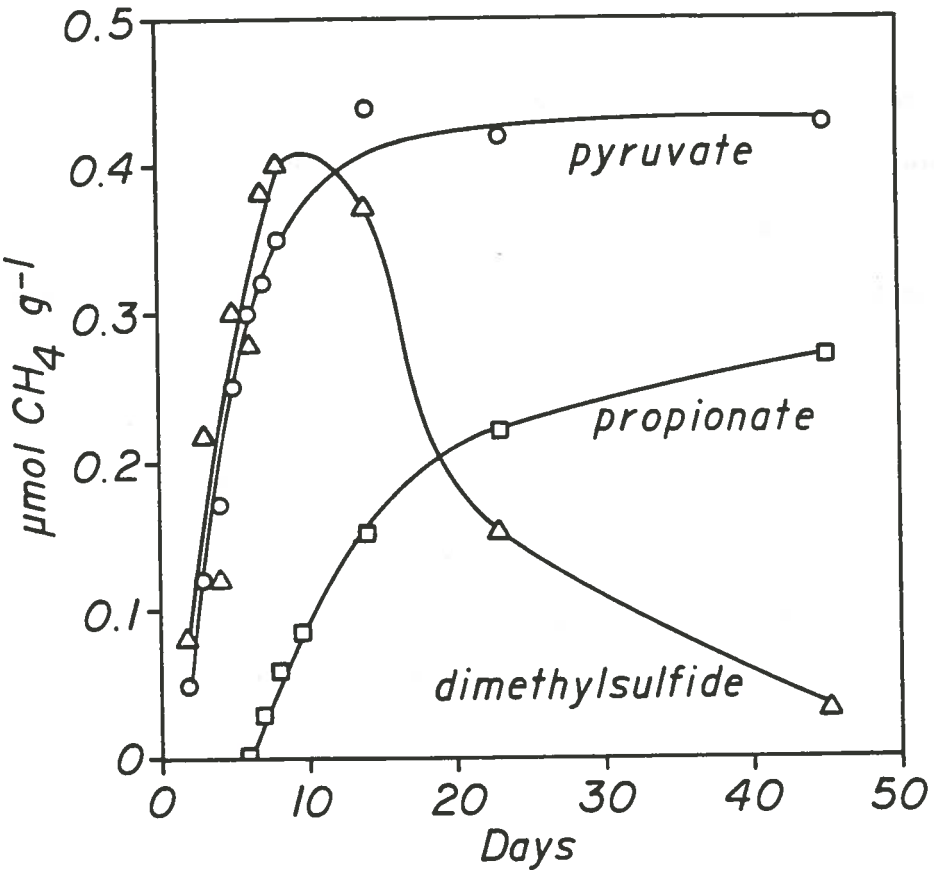
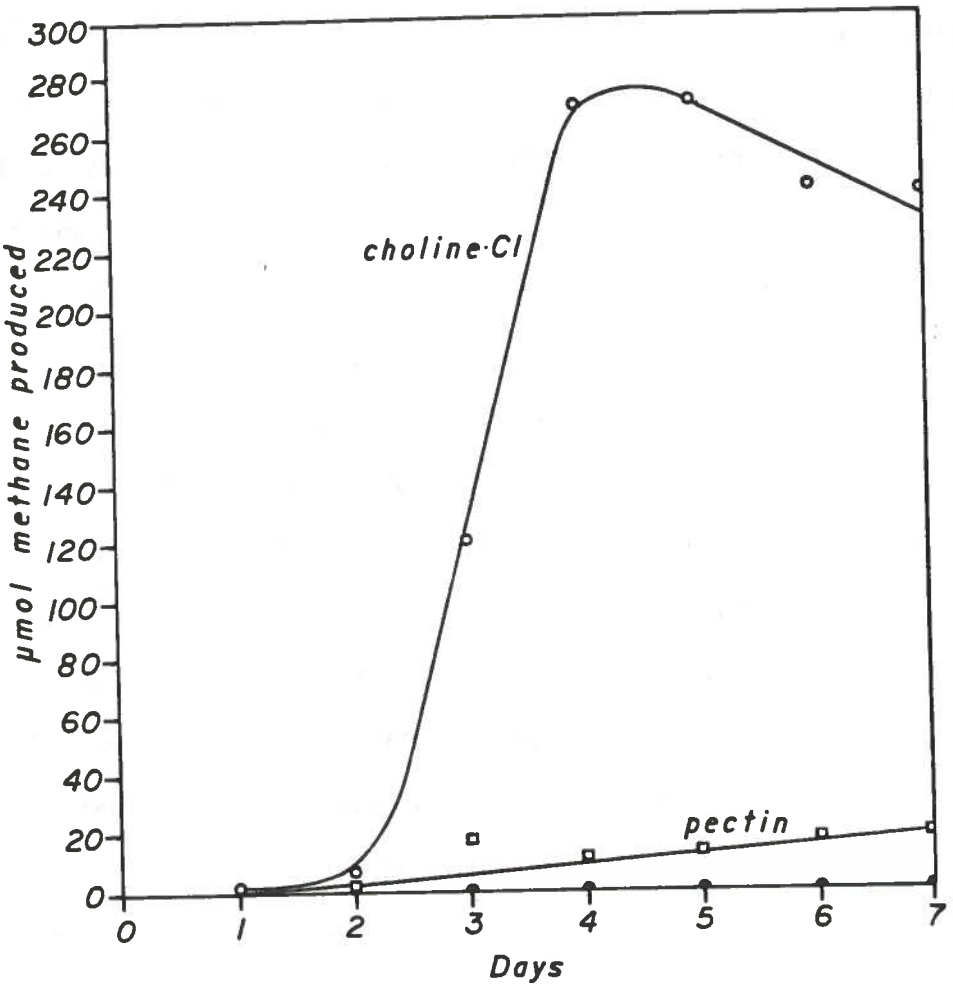

0.5
0.4
0.3
0.2
0.1
0
μmol CH_4 g^{-1}
pyruvate
propionate
dimethylsulfide
0
10
20
30
40
50
Days

Figure 11. *In vitro* methanogenesis with time from the addition of separate 100 µmol quantities of choline·Cl (○—○); and 0.014 g per tube quantities of pectin (□—□); and polypectate (●—●). Sediment slurries (50%) of Site 1, 0.2 m-deep, near-surface material were dispensed into triplicate sets of anaerobe tubes and monitored for methane production for 7 days. *In vitro* methanogenesis is given as total µmol methane produced at 30° C from the tube in each set yielding the maximum methane production. Each value was corrected for methanogenesis from endogenous precursors in triplicate control tubes that received no added substrates.


300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
µmol methane produced
choline·Cl
pectin
0
1
2
3
4
5
6
7
Days

Inhibition of Methanogenesis

The addition of 0.1% chloroform to Site 1 sediment samples (7.2 m-deep) caused a 90% decrease in methane production (Fig. 12). Inhibition of methanogenesis in samples from depths of 0.2 and 3.1 was also observed, but the degree of inhibition was of a lesser magnitude than in the 7.2 m sample. The addition of up to 0.1% β-fluoroacetate did not influence methanogenesis in samples from 0.2, 3.1, or 7.2 m (Fig. 13). Figure 14 shows that the addition of 0.1% β-fluoroacetate to 0.2 m-deep sediment did not influence the initial progress of methanogenesis (no lag period was observed), nor did it appear to diminish methanogenesis during an extended incubation of 16 days at 30^{o} C. Methanogenesis was, however, severely retarded in the presence of air, formaldehyde, and chloroform (Fig. 14). The addition of 5 mM KNO_3, and 2.5 mM bromoethanesulfonate (BrES) also inhibited methanogenesis (100%, and 82-100%, respectively), and it was not reversed by the presence of 100 µmol added $H_2 + CO_2$ (80%-20%, vol/vol) as a carbon and energy source (Fig. 15).

The effects of added sulfide, and sulfide plus $H_2 + CO_2$ are shown in Figures 15 and 16. The addition of 0.1 mM sulfide caused a slight stimulation (0.06 µmol CH_4/g sediment at 45 days) of methanogenesis above control values. Stimulation was greater with added sulfide concentrations ranging from 5-50 mM (0.4-0.5 µmol CH_4/g sediment at 45 days). The addition of 100 mM sulfide initially inhibited methanogenesis during a lag period lasting eight days. This was followed by a sizable stimulation of methanogenesis (0.8 µmol CH_4/g sediment at 45 days) when compared to unamended controls.

Figure 12. Inhibitory effect of chloroform on *in vitro* methanogenesis by Site 1 sediment samples. Chloroform, in concentrations from 0-0.5%, was added to triplicate anaerobe tubes containing 50% slurries of sediment taken from depths of 0.2 m (△—△); 3.1 m (□—□); 7.2 m (○—○); and 17.9 and 30.0 m (●—●). The tubes were incubated for 16 days at 30° C and then assayed for methane. *In vitro* methanogenesis is given as μmol methane produced per gram sediment, dry weight.

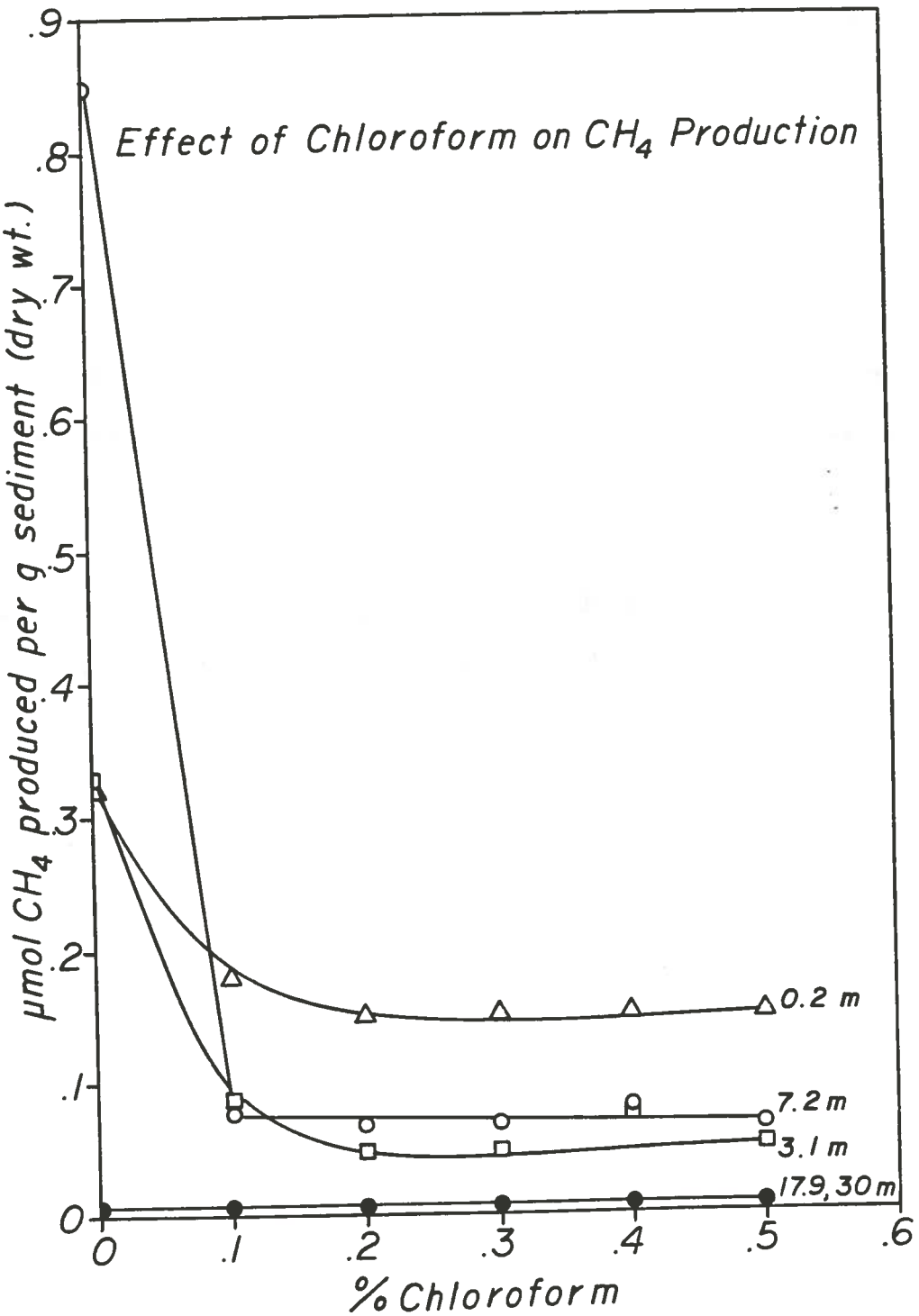

Effect of Chloroform on CH_4 Production
μmol CH_4 produced per g sediment (dry wt.)
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0
.1
.2
.3
.4
.5
.6
% Chloroform
0.2 m
7.2 m
3.1 m
17.9, 30 m

Figure 13. Effect of β-fluoroacetate on *in vitro* methanogenesis by Site 1 sediment samples. β-fluoroacetate, in concentrations ranging from 0-0.1%, was added to triplicate anaerobe tubes containing 50% slurries of sediments taken from depths of 0.2 m (□—□); 3.1 m (△—△); 7.2 m (○—○); and 17.9 and 30.0 m (●—●). The tubes were incubated for 16 days at 30° C and assayed for methane. *In vitro* methanogenesis is given as μmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production.

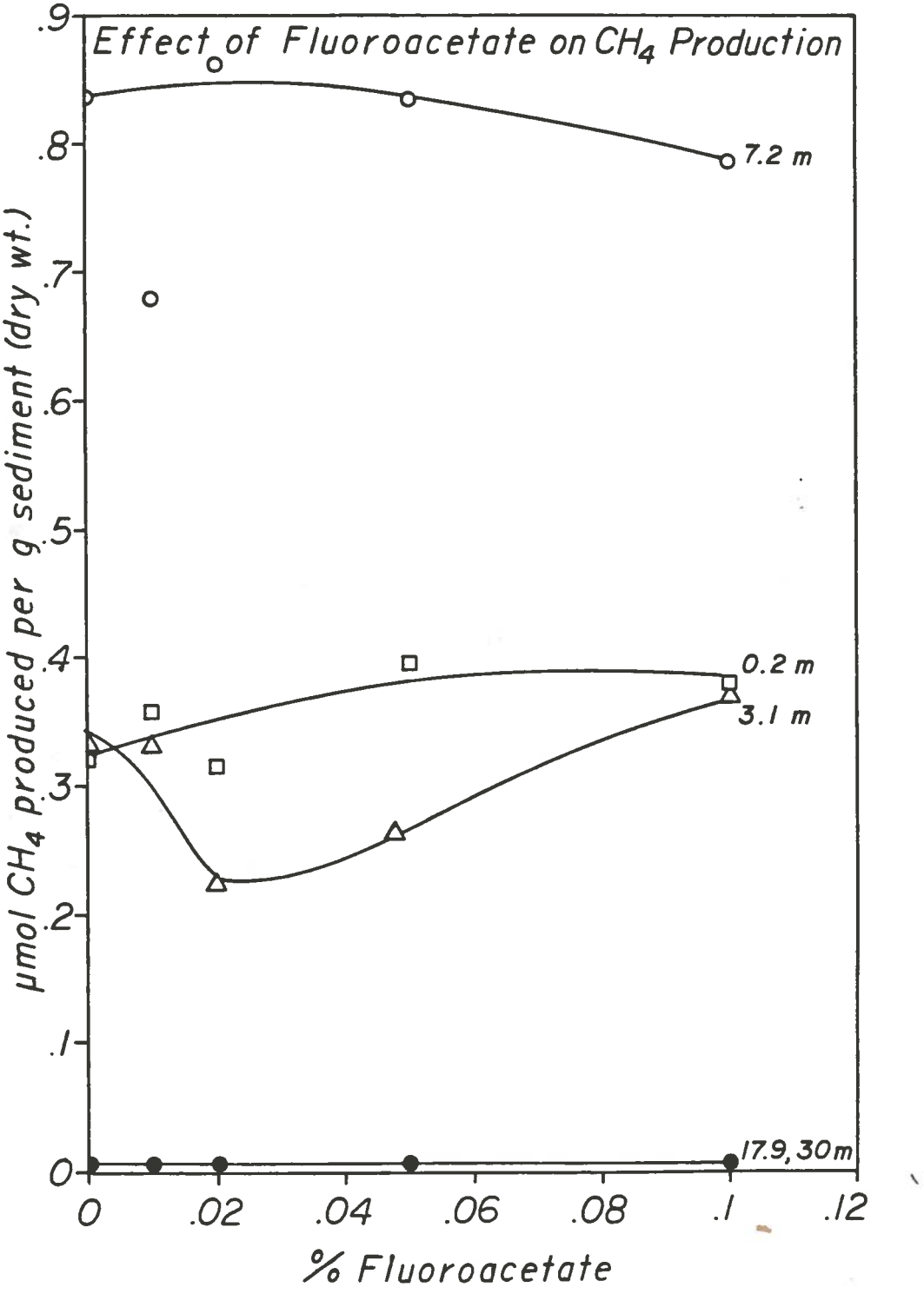

Effect of Fluoroacetate on CH_4 Production
7.2 m
0.2 m
3.1 m
17.9, 30m
μmol CH_4 produced per g sediment (dry wt.)
% Fluoroacetate
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
.02
.04
.06
.08
.12

Figure 14. Effect of methanogenesis inhibitors with time on methane production by Site 1, 0.2 m-deep, near-surface sediment samples. β-fluoroacetate (□—□), 0.1%; chloroform (●—●), 0.1%; formaldehyde (■—■), 0.1 ml of a 37% solution; and air (○—○), 17 ml, were each added to triplicate sets of anaerobe tubes containing 50% sediment slurries. *In vitro* methanogenesis (30° C) was monitored for 16 days and compared to methane production in triplicate control tubes that received no added inhibitors. Methanogenesis is given as nmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production.

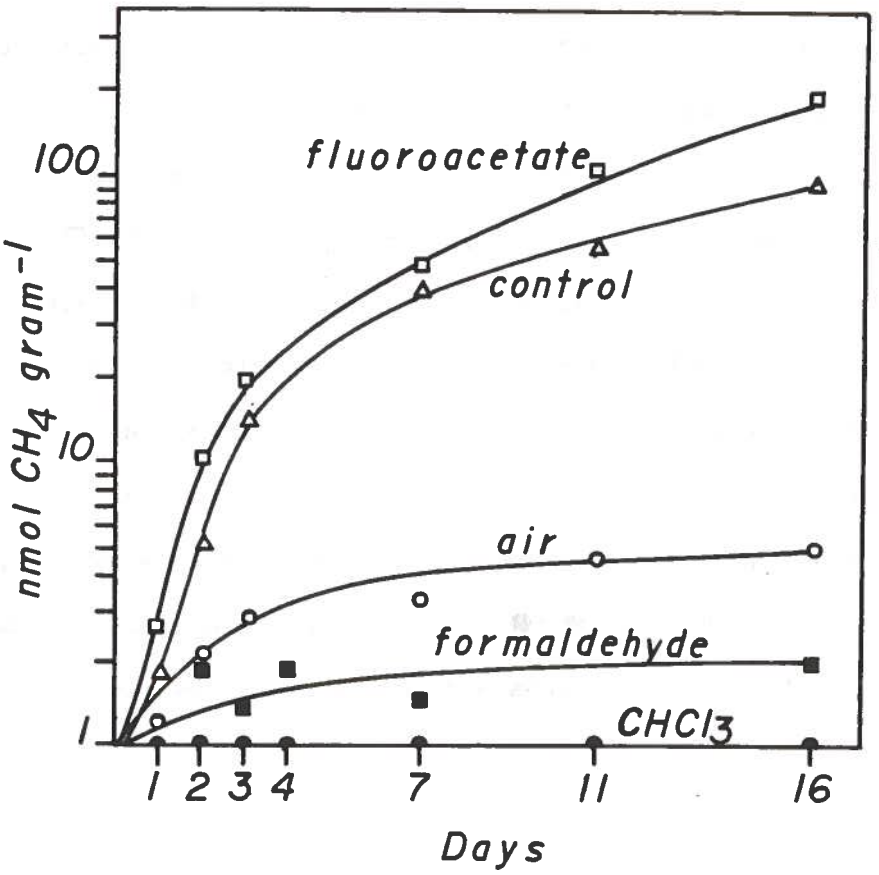

100
10
1
nmol CH_4 $gram^{-1}$
fluoroacetate
control
air
formaldehyde
$CHCl_3$
1 2 3 4 7 11 16
Days

Figure 15. Effect of methanogenesis inhibitors and potentially inhibitory compounds with time on methane production by Site 1, 0.2 m-deep, near-surface sediment samples. 2.5 mM bromoethanesulfonic acid, BrES (□—□); 5 mM KNO_3 (○—○); 100 mM sulfide (▲—▲); and 100 mM sulfate (■—■), were each added to triplicate 50% slurries of delta sediment containing 2.25 ml of H_2 + CO_2 (80%-20%, vol/vol) as added carbon and energy sources. *In vitro* methanogenesis (30° C) was monitored for 45 days and is given as μmol methane per gram sediment, dry weight, from the tube in each set yielding the maximum methane production. Control tubes (●—●) contained 2.25 ml of H_2 + CO_2 (80%-20%, vol/vol) without the addition of inhibitors.

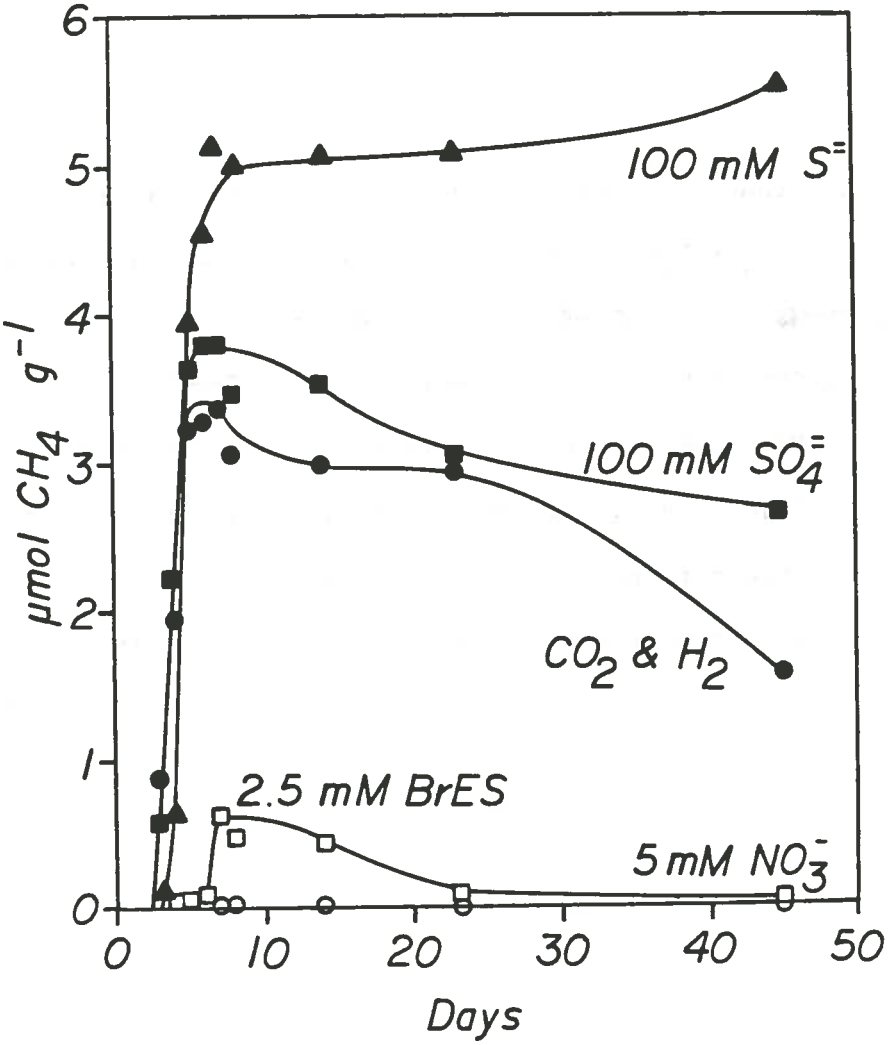
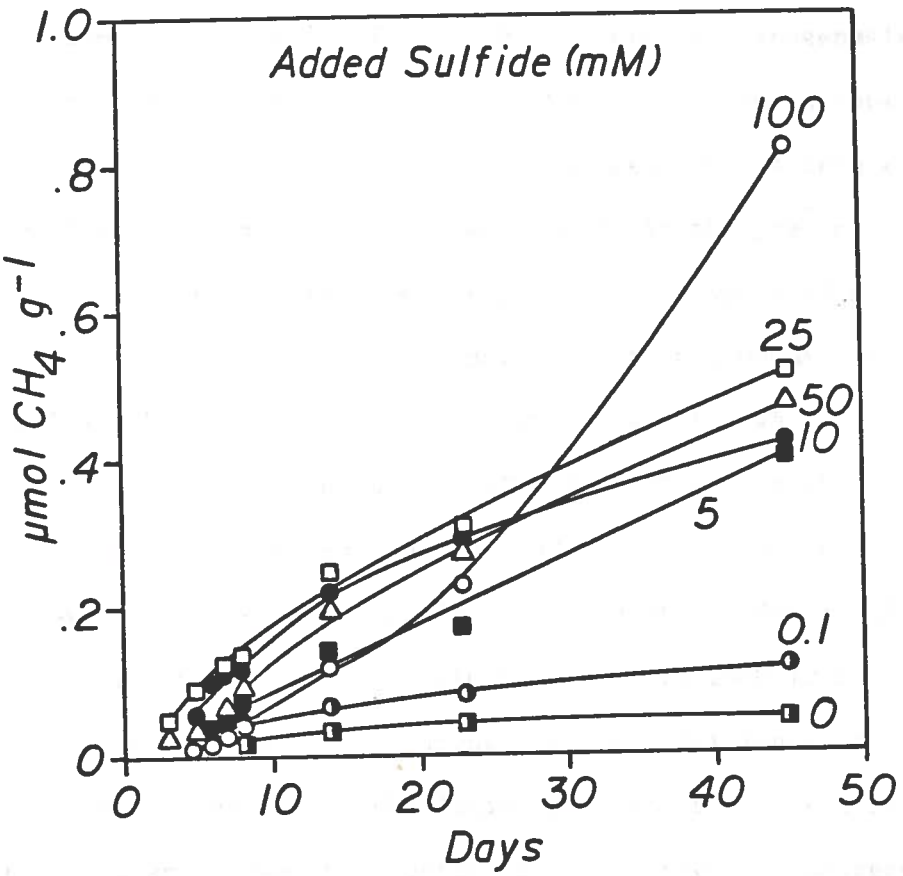

6
5
4
3
2
1
0
µmol CH_4 g^{-1}
100 mM $S^=$
100 mM $SO_4^=$
CO_2 & H_2
2.5 mM BrES
5 mM NO_3^-
0
10
20
30
40
50
Days

Figure 16. Effect of added sulfide on *in vitro* methanogenesis by Site 1, 0.2 m-deep, near-surface sediments. $Na_2S \cdot 9H_2O$, in concentrations of 0, control (◨—◨); 0.1 mM (◐—◐); 5 mM (■—■); 10 mM (●—●); 25 mM (□—□); 50 mM (△—△); and 100 mM (○—○), was added to triplicate sets of anaerobe tubes containing 50% sediment slurries. *In vitro* methanogenesis (30° C) was monitored for 45 days and is given as μmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production.


Added Sulfide (mM)
100
25
50
10
5
0.1
0
μmol CH4 g-1
1.0
.8
.6
.4
.2
0
0
10
20
30
40
50
Days

Added sulfate, in concentrations ranging from 0.1 to 100 mM, appeared to have no inhibitory effect on methanogenesis, but instead, stimulated methanogenesis (0.6-0.9 µmol CH_4/g sediment) above the control value (Fig. 17). With the addition of 100 mM sulfate, no lag period was evident.

The effect on methanogenesis by the addition of 50-5000 mM NaCl to 0.2 m-deep sediment is shown in Figure 18. Initial methanogenesis (days 0-8) appeared to be unaffected by added NaCl in concentrations ranging from 50-2000 mM. However, initial methanogenesis was severely inhibited by 3000 and 5000 mM NaCl. Methanogenesis in the presence of 3000 mM added NaCl recovered after about eight days and appeared not to be inhibited after 13 days of incubation. NaCl at a 5000 mM concentration totally inhibited methanogenesis throughout the 45 day incubation period. The results of added NaCl (50-2000 mM) in the presence of an added carbon and energy source (100 µmol H_2 + CO_2, 80%-20%, vol/vol) are shown in Figure 19. NaCl in the range of 50-1000 mM did not inhibit methanogenesis. However, methanogenesis in 2000 mM NaCl was totally inhibited. It is thus apparent that the addition of a carbon and energy source amplified the inhibitory effect of elevated NaCl concentrations, and showed that metabolically active methanogens were sensitive to 2M NaCl, a concentration which is approximately 4 times greater than the normal *in situ* concentration (0.5 M).

Optimum Temperature for Methanogenesis

Depending on the conditions under which the delta sediments were incubated, the optimum temperature for methanogenesis varied between

Figure 17. Effect of added sulfate on *in vitro* methanogenesis by Site 1, 0.2 m-deep, near-surface sediments. $MgSO_4 \cdot 7H_2O$, in concentrations of 0, control (◑—◑); 0.1 mM (□—□); 0.5 mM (△—△); 25 mM (●—●); and 100 mM (○—○), was added to triplicate sets of anaerobe tubes containing 50% sediment slurries. *In vitro* methanogenesis (30° C) was monitored for 45 days and is given as µmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production.

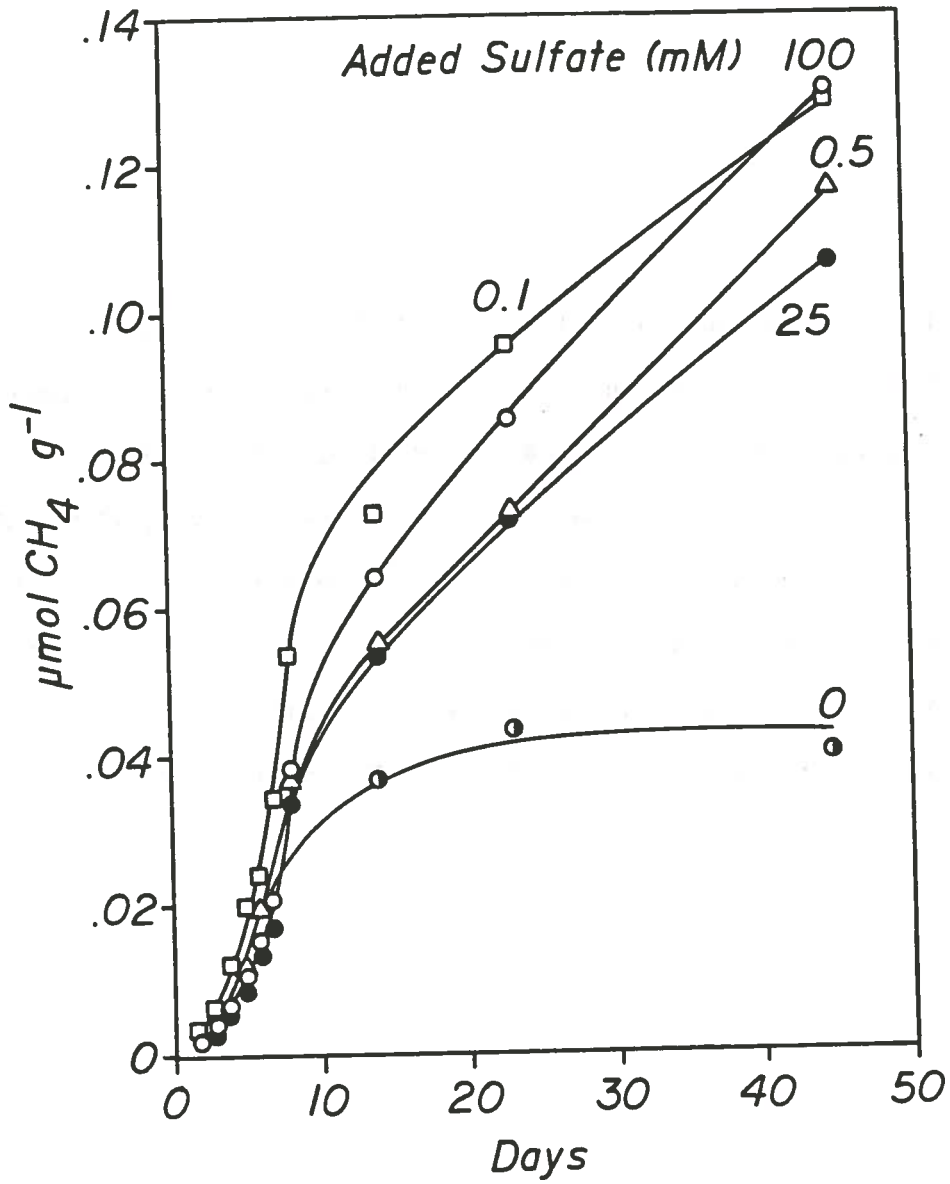

Added Sulfate (mM) 100
0.5
0.1
25
0
.14
.12
.10
.08
.06
.04
.02
0
µmol CH4 g-1
0
10
20
30
40
50
Days

Figure 18. Effect of added NaCl on *in vitro* methanogenesis by Site 1, 0.2 m-deep, near-surface sediments. NaCl, in concentrations of 50 mM (△—△); 1000 mM (□—□); 2000 mM (●—●); 3000 mM (○—○); and 5000 mM (□—□), was added to triplicate sets of anaerobe tubes containing 50% sediment slurries. *In vitro* methanogenesis (30° C) was monitored for 45 days and is given as μmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production. The results of the control tubes are shown in Fig. 17.

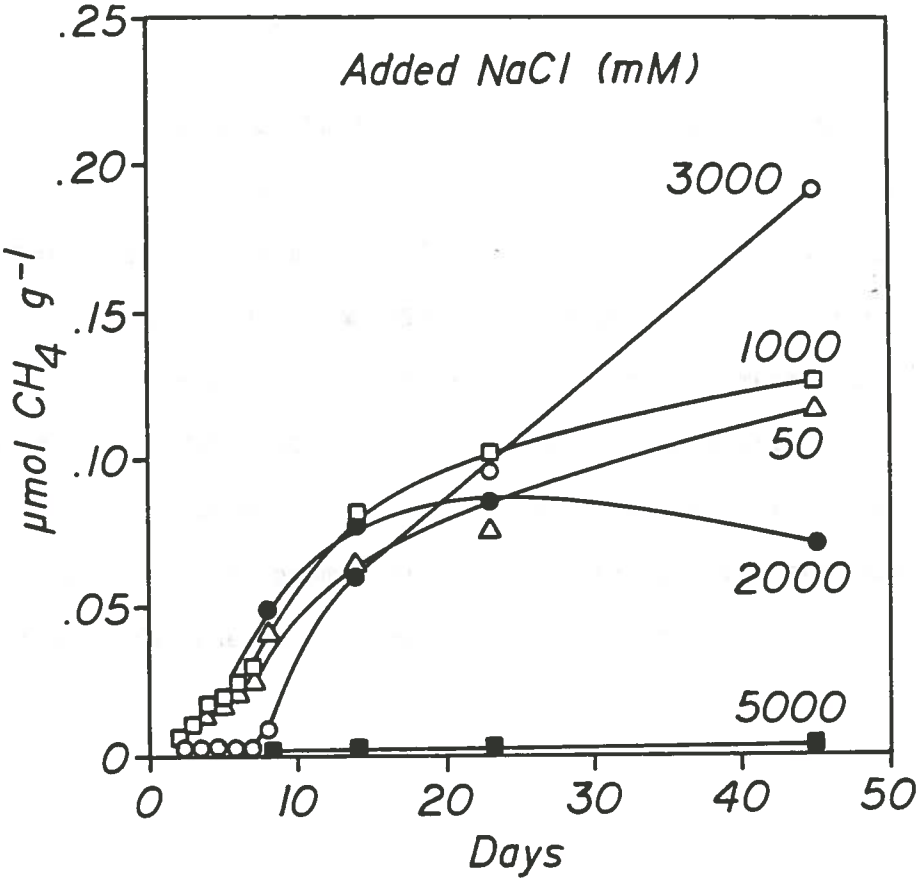
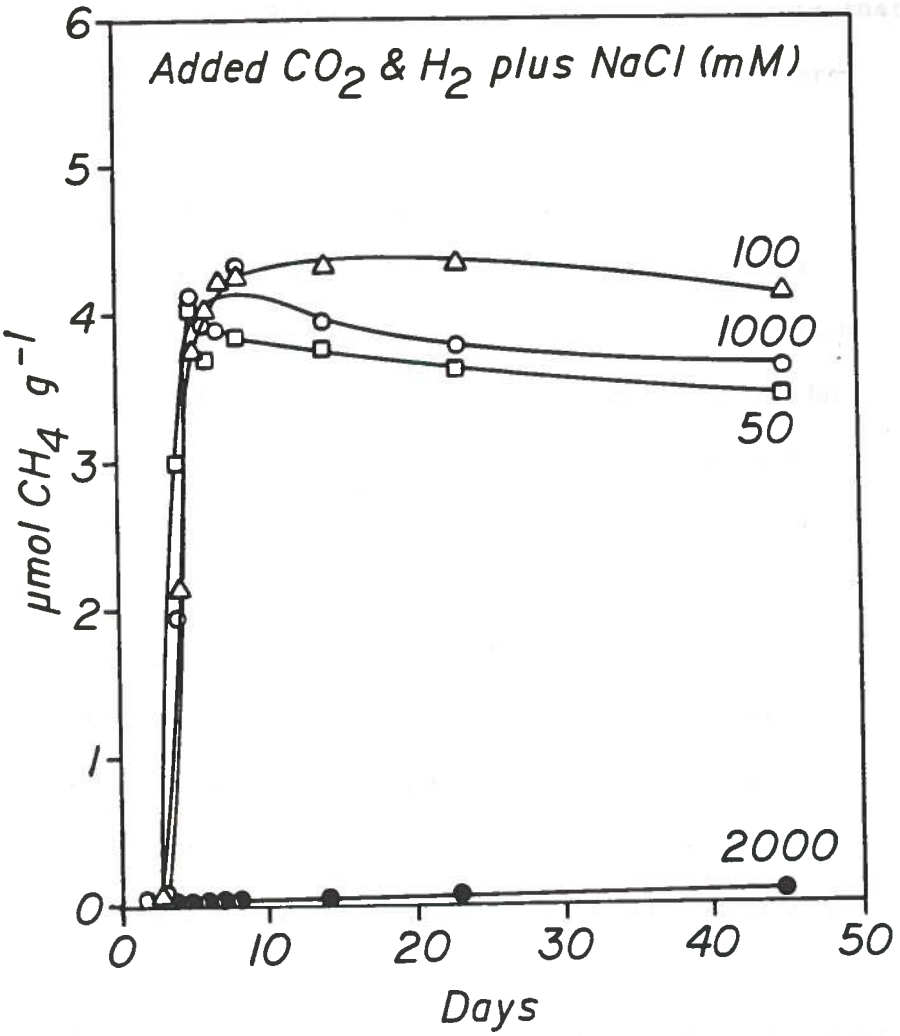

Added NaCl (mM)
3000
1000
50
2000
5000
.25
.20
.15
.10
.05
0
μmol CH_4 g^{-1}
0
10
20
30
40
50
Days

Figure 19. Effect of added NaCl on *in vitro* methanogenesis by Site 1, 0.2 m-deep, near-surface sediments in the presence of CO_2 plus H_2. NaCl, in concentrations of 50 mM (□—□); 100 mM (△—△); 1000 mM (○—○); and 2000 mM (●—●), was added to triplicate sets of anaerobe tubes containing 50% slurries of delta sediments plus 2.25 ml of H_2 + CO_2 (80%-20%, vol/vol) as carbon and energy sources. *In vitro* methanogenesis was monitored for 45 days and is given as µmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production.


Added CO_2 & H_2 plus NaCl (mM)
100
1000
50
2000
µmol CH_4 g^{-1}
0
1
2
3
4
5
6
0
10
20
30
40
50
Days

35 and 50° C. Figure 20 shows methanogenesis in unadulterated, 0.2 m-deep sediments that were incubated at temperatures ranging from 20-50° C. The optimum temperature in undiluted sediments was 45° C. After an incubation period of 18 days, the methane production at 45° C was 2000 times greater than in sediments incubated at the *in situ* temperature of 20° C. After 18 days of incubation, the sediments that were held at 45 and 50° C showed a decrease in methane levels, presumably due to anaerobic oxidation of methane (see below).

Methanogenesis in sediments that were diluted to a 50% slurry (as previously described) showed a slightly different response to temperature (Fig. 21). The optimum temperature for initial methanogenesis in 50% sediment slurries was 45-50° C. After about 10 days of incubation, the highest rate of methanogenesis occurred at 50° C. After 28 days of incubation, methanogenesis at 50° C was 45 times greater than at 20° C. Methane consumption in extended incubations at 45-50° C (longer than 30 days) was of a lesser magnitude in 50% sediment slurries than in undiluted sediments. Methane production in undiluted sediment at the optimum temperature of 45° C was 10 times greater than in 50% sediment slurries at an optimum temperature of 50° C (both values normalized to µmol CH_4 produced per g sediment, dry weight).

When 100 µmol H_2 + CO_2 (80%-20%, vol/vol) was added to 50% sediment slurries, the optimum temperature for methanogenesis was 35-40° C (Fig. 22). Methanogenesis was lower at temperatures of 20, 30, and 45° C, and extremely low at 50° C.

Figure 20. Optimum temperature for *in vitro* methanogenesis in unadulterated Site 1, 0.2 m-deep, near-surface sediments. *In vitro* methanogenesis was monitored in triplicate, undiluted sediment samples for 45 days at the following temperatures: 20° C (●—●); 30° C (■—■); 35° C (▲—▲); 40° C (□—□); 45° C (○—○); and 50° C (△—△). Methane production is given as μmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production.

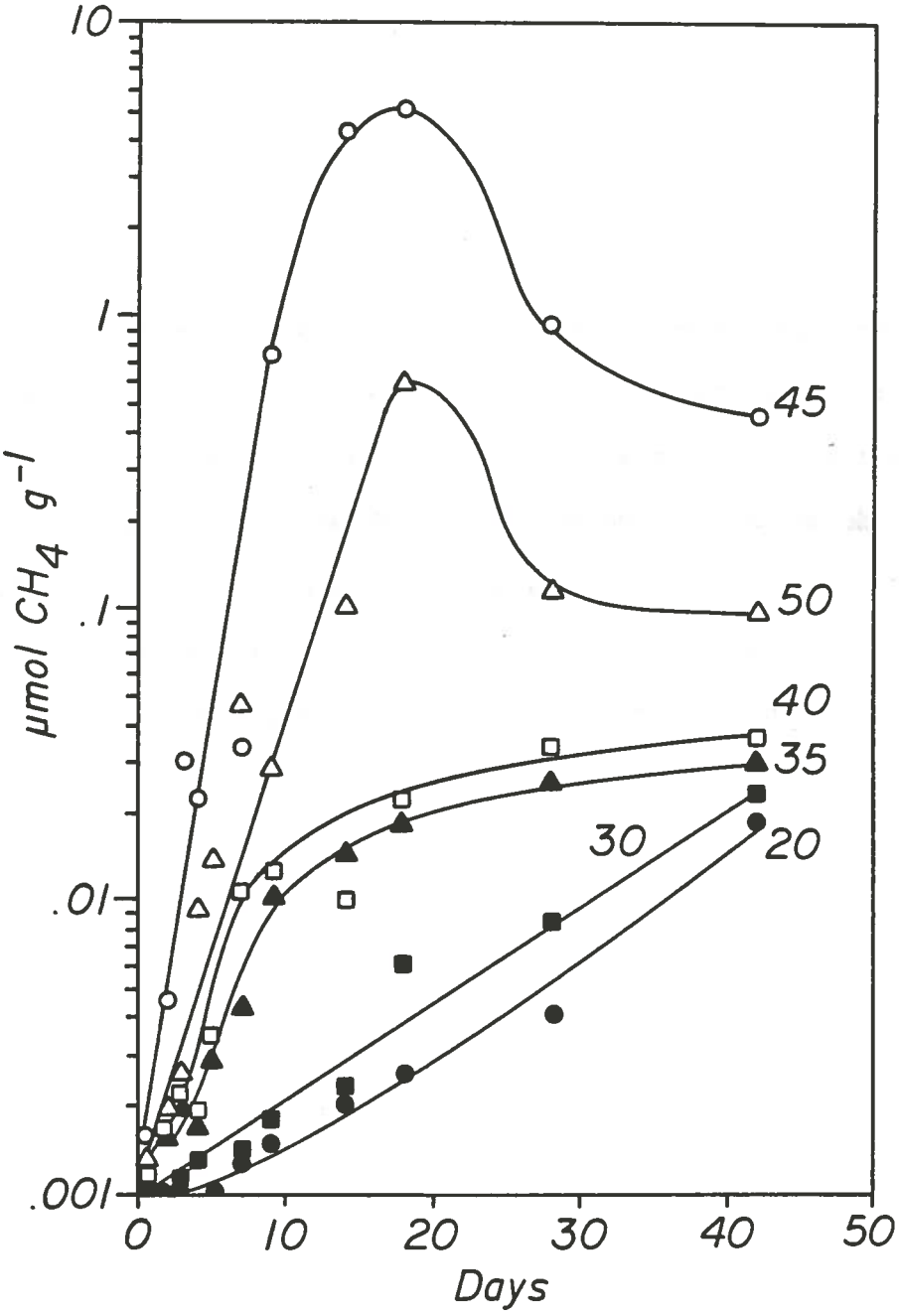

10
1
.1
.01
.001
µmol CH_4 g^{-1}
0
10
20
30
40
50
Days
45
50
40
35
30
20

Figure 21. Optimum temperature for *in vitro* methanogenesis in Site 1, 0.2 m-deep, near-surface sediment slurries. *In vitro* methanogenesis was monitored in triplicate 50% sediment slurries for 45 days st the following temperatures: 20° C (●—●); 30° C (■—■); 35° C (▲—▲); 40° C (□—□); 45° C (○—○); and 50° C (△—△). Methane production is given as μmol methane produced per gram sediment, dry weight, from the tube in each set yielding the maximum methane production.

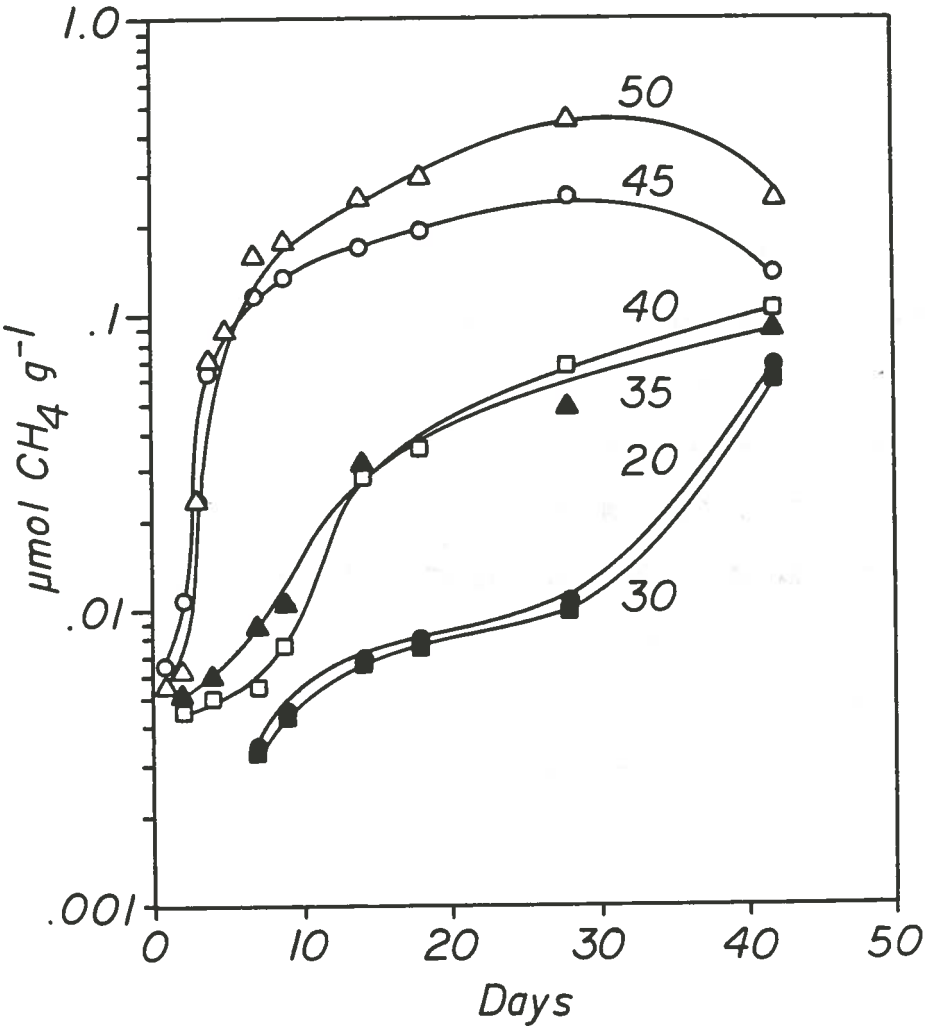
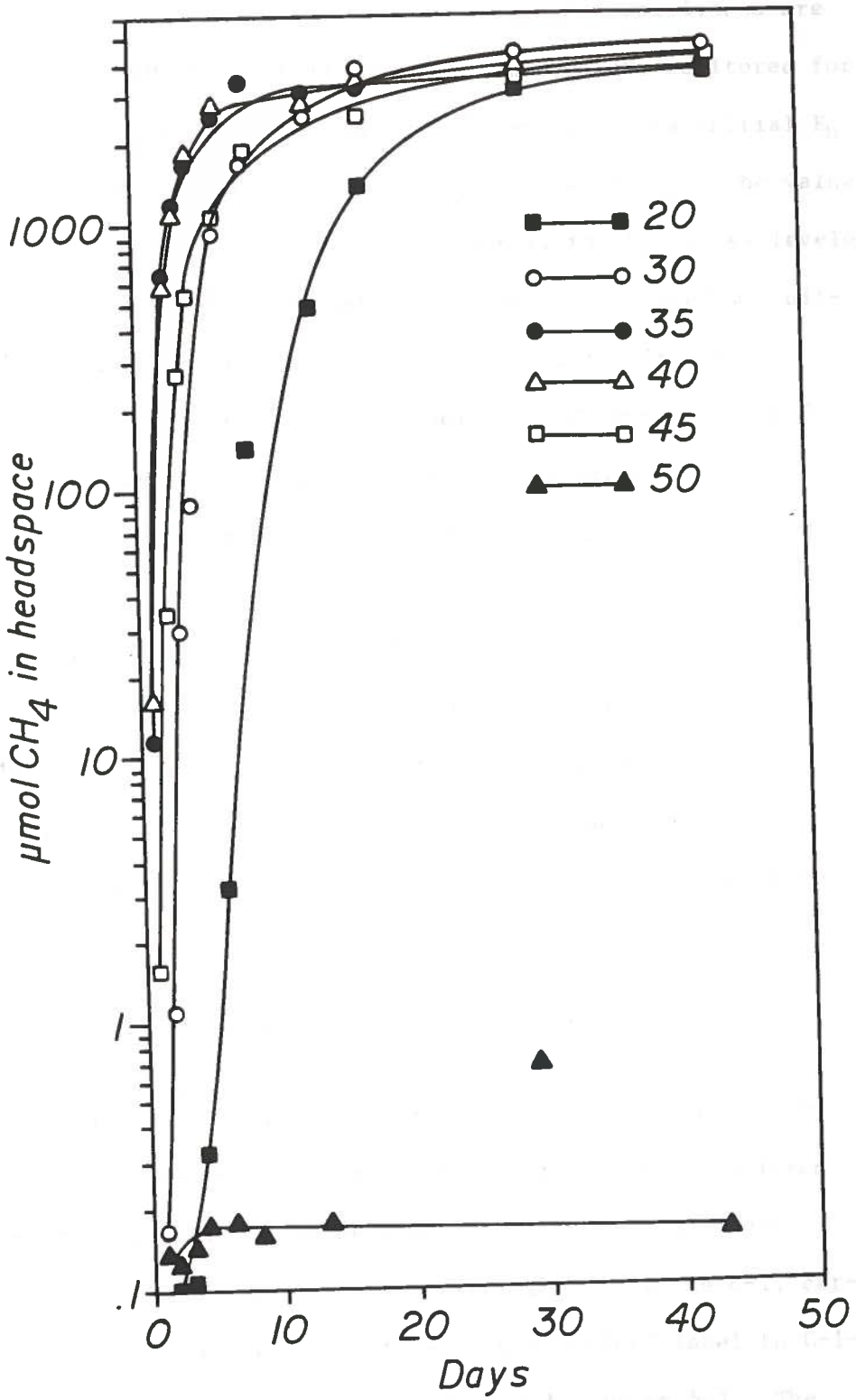

1.0
.1
.01
.001
µmol CH_4 g^{-1}
50
45
40
35
20
30
0
10
20
30
40
50
Days

Figure 22. Optimum temperature for *in vitro* methanogenesis in Site 1, 0.2 m-deep, near-surface sediment slurries containing added $CO_2 + H_2$ as carbon and energy sources. *In vitro* methanogenesis was monitored in triplicate sets of 50% sediment slurries for 45 days at the following temperatures: 20° C (■—■); 30° C (○—○); 35° C (●—●); 40° (△—△); 45° C (□—□); and 50° C (▲—▲). The sediment slurries each received 2.25 ml $H_2 + CO_2$ (80%-20%, vol/vol). Methane production is given as total µmol methane produced from the tube in each set yielding the maximum methane production.


1000
100
10
1
.1
µmol CH_4 in headspace
0
10
20
30
40
50
Days
20
30
35
40
45
50

Changes in pH and E_h During Methanogenesis

The results of four separate sediment microcosm experiments are shown in Figure 23. Methane production, pH, and E_h were monitored for 27 days in four reaction vessels. In each experiment, the initial E_h was within the range of -300 to -350 mV, which is well below the value necessary to promote methanogenesis. In general, the E_h values leveled off between -300 to -375 mV. The pH in each reaction vessel was different, and generally remained constant throughout the incubation period. The pH in the four reaction vessels ranged from 6.9 to 8.2. Methane production in each reaction vessel peaked initially after 5 days of incubation, and then decreased markedly. Methane production in reaction vessel 1 continued to decline, while in reaction vessel 2 it remained constant throughout the 27 day incubation period. In reaction vessels 3 and 4, however, methane production resumed after 8 days of incubation and continued to increase throughout the incubation period. Only in reaction vessel 4 did methane production at the end of the 27 day incubation period exceed that of the initial peak on day 5.

Methanogenesis from ^{14}C-acetate

The production of $^{14}CH_4$ from ^{14}C-1-acetate and ^{14}C-2-acetate by East Bay sediment is shown in Table 7. In 5 out of 7 samples (from depths of 0.075-29.0 m below the sediment-water interface), more $^{14}CH_4$ was derived from the C-2, methyl carbon than from the C-1, carboxyl carbon of acetic acid. The ratios of C-2-derived label to C-1-derived label in these 5 samples ranged from 6.1:1 to 46.5:1. The

Figure 23. Measurement of changes in redox potential (E_h) and pH during *in vitro* methanogenesis by Site 1, 0.2 m-deep, near-surface sediments. *In vitro* methanogenesis was monitored for 27 days in four one-liter reaction vessels constructed according to the design of Patrick, *et al.* (45). Methane production (○—○); E_h (□—□); and pH (△—△) were each monitored on a daily basis. Each reaction vessel contained 400 g of a 50% sediment slurry maintained under a N_2 atmosphere at 30^{o} C.

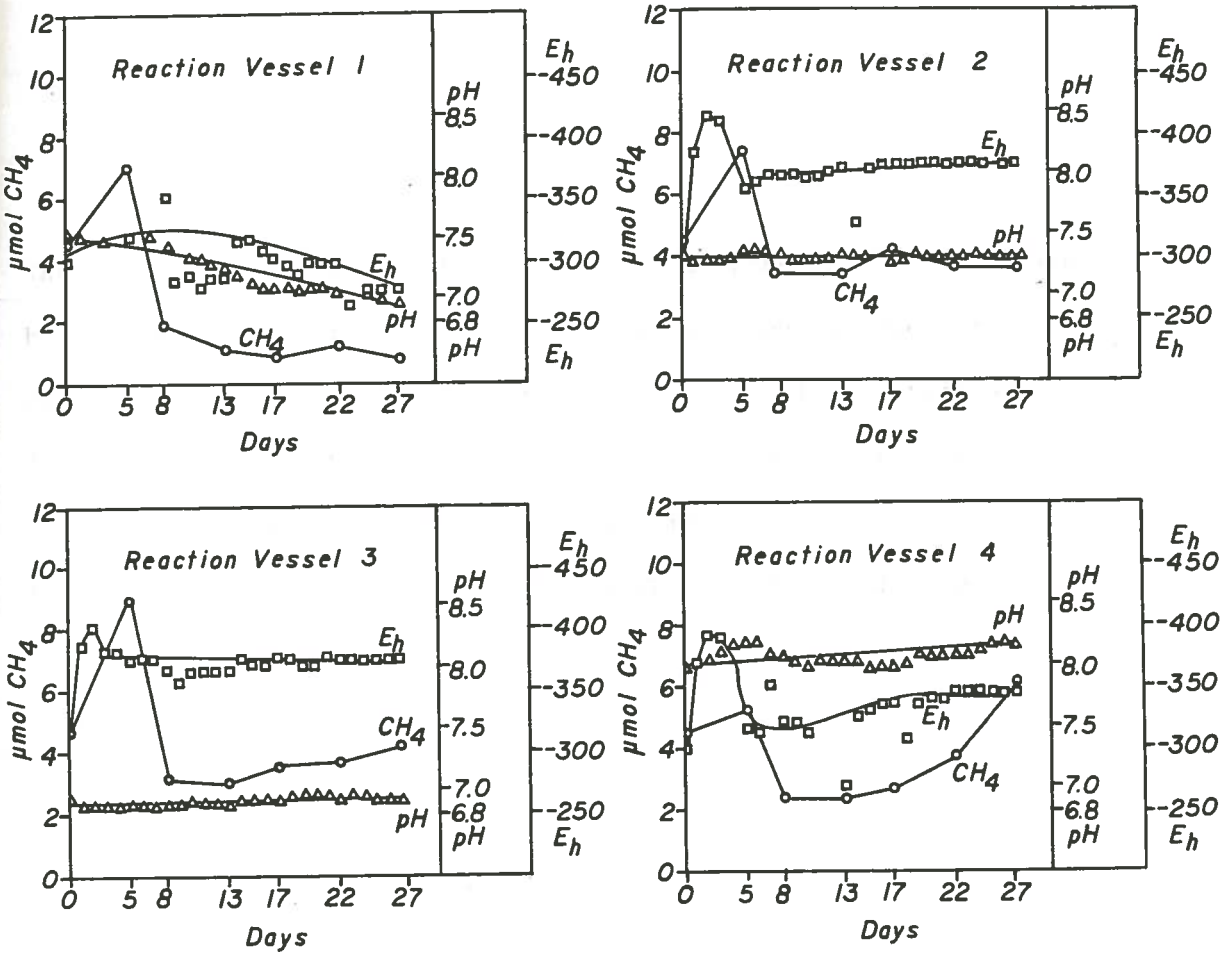

Reaction Vessel 1
Reaction Vessel 2
Reaction Vessel 3
Reaction Vessel 4
µmol CH4
Days
Eh
pH
CH4
0 5 8 13 17 22 27
0 2 4 6 8 10 12
pH 8.5 8.0 7.5 7.0 6.8
Eh -450 -400 -350 -300 -250

Table 7. Production of $^{14}CH_4$ from ^{14}C-1-acetate and ^{14}C-2-acetate by methanogenic delta samples. East Bay sediment samples from depths ranging from 0.075-29.0 m were given labeled acetate and incubated for 10 days at 30° C. The production of $^{14}CH_4$ from the C-1 and C-2 labeled carbons of acetate are given as dpm, corrected for background. The ratio of C-2/C-1 dpm are listed in the column to the far right.

Production of $^{14}CH_4$ from ^{14}C-1-acetate and ^{14}C-2-acetate by Methanogenic Mississippi River Delta Samples

	$^{14}CH_4$ Produced from:		
Depth (m)	^{14}C-1-acetate	^{14}C-2-acetate	C-2/C-1 Ratio
0.075	15	11	0.7
0.75	39	457	11.7
1.5	65	53	0.8
2.9	35	1005	28.7
3.6	561	6194	11.0
11.2	13	604	46.5
29.0	17	103	6.1

greatest formation of $^{14}CH_4$ from both ^{14}C-1-acetate and ^{14}C-2-acetate occurred in the sediment samples from a depth of 3.6 m. The high C-2/C-1 ratios indicate that acetate-utilizing methanogens were present in the sediment samples, and that the methyl carbon of acetate was the preferred carbon for reduction to methane.

Physiological Characterization of *Desulfovibrio* and *Desulfotomaculum* Isolates

The growth characteristics of the 75 *Desulfovibrio* and 5 *Desulfotomaculum* isolates are listed in Tables 8 and 9, respectively. In addition to growth on lactate plus sulfate, all 75 *Desulfovibrio* isolates grew on pyruvate and malate. Only 17 of the 75 isolates were able to grow on choline. None were able to utilize acetate or formate. All 75 isolates were highly resistant to the antibiotic Hibitane, and all produced the pigment desulfoviridin. None of the isolates grew in the absence of 3% NaCl, or at 55° C.

Table 9 lists similar tests on the growth characteristics of the 5 *Desulfotomaculum* isolates. Growth occurred with lactate, pyruvate, and malate, but only in the presence of sulfate. No growth occurred on choline, acetate, or formate. The 5 *Desulfotomaculum* isolates were very sensitive to Hibitane. Four out of five were unable to tolerate 2.5 µg/ml Hibitane, and none grew in 10 µg/ml. None of the *Desulfotomaculum* isolates produced desulfoviridin, grew in media without added NaCl, or at 55° C.

The minimum NaCl requirement of a representative strain of the *Desulfovibrio* and *Desulfotomaculum* isolates is shown in Figure 24. *Desulfovibrio* failed to grow in 3% NaCl, or less. Growth in 3.5%

Table 8. Physiological characteristics of *Desulfovibrio* isolates (75 strains). The *Desulfovibrio* isolates were obtained from Site 1, 0.2 m-deep, near-surface sediments and were tested for their ability to grow on five different carbon sources (in addition to lactate) based upon the taxonomic criteria of Postgate and Campbell (50). Growth of the isolates was accomplished in anaerobic broth tubes supplemented with substrates, as indicated. Positive growth resulted in turbidity and/or blackening of the medium.

Physiological Characteristics of *Desulfovibrio* Isolates (75 strains)

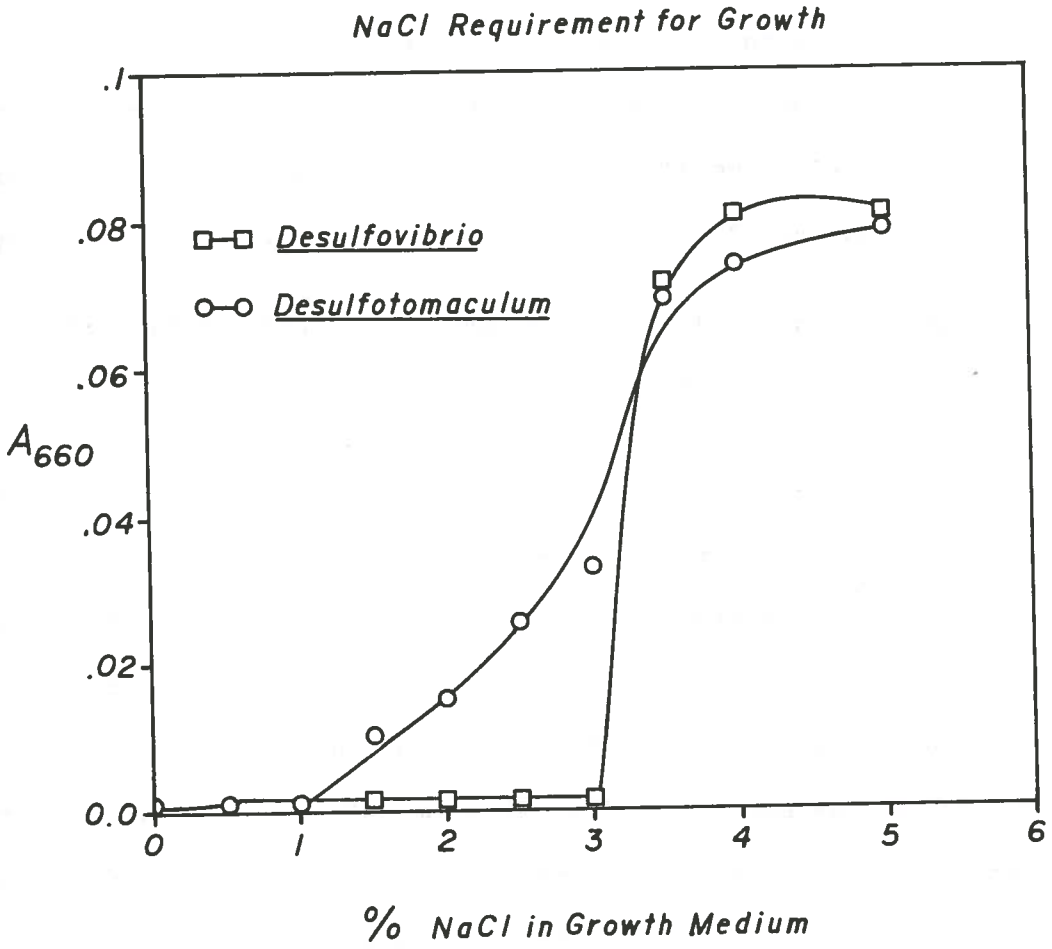
Growth Conditions	Number of Isolates
pyruvate plus sulfate	75
pyruvate w/o sulfate	75
malate plus sulfate	75
choline w/o sulfate	17
acetate plus sulfate	0
formate plus sulfate	0
resistance to 1000 µg/ml Hibitane	75
production of desulfoviridin	75
growth in NaCl-free media	0
growth at 55°C	0

Table 9. Physiological characteristics of *Desulfotomaculum* isolates (5 strains). The *Desulfotomaculum* isolates were obtained from Site 1, 0.2 m-deep, near-surface sediments and were tested for their ability to grow on five different carbon sources (in addition to lactate) based upon the taxonomic criteria of Postgate and Campbell (50). Growth of the isolates was accomplished in anaerobic broth tubes supplemented with substrates, as indicated. Positive growth resulted in turbidity and/or blackening of the medium.

Physiological Characteristics of *Desulfotomaculum* Isolates (5 strains)

Growth Conditions	Number of Isolates
pyruvate plus sulfate	5
pyruvate w/o sulfate	0
malate plus sulfate	5
choline w/o sulfate	0
acetate plus sulfate	0
formate plus sulfate	0
Hibitane resistance: 0.25 µg/ml	5
1.0	5
2.5	1
10	0
25	0
1000	0
production of desulfoviridin	0
growth in NaCl-free media	0
growth at 55°C	0

Figure 24. Requirement of NaCl for growth of representative *Desulfovibrio* and *Desulfotomaculum* isolates. The isolates were grown at 35° C in Postgate's enrichment medium (49) containing lactate plus sulfate with NaCl concentrations ranging from 0-5%. Absorbance at 660 nm (A_{660}) of ninety-six hour old cultures was measured with a Bausch and Lomb Spectronic 20 spectrophotometer.


NaCl Requirement for Growth
.1
.08
.06
.04
.02
0.0
A_{660}
Desulfovibrio
Desulfotomaculum
0
1
2
3
4
5
6
% NaCl in Growth Medium

NaCl (the approximate *in situ* concentration in the delta sediments) was nearly as great as that at the optimum NaCl concentration of 4%. The minimum NaCl requirement of the *Desulfotomaculum* isolates was not as restrictive. It failed to grow in 1% NaCl, but grew slowly in 1.5% NaCl. Optimum growth occurred in 3.5% NaCl, and higher.

The optimum growth temperature of the representative *Desulfovibrio* and *Desulfotomaculum* isolates is shown in Figure 25. Growth of each occurred in the range of 20-40° C, with an optimum growth temperature of 30° C. No growth occurred at 50° C, or higher.

Photo- and Electron-Microscopy of Sulfate-Reducer Isolates

All 75 *Desulfovibrio* isolates were vibrio-shaped cells and measured 3-5 µm long by 0.6 µm wide. Cells from older cultures (one week) were somewhat longer and spiral-like. Morphologically, all 75 *Desulfovibrio* isolates were identical to the cells shown in Figure 26, A and B. Electron microscopic examination of cells, negatively stained with 1% phosphotungstate (Fig. 26A), showed that the *Desulfovibrio* isolate had a single, polar flagellum. A Nomarski interference photomicrograph of a representative, 96 hour old culture of *Desulfovibrio* is shown in Figure 26B. The *Desulfovibrio* isolates often contained 1-2 electron-dense bodies of unknown composition (Fig. 26A).

The five *Desulfotomaculum* isolates were rod-shaped cells measuring 3-6 µm long by 1.5 µm wide. The cells had blunt, rounded ends, and were often seen in pairs (Fig. 27). Electron microscopic examination of cells negatively stained with 1% phosphotungstate (Fig. 27A) showed that the *Desulfotomaculum* isolate was peritrichously flagellated.

Figure 25. Optimum growth temperature of representative *Desulfovibrio* and *Desulfotomaculum* isolates. The isolates were grown in Postgate's enrichment medium (49) containing lactate plus sulfate at temperatures ranging from 20-60° C. Absorbance at 660 nm (A_{660}) of ninety-six hour old cultures was measured with a Bausch and Lomb Spectronic 20 spectrophotometer.

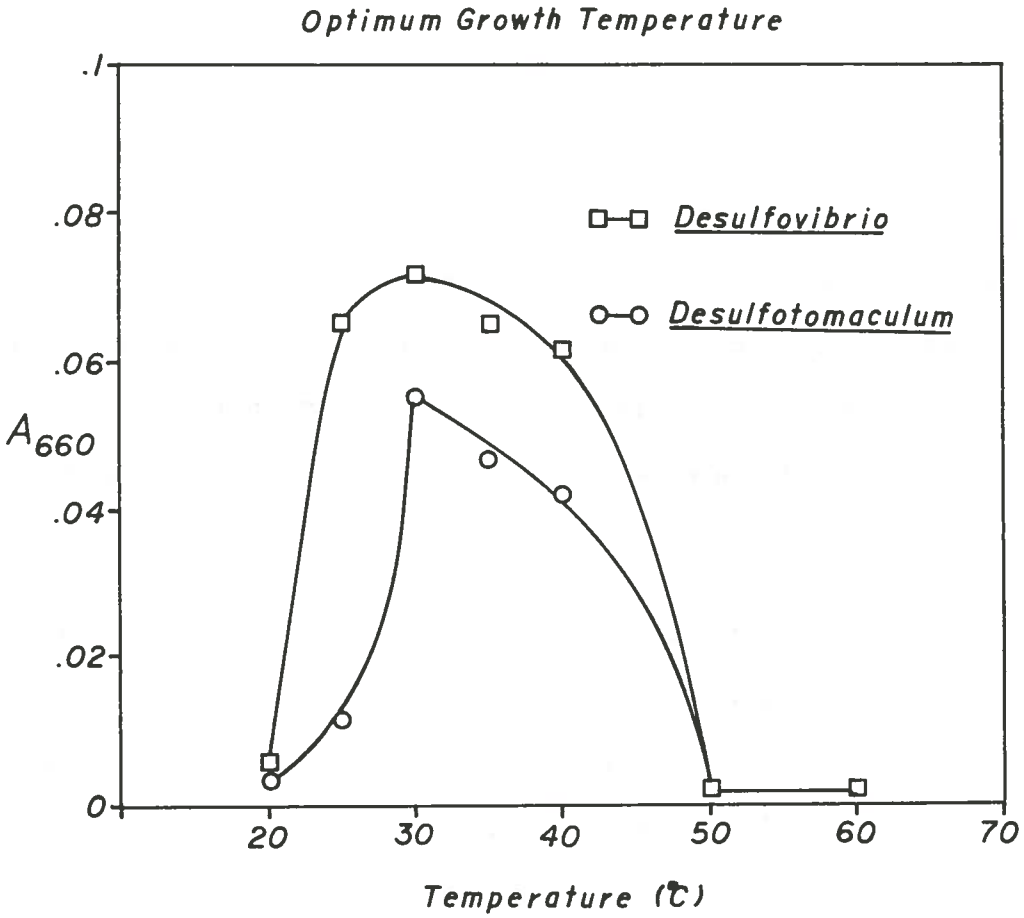

Optimum Growth Temperature
.1
.08
.06
.04
.02
0
A_{660}
Desulfovibrio
Desulfotomaculum
20
30
40
50
60
70
Temperature (°C)

Figure 26 A. Electron micrograph of a representative *Desulfovibrio* isolate. The cell was negatively stained with 1% phosphotungstate and viewed in a JEL 100CX transmission electron microscope at a magnification of 23,000 X. The cell possessed a single polar flagellum. The bar represents 1.0 µm.

Figure 26 B. Nomarski interference photomicrograph of a representative 96 hour old culture of a *Desulfovibrio* isolate grown in Postgate's enrichment medium (49). The isolate was vibrio-shaped and measured 3-5 µm by 0.6 µm, and was highly motile. The bar represents 10 µm.

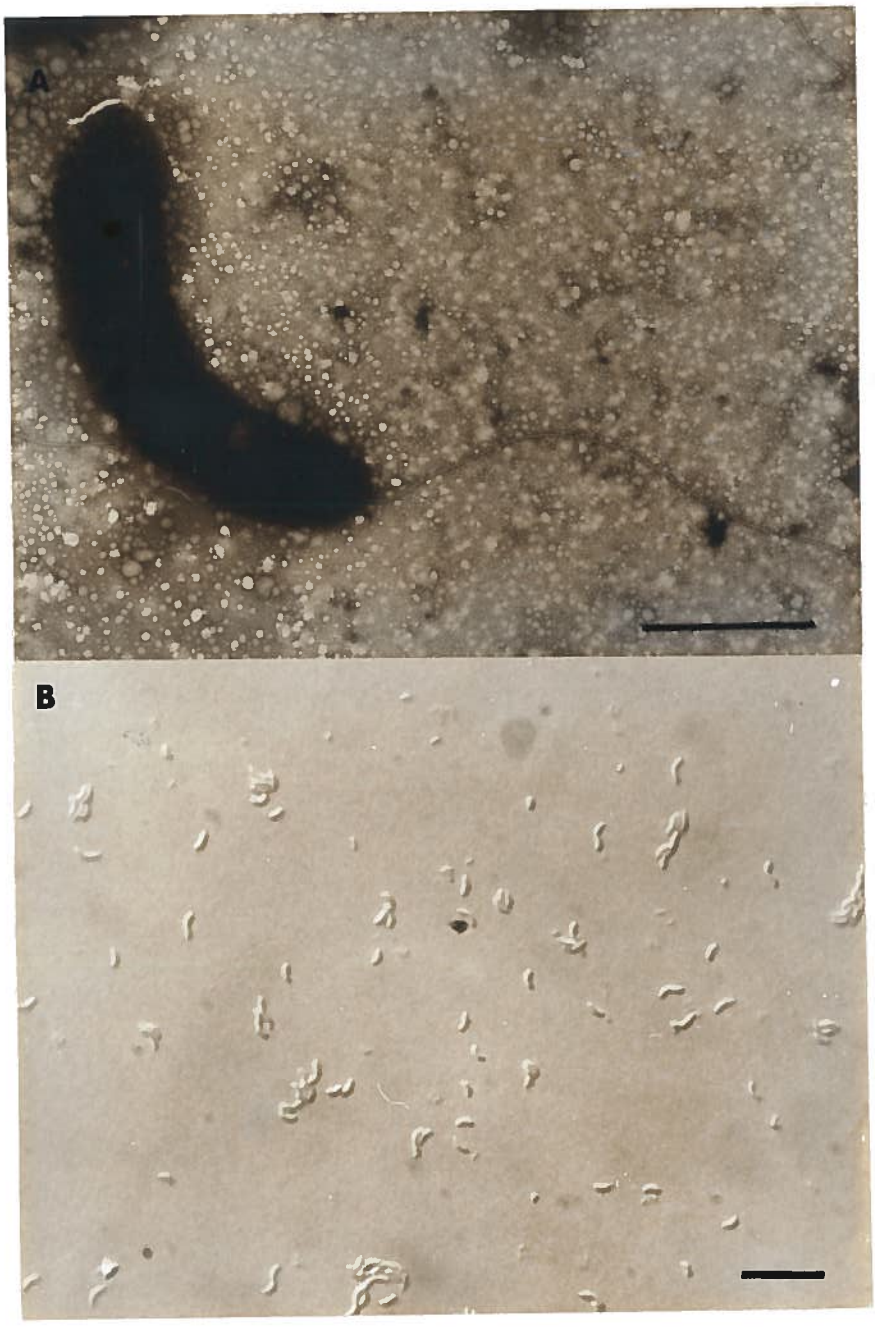

A
B

Figure 27 A. Electron micrograph of a representative *Desulfotomaculum* isolate. The cell was negatively stained with 1% phosphotungstate and viewed in a JEL 100CX transmission electron microscope at a magnification of 8,600 X. The cells often occurred in pairs and were peritrichously flagellated. The flagella were extremely fragile and some were broken off during specimen preparation. The bar represents 1.0 µm.

Figure 27 B. Nomarski interference photomicrograph of a representative 96 hour old culture of a *Desulfotomaculum* isolate grown in Postgate's enrichment medium (49). The isolate was rod-shaped and measured 3-6 µm by 1.5 µm, and exhibited a slow, tumbling motility. The bar represents 10 µm.

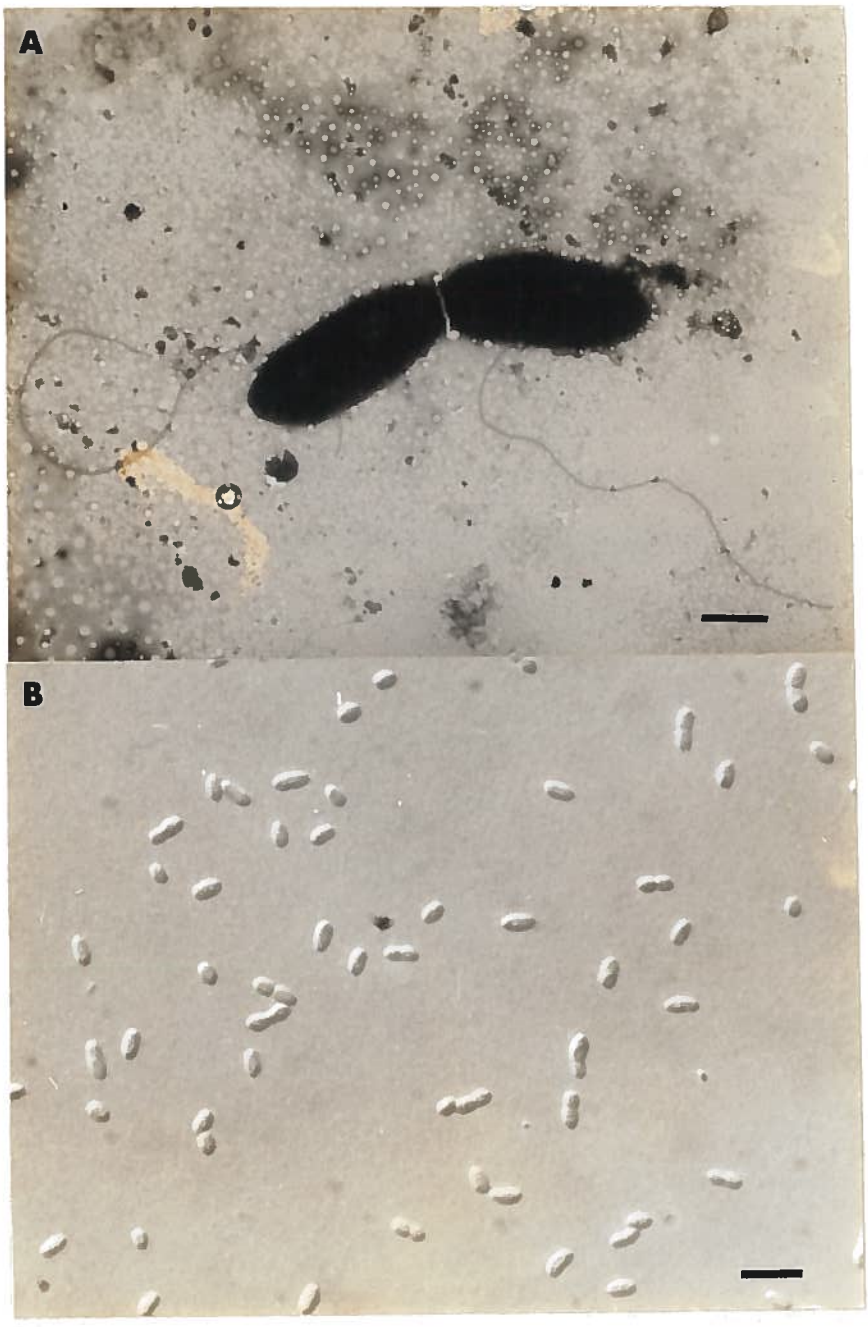

A
B

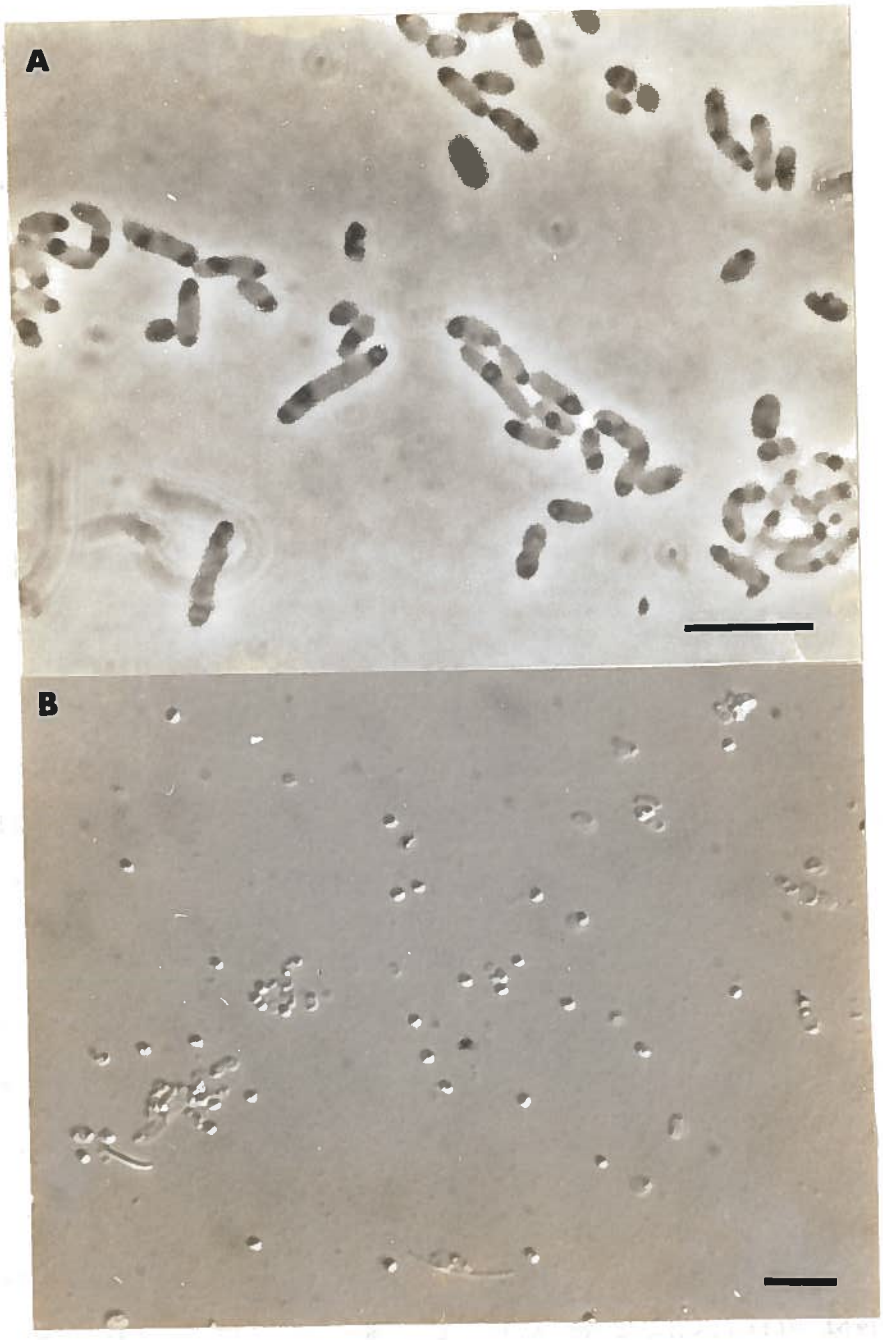
Figure 27B is a Nomarski interference photomicrograph of a representative 96 hour old culture of a *Desulfotomaculum* isolate. Figure 28A is a phase contrast photomicrograph of a week old culture showing that terminal to subterminal endospores had formed within some of the cells. Phase-transparent regions of unknown composition were also observed in some cells. The cells in 7-10 day old cultures usually became rounded and later lysed, releasing spherical, refractile endospores (Fig. 28B).

Attempted Isolation of Methanogens

Attempts to isolate methanogens from numerous samples of delta sediments were unsuccessful. The media used in these attempts (Tables 1-3) contained a variety of substrates and other components, and basically, consisted of variations of the following four constituents: (i) a carbon source; acetate, bicarbonate, formate, N-methyl compounds, or methanol; (ii) vitamins and/or other growth factors; yeast extract, trypticase, and HS-CoM; (iii) salts and minerals; and (iv) reducing agents and a redox indicator; sulfide, cysteine, and thioglycollate, plus resazurin. Several experiments were undertaken to isolate pure cultures of delta methanogens employing variations of these media (Tables 1-3). Nearly all were useful in enriching for methanogenic bacteria in broth culture, but all failed to support growth of methanogens in extinction dilution tubes, roll tubes (25), and anaerobic streak plates (21). It became apparent that some unknown growth factor was missing from these media, and that it was very likely that some non-methanogenic organisms growing in the mixed-population

Figure 28 A. Phase contrast photomicrograph of a representative 96 hour old culture of a *Desulfotomaculum* isolate grown in Postgate's enrichment medium (49). Some cells formed terminal to subterminal endospores. The bar represents 10 µm.

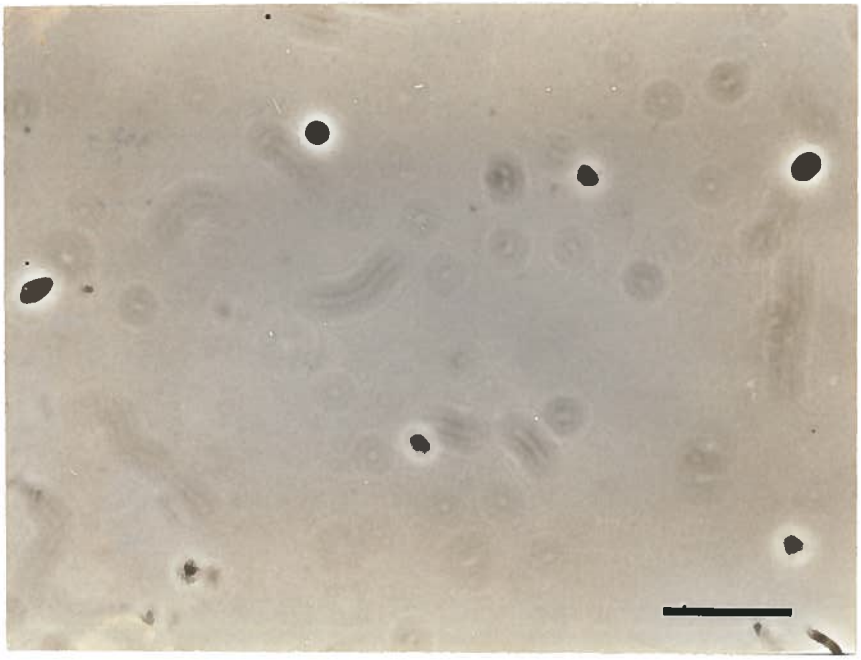
Figure 28 B. Nomarski interference photomicrograph of a representative *Desulfotomaculum* isolate grown in Postgate's enrichment medium (49). The rods rounded up and lysed, releasing spherical endospores. The bar represents 10 µm.


A
B

methanogenic enrichments were supplying the unknown growth factor(s). To test this possibility, methanogenic enrichments were grown at 35° C for 2 weeks and centrifuged in N_2-flushed, anaerobe tubes to remove all cells. The anaerobic supernatant fluid was filter sterilized and added to extinction dilution tube media. The addition of cell-free, spent enrichment media to extinction dilution, roll tube, and anaerobic streak plate media did not aid in the isolation of delta methanogens. The addition of D-cycloserine and penicillin (27) to enrichment media in order inhibit non-methanogenic contaminants also failed to select for methanogens. At the time of this writing, another attempt to supply unknown growth factors to isolation media is underway using clarified rumen fluid. Whether or not this addition will be useful is at present unknown.

From the very first successful methanogenic enrichments of delta sediments, it was obvious that a single morphological group of microorganisms dominated the enrichment cultures. The microorganisms were irregularly coccoid in shape, appeared phase-dense under phase contrast microscopy, and measured 1-3 μm in diameter. The coccoid cells were especially prevalent in media containing methanol, and N-methyl compounds (methylamine, trimethylamine). The cells were extremely sensitive to manipulation during preparation for photomicrographic examination, and readily lysed. Figure 29 is a phase contrast photomicrograph of a methanogenic enrichment culture dominated by iregularly coccoid microorganisms that were morphologically identical to those observed in numerous other methanogenic enrichments using a variety of different growth media.

Figure 29. Phase contrast photomicrograph of a representative methanogenic enrichment culture grown in Methylamine-Methanol (MM) medium (see Table 2). The culture was dominated by irregularly coccoid cells measuring 1.5-3.0 µm in diameter, and are presumably the methanogenic organisms in the mixed culture. The bar represents 10 µm.



DISCUSSION

Ecology of Methanogens and Sulfate-Reducers in Delta Sediments

Methanogenesis in the sediments of the Mississippi River delta is an important diagenetic process of this anaerobic ecosystem. Not only does methanogenesis play a significant role in anaerobic carbon cycling in this environment, but it also results in the accumulation of large quantities of methane gas within the sediments. The methane gas is, in large part, responsible for the unconsolidation and instability that is so characteristic of alluvial delta sediments. This study investigated the distribution and physiology of the obligately anaerobic microorganisms that are believed to be mainly responsible for the final processes of anaerobic degradation of organic materials in the delta: methanogens and sulfate-reducers.

This was the first attempt to determine the extent of the depth distribution of methanogens and sulfate-reducers in a series of deep core samples from a marine environment. The depth distribution of methanogens and sulfate-reducers in the delta sediments was extensive. Methanogens were observed in high numbers in the upper 7.5 m of Site 1 sediments, and in the upper 11.3 m of Site 2 sediments. Viable methanogens were found as deep as 29.3 m below the sediment-water interface at Site 2. Similarly, sulfate-reducers existed in high numbers in the upper 3 m of Site 1 sediments, and in the upper 45.1 m of Site 2 sediments. They reached a maximum depth of 75.0 m at Site 3.

Previous studies on the depth distribution of methanogens and sulfate-reducers in marine environments (28, 56) were limited to the

examination of the upper 5 m of sediment. Methanogens in stable sediments of the Cariaco Trench were restricted to the upper 81 cm of sediment (MPNs of 20-200 cells per cm^3), and were absent at depths of 100-500 cm (56). Similarly, methanogens were observed only in the top 20 cm of stable Black Sea sediments, and were absent in 30-100 cm-deep sediments (56). The work of Romesser, *et al.* (56) is the only report on the extent of the depth distributions of methanogens in a marine environment. Assuming that they are fairly representative of stable marine sediments, it appears that the depth to which viable methanogens were found in the unstable delta sediments exceeded that of stable sediments by 2-6 times. The extreme depth in which methanogens occurred in the delta sediments was probably due to at least two factors: (i) the rapid deposition of organic-rich, sedimentary material which buried viable methanogens under deep strata, and (ii) the instability of the sediments, exemplified by the numerous undersea landslides and collapse depressions, which caused a high degree of sediment mixing and resulted in the burial of viable methanogens under layers of shifted sediment. The compact distributional patterns of methanogens in stable marine sediments (Cariaco Trench, Black Sea) resulted from the diffusion of methane precursors and growth factors into the underlying sediments from the relatively organic-rich upper strata, and were probably not affected by sediment movement or bioturbation.

The only other attempt to quantitate methanogens in a marine sediment was that of Jones and Paynter (28). They observed methanogens in the upper 36 cm of salt marsh sediments, with maximal numbers

in the upper 0-7 cm. The *Spartina* marshes in their study are highly productive ecosystems, and it appeared that the high numbers of methanogens present in the salt marsh sediments were dependent upon the decomposition of *Spartina* cellulose by clostridia. This eventually provided usable methane precursors for the methanogens. The highest methanogen MPNs occurred in "short *Spartina*" regions of the salt marsh where the *Spartina* roots were densely packed, and where intertidal flushing of the area was minimal. In this environment, dying *Spartina* plants contributed a heavy nutient load to a narrow band encompassing the dense root system of the plants. The high methanogen MPNs in this region were directly attributable to the nutrient load. The same phenomenon can be seen in the upper delta sediments. The highest MPNs of methanogens and sulfate-reducers occurred in the uppermost samples where the highest levels of dissolved organic carbon (DOC) and dissolved inorganic carbon (ΣCO_2) occurred.

The coring methods used in the present study permitted the examination of deeply buried sediments. This was unusual for a microbiological study, in as much as most such endeavors employ gravity corers that limit the investigator to the study of the upper (and most accessible) sediments. In addition, a major problem arising from the use of long gravity corers is that of compression of the samples. This was not a problem in the present study since only 0.6 m-long sample cores were removed at any one time. It also allowed a far greater penetration of the sediments, a necessary prerequisite for studying highly unstable, mixed sediments.

Analysis of the MPN results of methanogen and sulfate-reducer

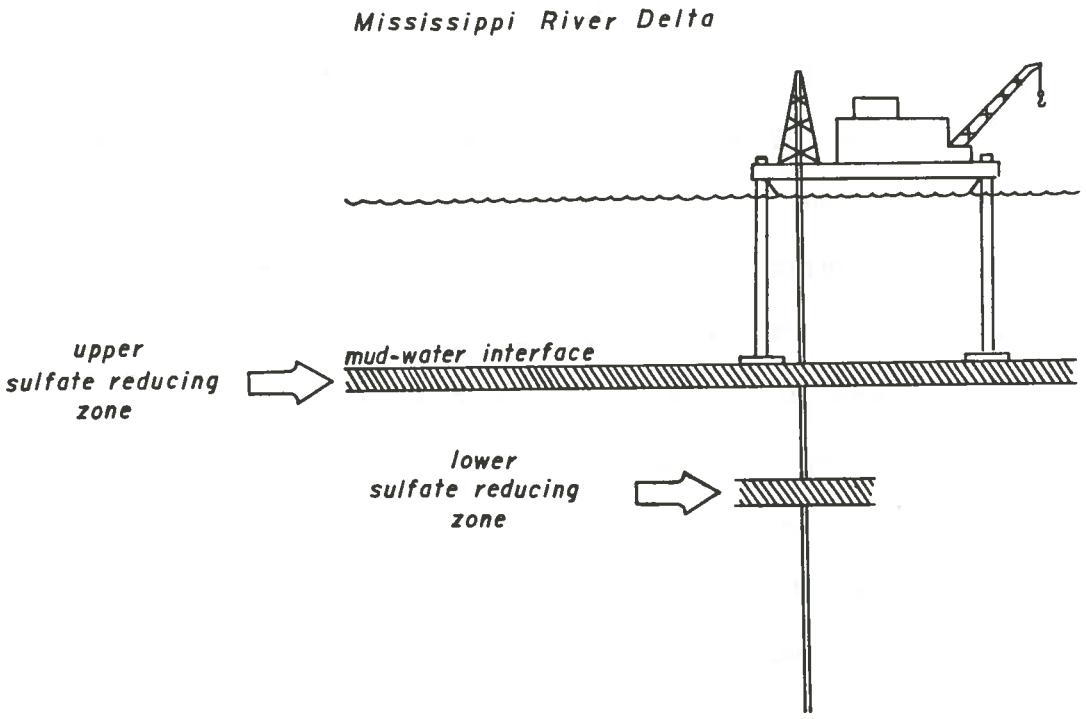
populations in Site 4 delta sediments showed that no apparent unilateral stratification of the populations of these two organisms existed. Cappenberg (11) had shown that methanogens and sulfate-reducers were each most numerous in separate strata of the freshwater sediments of Lake Vechten. Sulfate-reducers occupied the upper strata, and methanogens were found below. The structures of these communities in Lake Vechten and the Mississippi River delta are quite dissimilar. The layering of the two communities did not occur in the delta sediments. Instead, separate strata of the two types of bacteria were intermingled. Peaks of methanogens occurred at depths of 0.165, 0.355, and 0.770 m, while peaks of sulfate-reducers occurred at depths of 0.100, 0.225, and 0.420 m. Visual inspection of the data did not indicate that the methanogen and sulfate-reducer populations were mutually exclusive: i.e., high numbers of methanogens did not preclude the presence of high numbers of sulfate-reducers (e.g., Table 5, 0.100 and 0.420 m). Instead, the structure of the microbial communities appeared to be random, and was probably formed as a result of sediment mixing. Thus, if any original unilateral stratification of the methanogens and sulfate-reducers did exist, it was masked by sediment movement.

It should be pointed out that the MPN technique is not the only method that has been used to enumerate methanogens in sediments. The fluorescent antibody (FA) technique has been used successfully to enumerate methanogenic isolates in freshwater lake sediments of Wintergreen Lake, Michigan (60), and Lake Erie (67). Comparison of FA counts with MPN counts in Wintergreen Lake sediments showed that the FA method produced counts at least one order of magnitude higher

than MPN counts (60). However, the FA method is contingent upon obtaining one or more isolated methanogens that are derived from the sediments under study. The FA method should prove useful in the enumeration of delta methanogens, but it will first be necessary to obtain delta isolates with which to prepare the FA.

When the Site 1 depth distribution profiles of methanogens and sulfate-reducers are compared to depth profiles of dissolved methane and dissolved sulfate (Fig. 5), two clearly apparent relationships can be seen: (i) the presence of sulfate-reducing bacteria was directly related to the presence of dissolved sulfate; and (ii) the presence of sulfate-reducing bacteria was inversely related to the presence of high concentrations of dissolved methane. The occurrence of sulfate-reducers in zones of high dissolved sulfate is indicative of the presence of a sulfate-reducing zone (18, 32, 66). The delta sediments at Site 1 clearly exhibited two sulfate-reducing zones at depths of 0-2 and 10-12 m. Due to the high concentration of sulfate in seawater, 28 mM, most marine sediments have a single sulfate-reducing zone restricted to the upper 2-3 m of sediment (18). As sulfate diffuses downward into the sediments, it is usually depleted within a short depth below the sediment-water interface. In the delta sediments, the second sulfate-reducing zone (illustrated diagramatically in Figure 30) at 10-12 m probably originated at the sediment-water interface. Its present position, buried well below the sediment-water interface, resulted from sediment instability and movement. The approximately 10 m-thick layer of material covering the lower sulfate-reducing zone probably arose from a combination of slumping sediment and natural

Figure 30. Diagrammatic illustration of the sediments of the Mississippi River delta at Site 1. Two sulfate-reducing zones were present. The upper zone occupied the upper 0-2 m of sediment, while the lower sulfate-reducing zone was 10-12 m below the sediment-water interface. The horizontal extent of the lower sulfate-reducing zone is unknown, but probably did not exceed more than several meters.


Mississippi River Delta
upper
sulfate reducing
zone
mud-water interface
lower
sulfate reducing
zone

accretion of delta silt deposits.

The inverse relationship between sulfate-reducers and dissolved methane is indicative of anaerobic oxidation of methane in the sediments possessing high numbers of these organisms. At Site 1, the sample just below the sediment-water interface contained the highest inorganic and organic carbon loads, in addition to the highest numbers of methanogens and sulfate-reducers, yet the level of dissolved methane was quite low. While some studies have provided indirect evidence of anaerobic methane oxidation in marine sediments (5, 54, 55, and the present study), recent efforts show clearly that anaerobic consumption of methane occurs in both freshwater (78) and marine sediments (32). Based upon radiotracer data ($^{14}CH_4$ produced from ^{14}C-U-lactate or ^{14}C-2-acetate), Kosiur and Warford (32) showed that methanogenesis occurred in the sulfate-reducing zone of a marine sediment, and that the low level of dissolved methane in this zone was due to a balance between methane production and anaerobic oxidation. The upper sulfate-reducing zone of the delta sediments was roughly analogous to that of the Santa Barbara Basin (32). Kosiur and Warford (32) observed significant methane oxidation in the upper 1.2-1.3 m of sediment, with the greatest oxidation occurring at a depth of 0.3-0.35 m. This is comparable to the extremely low concentration of dissolved methane in the 0.2 m-deep delta sediment. Zehnder and Brock (78) suggested that, in addition to sulfate-reducers, methanogens themselves may be partly responsible for anaerobic methane oxidation.

In Vitro Utilization of Methane Precursors

The most readily utilized methane precursors in the delta sediments

were methylamine, dimethylamine, trimethylamine, methanol, and $CO_2 + H_2$. The extremely rapid conversion of these compounds to methane supports the argument that they were directly reduced to methane by methanogens. Methanogenesis from N-methyl compounds has been reported in pure cultures of *Methanosarcina barkeri* (24). *M. barkeri* is the most versatile methanogen known, being the only organism of the group that is capable of methanogenesis from all the substrates listed above (46, 59). Hippe, *et al.* (24) showed that methanol and N-methyl compounds were converted to methane by samples of freshwater muds. So far, *M. barkeri* has not been isolated from marine sediments, although it is common in sewage sludge digestors, freshwater sediments, and the rumen (46, 80).

Methanethiol and methylhydrazine were both converted to methane in delta sediment samples. The utilization of methanethiol in freshwater sediments has been previously reported (83), but the organism, or organisms, responsible for the reduction is, at present, unknown. The conversion of methylhydrazine to methane has not been previously reported. Since no information is available on these reactions, one can only speculate that the reductions of these substrates to methane might be carried out by a physiologically versatile methanogen of the methanosarcinae type.

Methanogenesis in the delta sediments was not appreciably stimulated by acetate or formate, two known immediate methane precursors, or by lactate, which promoted methanogenesis in freshwater sediments (12, 72, 74, 75). It can be inferred, therefore, that these substrates were not limiting factors in methanogenesis in the delta

sediments. The failure of either acetate or lactate to stimulate methanogenesis poses the question of whether or not acetate is a major methane precursor in the delta sediments. Although methanogenesis from lactate (through acetate as an intermediate) has been shown in freshwater sediments (12, 13, 15), this may not be the dominant pathway for methanogenesis in marine sediments (18, 66). Radiotracer studies with ^{14}C-acetate showed that aceticlastic methanogens were present in the delta sediments, yet unlabeled acetate was poorly utilized by delta sediments. Claypool and Kaplan (18) and Warford, *et al.* (66) suggested that the fate of acetate in marine sediments is conversion to CO_2, and that the major methane precursor in marine sediments is CO_2. The following observations in the delta sediments tend to support this argument. First, the addition of CO_2 (with H_2) was highly stimulatory to methanogenesis (whereas H_2 alone was not). Second, the level of ΣCO_2 decreased by about 50% from the uppermost Site 1 sediment sample to the next sample below, with a concomitant decrease in methanogen MPNs. Third, enzymatic assays of acetate in delta sediments (D. Sarkar, unpublished data) showed that the concentration of acetate was below detectable limits. It is assumed that if the extremely low level of acetate in the sediments was limiting growth and methanogenesis, then one would expect to see an increase in methanogenesis with added acetate. This did not occur. Fourth, the addition of β-fluoroacetate, a known inhibitor of methanogenesis in freshwater systems (12) did not influence methanogenesis in the delta sediments. In order to determine the role of CO_2 in delta methanogenesis, it will be necessary to perform future experiments using radiotracers, and to

follow changes in specific activity of $^{14}CO_2$ and $^{14}CH_4$.

Two potential sources of CO_2 exist in the delta sediments: (i) CO_2 derived from dissolved organic carbon (DOC); and CO_2 derived from dissolved inorganic carbon (ΣCO_2). The total carbon concentration in the upper sediments approached 0.1 g/l of interstitial water, and represents a sizable reservoir of potential methane precursors.

Although the concentrations of methanol, methylamine, dimethylamine, and trimethylamine were not measured in delta sediment samples, it is unlikely that they would exist in high concentrations in this environment because they were so readily reduced to methane in *in vitro* tests. The presence of methanogens in the delta sediments that were capable of utilizing methyl- and N-methyl compounds may infer that these substrates occur naturally in this environment. Methanol is a microbially-produced end product of pectin degradation (58). Pectin is a ubiquitous component of plant tissue that may provide an important source of methanol for the growth of methylotrophic and methanogenic bacteria (58). Pectinolytic organisms such as *Clostridium butyricum, Erwinia chrysanthemi,* and *Pseudomonas marginalis* are capable of a stoichiometric formation of methanol during pectin metabolism (58). If these organisms are, in fact, ecologically important, they may depend upon methylotrophs and/or methanogens for the removal of methanol which they cannot consume and which is toxic (58). Pectin was slowly converted to methane by the delta sediments, but in order to demonstrate methanogenesis from pectin via a methanol intermediate, it would be necessary to follow a radiotracer from pectin, through methanol, to methane.

The production of trimethylamine within the delta sediments could occur via at least two different pathways: (i) the degradation of choline by the reaction:

$2\ (CH_3)_3N^+CH_2CH_2OH + H_2O \rightarrow 2\ (CH_3)_3N^+H + CH_3COOH + CH_3CH_2OH$ (23); and (ii) the reduction of trimethylamine oxide (TMAO) by various bacteria (usually gram-negative) that utilize TMAO as a terminal electron acceptor via the following reaction:

$$(CH_3)_3NO + AH_2 + H^+ \rightarrow (CH_3)_3N^+H + A + H_2O$$ (62, 63, 64).

Choline is released upon the degradation of phosphatidyl choline, a major component of cell membrane material in plant (and animal) tissue (42). TMAO is a constituent of the tissue of several marine fish and invertebrates (63). It is possible that after the death of certain marine organisms (fish, seaweed, benthic invertebrates), they may contribute a portion of the organic nutrient load in the delta sediments, and may provide a source of N-methyl compounds that serve as immediate methane precursors in this environment.

Another potential source of methane precursors is cellulose. If one assumes that decaying plant material constitutes a significant portion of the organic nutrient load in the delta sediments, then the role of cellulolytic organisms within the sediments becomes an important consideration in the food chain. Weimer and Zeikus (68) found that cellulose degradation by a monoculture of *Clostridium thermocellum* began after a considerable lag period. The lag was lessened in the presence of a methanogenic organism, *Methanobacterium thermoautotrophicum*. Addition of cellulose to delta sediments resulted in active methanogenesis after a lag period of 13 days. Cellulose

degradation by *Clostridium* (68) and *Ruminococcus* (35) resulted in the production of acetate, succinate, formate, ethanol, and CO_2 + H_2. Of these, CO_2 and H_2 could serve as readily-utilizable methane precursors in the delta sediments, with acetate and formate as additional, but less usable, substrates.

Factors Affecting Delta Methanogenesis

Several compounds have been implicated in the inhibition of methanogenesis in natural sediments: nitrate (4); sulfide (11); sulfate (73); and NaCl (44). Other compounds not found in natural sediments that have been shown to be potent inhibitors of methanogenesis include: chloroform (12); β-fluoroacetate (12); 2-bromoethanesulfonate (77); and nitrapyrin (57). Delta methanogenesis was inhibited by chloroform, BrES, O_2, formaldehyde and nitrate, but not by β-fluoroacetate, sulfide, or sulfate. When low concentrations of added sulfide were introduced to delta sediments, methanogenesis was either unaffected, or it was stimulated. However, with the addition of 100 mM sulfide, methanogenesis was inhibited for a few days, followed by an increase in methane production. Sulfide is an extremely toxic substance, and it is reasonable that high levels would be bactericidal or bacteriostatic. But in an anaerobic sediment, most of the sulfide that is produced is precipitated as FeS (8), or FeS_2 (7). Recovery of methanogenesis in the delta sediments 6 days after the addition of 100 mM sulfide indicates that the natural accumulation of sulfide is not responsible for inhibition of methanogenesis in these sediments. Sulfide, at a concentration of 100 mM, is far above the concentration

encountered in the delta sediments (sulfide concentration in Site 1, 0.2 m-deep sediment was 0.32 µg/ml, measured spectrophotometrically by the methylene blue method). The temporary inhibition of methanogenesis by 100 mM sulfide was probably terminated after most of the sulfide was precipitated by ferrous iron, or immobilized in some manner, within the sediments. The addition of sulfate to delta sediments failed to inhibit methanogenesis. In fact, methanogenesis was uniformly stimulated by sulfate concentrations of 0.1-100 mM. When carbon and energy sources were supplied to delta sediment samples, high levels (100 mM) of sulfide and sulfate still failed to inhibit methanogenesis.

Several studies have indicated that methanogenesis is inhibited in the presence of dissimilatory sulfate reduction (11, 13, 39), yet the bacterial depth distribution data and added inhibitor experiments on delta sediments indicated otherwise. Another possibility of methanogenesis inhibition that was not explored in the present study is the competition between methanogens and sulfate-reducers for H_2. Abram and Nedwell (1) showed that a hydrogen-utilizing sulfate-reducer was able to out-compete *Methanococcus mazei* in its ability to scavenge H_2 in a mixed culture. Thus, methanogenesis occurred only if the sulfate-reducer was absent. Such a competition could easily occur in the delta sediments. If, as some studies indicate (18, 66), CO_2 is the major methane precursor in marine environments (such as the Mississippi River delta) then methanogenesis would be highly dependent upon, and perhaps limited by, the availability of H_2 in the sediments. Studies of H_2 utilization in freshwater sediments show that H_2 is

present in extremely minute amounts. Low partial pressures of H_2 limit the rate of methanogenesis in Lake Mendota sediments (72). Heterotrophic bacteria that possess hydrogenases and utilize protons as electron sinks, are, in turn, dependent upon H_2-utilizers to keep the partial pressure of H_2 low, and in effect, allow the thermodynamically unfavorable reaction to proceed (80). This process of "interspecies H_2 transfer" is extremely important to CO_2-reducing methanogens, since they require 4 moles of H_2 for every mole of CO_2 reduced (80). Their rapid use of H_2 for methane production benefits the H_2-producing organisms and ensures a continuing supply of reducing power. If methanogens were found to compete with sulfate-reducers for available H_2, the respiratory process deriving the greatest energy (sulfate-reduction) would dominate, and as a result, methanogenesis would be inhibited (18, 65, 73).

Very little is known about the effect of NaCl on methanogenic bacteria. In one study of freshwater methanogens, methane production was inhibited by 97.3 mM NaCl (44). However, *Methanogenium cariaci* and *M. marisnigri*, two marine methanogens, have optimum NaCl requirements of 46 and 10 mM, respectively (56). Methanogenesis in the delta sediments with added carbon and energy sources was severely inhibited by 2000 mM NaCl, and enrichments in broth media without added NaCl showed no methanogenic activity (data not shown). It thus appears that the delta methanogens were obligate NaCl-requirers, but their optimum NaCl concentration for growth remains to be determined. It is not surprising that delta methanogenesis was NaCl-dependent, since many bacteria isolated from marine habitats require elevated NaCl

concentrations. No methanogens isolated from any source, to date, possess peptidoglycan in their cell walls (30, 31), and some, including those in the delta sediments, appear to possess very fragile cell walls (27, 56). It is possible that the structural integrity of these methanogens is dependent upon elevated NaCl concentrations, analogous to that seen in the obligate halophile, *Halobacterium*.

The optimum temperature for methanogenesis in unadulterated sediments was 45° C. However, in enrichments that selected specifically for methanogens, the optimum temperature for methanogenesis was 35-40° C. This indicates that the *in situ* temperature of 20° C was rate-limiting. A similar phenomenon was seen in freshwater sediments of Lake Mendota (81). The optimum temperature of 35-42° C was considerably higher than the maximum *in situ* temperature of 28° C (81). In the delta sediments, incubation at 45° C may have favored the growth of some bacteria that provided methane precursors (or other growth factors) to the methanogens. Thus, methanogenesis would proceed maximally as a result of increased substrate availability, and not necessarily because it was the optimum growth temperature for the delta methanogens.

Methanogenesis in unadulterated, 0.2 m-deep, Site 1 sediments at 20° C amounted to 0.24 nmol CH_4 per gram sediment, dry weight, per day. This is equivalent to 0.003 ml CH_4 (at S.T.P.) per liter of wet sediment per day, or roughly 1 ml CH_4/l/year. This value compares favorably with methane production rates measured in other marine sediments: 0.035-0.041 nmol CH_4/g/day in the Santa Barbara Basin (5, 32); and 0.029 nmol CH_4/g/day in tropical Florida Keys sediments (43).

Methanogenesis in the delta sediments occurred at a rate 6-8 times that observed in other marine sediments. This may have been due to the large nutrient load found in the delta sediments near the mouth of the Mississippi River, as compared to the open ocean sediments in the other studies. The rates of methanogenesis in Lake Mendota, 12.2 nmol CH_4/g/day (74); and Lake Vechten, 34.0 nmol CH_4/g/day (14) were 50-140 times greater than in the delta. Wintergreen Lake, a hyper-eutrophic lake, exhibited an enormous rate of methanogenesis, 15,360 nmol CH_4/g/day, or about 6.4 X 10^4 times greater than in the delta (61).

Methanogenesis by 0.2 m-deep sediments in the one-liter reaction vessels exhibited a response that is typical of methanogenesis in other systems (76, 80). Methane production occurred at E_h values ranging from -300 to -350 mV. The biological generation of methane requires that the E_h of the system be at or below approximately -200 mV. The reduced delta sediments were below the limit needed for active methanogenesis. In each reaction vessel, the E_h initially dropped to a more negative value (-350 to -420 mV). After 8 days, it became slightly more positive and leveled off at -300 to -375 mV. The dip in E_h early in the incubation period was probably a response to the release of sulfide during sulfate-reduction. The sediments became visibly blackened during this period. Later, sulfide was precipitated as FeS, and the E_h reached a plateau. The high buffering capacity of the delta sediments maintained the pH at a constant value throughout the experiment, although the pH was different in each vessel.

Identification of Delta Methanogens and Sulfate-Reducers

It is impossible to identify the delta methanogens that were observed in enrichment cultures without the benefit of pure cultures. However, based upon cellular morphology, and the response of the delta sediments to added substrates, it is possible to speculate on the physiological types of methanogens that may be present in the delta sediments. Virtually all methanogenic enrichments were dominated by a single morphotype, an irregularly coccoid cell. Morphologically, it appeared to be similar to the genus *Methanogenium*. All members of this genus, *M. cariaci* (56), *M. marisnigri* (56), and *M. paludis-maritimae* (29) are marine methanogens. These species utilize $CO_2 + H_2$ and formate for growth and methane production, but do not utilize methanol or N-methyl compounds. Mah (37) recently described the isolation and characterization of *Methanococcus mazei* from a laboratory sewage sludge digestor. This organism undergoes a life cycle in which the methanogen (in young cultures) forms a large cell aggregate typical of the genus *Methanosarcina* (37). The cell aggregates later give rise to cyst-like structures that contain hundreds of irregularly coccoid cells (37). The coccoid cells resemble *Methanococcus vannielii, Methanogenium cariaci,* and *M. marisnigri* (37). The irregularly coccoid cells observed in the delta-derived enrichments also morphologically resembled *Methanococcus mazei*. *M. mazei* possesses the same physiological characteristics of the methanosarcinae, i.e., it uses acetate, methanol, methylamine, and trimethylamine for growth and methanogenesis (37). The rapid production of methane, both in delta sediment slurries and in enrichment cultures, from methanol, N-methyl compounds, and $CO_2 + H_2$ (as well as $^{14}CH_4$ produced from ^{14}C-2-acetate) indicates that

methanogens which are physiologically related to *Methanosarcina barkeri* and *Methanococcus mazei* occurred in the delta sediments. There is a good probability that the irregularly coccoid cells that dominated the methanogenic delta-derived enrichment cultures were, in fact, the methanogenic species of the mixed cultures. It will be necessary to obtain pure cultures of the delta methanogens in order to confirm their identity. At this point, it is possible to speculate that the delta isolate would be closely related, physiologically, to the methanosarcinae, and morphologically similar to the methanococci. It would probably most closely resemble, over all, the organism, *Methanococcus mazei*, with the exception that the delta methanogens require an elevated NaCl concentration for growth. In addition, the alleged delta methanogen did not appear to form *Methanosarcina*-like cell aggregates in young broth cultures. The coccoid delta organisms would probably constitute a new taxon, since all known marine methanogens (*Methanogenium* sp.) utilize formate for growth and methanogenesis. Formate had no effect on delta methanogenesis. The delta methanogens were readily grown in mixed cultures, but it will require the addition of certain unknown growth factors in order to obtain these organisms in pure culture.

The isolation and characterization of delta sulfate-reducers posed no insurmountable problem. Of the 80 strains that were isolated, 75 belonged to the genus *Desulfovibrio*, and 5 belonged to the genus *Desulfotomaculum*. Based upon cell morphologies and physiological characteristics, all 75 strains of *Desulfovibrio* appeared to be representatives of *Desulfovibrio salexigens*. Two salient characteristics

of *Dv. salexigens* are: (i) extreme resistance to Hibitane; and (ii) an absolute requirement for NaCl for growth (50). All 75 delta desulfovibrios shared these characteristics. However, in some respects, the delta isolates physiologically resembled *Dv. desulfuricans* in their ability to grow on pyruvate without sulfate. Also, 17 of the 75 isolates were able to grow on choline without sulfate, another feature in commom with *Dv. desulfuricans*. It will be necessary to determine the mol% guanine plus cytosine (G + C) content in the DNA of the 75 isolates in order to more clearly classify the organisms. *Dv. salexigens* contains 46.1 mol% G + C, while *Dv. desulfuricans* contains 55.3 mol% G + C (50).

The 5 *Desulfotomaculum* isolates did not appear to closely resemble any described taxon. Of the three recognized species of spore-forming sulfate-reducers, two can be eliminated as possible matches. First, *Desulfotomaculum ruminis* and *Dt. nigrificans* are both capable of growth on pyruvate minus sulfate (50). The 5 delta isolates were not. Second, a salient characteristic of *Dt. nigrificans* is growth at 55^{o} C (50). The 5 delta isolates failed to grow at this temperature. Third, *Dt. ruminis* is capable of growth on formate plus sulfate (50). The 5 delta isolates failed to grow in this medium. Based on these considerations, *Dt. ruminis* and *Dt. nigrificans* were ruled out as possible identities of the 5 delta isolates. The remaining known member of the genus *Desulfotomaculum* is *Dt. orientis* (50). Some physiological features of this organism are shared with the 5 delta isolates, however, some striking features of each organism tend to differentiate them. *Dt. orientis* is a curved rod, and none of the

five delta isolates were curved. Also, the 5 delta isolates required NaCl for growth, whereas *Dt. orientis* does not (50). It thus appears that the 5 delta isolates probably constitute a new species of the genus *Desulfotomaculum*, but may be related to *Dt. orientis*.

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