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Inhibition Experiments on Anaerobic Methane Oxidation†

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Anaerobic methane oxidation is a general process important in controlling fluxes of methane from anoxic marine sediments. The responsible organism has not been isolated, and little is known about the electron acceptors and substrates involved in the process. Laboratory evidence indicates that sulfate reducers and methanogens are able to oxidize small quantities of methane. Field evidence suggests anaerobic methane oxidation may be linked to sulfate reduction. Experiments with specific inhibitors for sulfate reduction (molybdate), methanogenesis (2-bromoethanesulfonic acid), and acetate utilization (fluoroacetate) were performed on marine sediments from the zone of methane oxidation to determine whether sulfate-reducing bacteria or methanogenic bacteria are responsible for methane oxidation. The inhibition experiment results suggest that methane oxidation in anoxic marine sediments is not directly mediated by sulfate-reducing bacteria or methanogenic bacteria. Our results are consistent with two possibilities: anaerobic methane oxidation may be mediated by an unknown organism or a consortium involving an unknown methane oxidizer and sulfate-reducing bacteria.

Studies employing three different approaches, geochemical modeling, tracer experiments, and changes in stable carbon isotope ratios, provide evidence that methane is oxidized in anoxic marine sediments. The geochemical models (1, 4, 11, 12a, 14, 20) show that net consumption of methane is required to account for the shape of methane depth distributions. The tracer experiments (1, 10, 12, 12a, 21) show that $^{14}CH_4$ is oxidized to $^{14}CO_2$ at depths and rates consistent with the geochemical models. The stable carbon isotope studies show that methane becomes enriched in ^{13}C as a result of isotopic fractionation during oxidation (1). A minimum in the $\delta^{13}CO_2$ depth distribution (1, 7) indicates that the CO_2 pool is modified by input of isotopically light methane-derived CO_2. Reviews of several studies (1, 24) indicate that anaerobic methane oxidation is a general process that occurs in a variety of freshwater, marine, and hypersaline water column and sediment environments. Anaerobic methane oxidation effectively restricts the flux of methane from anoxic environments (22) and appears to be an important methane sink in the global methane budget.

The organism responsible for anaerobic methane oxidation has not yet been identified or isolated. Laboratory studies of methanogens and sulfate reducers have shown that both groups are capable of oxidizing small quantities of methane, but it is not clear whether either group is responsible for the net consumption observed in nature. Zehnder and Brock (28) have shown that nine strains of methanogenic bacteria are able to oxidize methane, but only a small fraction (<1%) of the methane produced is oxidized. They suggested (29) that net methane oxidation could result from a bacterial consortium consisting of a methanogen and another bacterium. They hypothesized that acetate, methanol, or hydrogen produced during methane oxidation by the methanogen was consumed by an organism able to use MnO_2 or sulfate as an electron acceptor. Sulfate-reducing bacteria are unable to oxidize methane when it is the sole carbon source (18, 25) but can oxidize methane when an additional electron donor is present (9). Recent work on four pure cultures of *Desulfovibrio* spp. (N. Iversen, Ph.D. thesis, Aarhus University, Aarhus, Denmark, 1985) showed oxidation of methane at rates 0.01 to 0.2% of the sulfate reduction rate when an additional electron donor was present. Field evidence of a link between anaerobic methane oxidation and sulfate reduction comes from observations of coincident maxima in both rates in several marine systems (11, 12a).

In this study, we examined the effects of specific inhibitors on anaerobic methane oxidation in anoxic marine sediments. This approach permits us to determine whether sulfate-reducing bacteria or methanogenic bacteria, acting alone or as a consortium, are responsible for anaerobic methane oxidation in coastal marine sediments. Molybdate, an inhibitor of sulfate reduction (3), 2-bromoethanesulfonic acid (BES), an inhibitor of methanogenesis and methane oxidation by methanogens (28), and fluoroacetate, an inhibitor of acetate utilization (2), were added to slurried and intact sediments in which anaerobic methane oxidation was observed. Rates of methane oxidation and sulfate reduction were measured to determine the effect of the inhibitors on these processes.

MATERIALS AND METHODS

Study site and sediment sampling. The sediment samples used in this study were collected with a box corer at the deepest part of Skan Bay (65 m). Skan Bay is a silled basin on the northwest side of Unalaska Island in the Aleutian Islands (53° N, 167° W) with rapidly accumulating (~1 cm yr^{-1}), organic-rich (4% carbon) sediments. The sediments are permanently anoxic and are not bioturbated by benthic fauna. As many as nine subcores were taken from each box core by gently pushing transparent plastic tubes (7.5-cm diameter) into the box core surface. The subcores were capped (45A) and stored vertically in a water bath at in situ (4°C) temperature.

Reagents. Biosynthesized (8) $^{14}CH_4$ (~45 mCi/mmol) was

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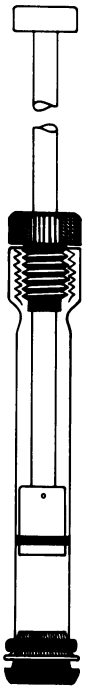

FIG. 1. Syringe for headspace-free incubations.

kindly supplied by K. Sandbeck and M. Lidstrom. It was stored in a 35-ml serum vial containing anoxic 1 N NaOH to remove CO_2. The $^{14}CH_4$ was checked for purity with a gas chromatograph-proportional counter and was found to contain trace amounts of $^{14}C_2H_6$ ($^{14}C_2H_6/^{14}CH_4$ = 0.00031). The $^{35}SO_4^{2-}$ (Amersham Corp., Arlington Heights, Ill.) was obtained carrier free. It was diluted with an anoxic 32 g liter^{-1} NaCl solution and stored in 5-ml serum vials. Stock solutions of the sodium salts of BES (Eastman Kodak Co., Rochester, N.Y.), fluoroacetate (Sigma Chemical Corp., St. Louis, Mo.), and molybdate (Mallinckrodt Chemical Works, St. Louis, Mo.) were prepared in an anoxic solution containing 32 g liter^{-1} NaCl–2 mM Na_2SO_4. These solutions were made anoxic by stripping with Hungate furnace-treated N_2 and matched the interstitial water salinity and sulfate content in the zone of methane oxidation.

Incubation syringes. Syringes capable of long-term headspace-free incubations were made for this study and are shown in Fig. 1. The syringes (14-mm inner diameter) were fitted with O-ring seal Teflon pistons. A threaded tubing connector (no. 7 Ace Thred tube adapter; Ace Glass, Inc., Vineland, N.J.) was welded to one end of the barrel and used to immobilize the piston rod; the other end was flanged to accept a standard butyl rubber serum stopper. The rubber O ring located behind the flanged end of the barrel allowed use of aluminum crimp seals. The syringes permitted analyses of pool size, added tracer, and reacted tracer on the same sample. Tests showed that the syringes were gas tight and could be autoclaved without methane loss.

Rate depth distributions. Depth distributions of methane, sulfate, methane oxidation rate, and sulfate reduction rate were measured to locate the methane oxidizing zone for the inhibition experiments. The tubing used for subcoring contained holes spaced at 3-cm intervals that were covered with electrical tape. The incubation syringes were used as piston corers; the tape was removed, and each syringe was filled by holding the piston stationary and inserting the barrel into the sediment. Samples for methane and sulfate concentration were collected in the same manner on parallel cores with 3- or 10-ml plastic syringes with the tips removed. Incubation syringes were injected with 50 μl of $^{14}CH_4$ (1 μCi) or $^{35}SO_4^{2-}$ (5 μCi). The $^{14}CH_4$ tracer was maintained at constant pressure in a serum bottle; an equal volume of Hg replaced each portion removed. The syringes were maintained at in situ temperature in a rotating Ferris wheel-type rack submerged in a plastic garbage can. Seawater from a submersible pump suspended at a depth of 45 m supplied constant-temperature water, which was also used to rotate the incubator. The syringes were incubated for 20 to 25 h and quick frozen in an isopropanol bath at −60°C to stop biological activity. The syringes were stored frozen until they were stripped and analyzed.

Inhibition experiments. The inhibition experiments were performed on sediments from the zone of methane oxidation in Skan Bay, which was located by the rate depth distributions. These inhibition experiments were conducted on both slurried and intact sediments. Slurrying the sediment ensures uniform distribution of inhibitor and eliminates natural sediment heterogeneity but has been observed to decrease turnover rates (6, 13), presumably due to the effects of manipulating and diluting the sediment. Slurrying could reduce rates below the detection limit for a process with a relatively low fractional turnover rate, like anaerobic methane oxidation. Recent work has shown that microbial processes can be inhibited in intact sediment (5), but natural heterogeneity leads to more scatter.

Slurried sediment. The sediment slurry was prepared by mixing sediment from the 22- to 32-cm depth interval with an equal volume of an anoxic solution containing 32 g liter^{-1} NaCl–2 mM Na_2SO_4. This mixture was homogenized in a closed container with a paddle-type stirrer while methane was bubbled through the slurry. Portions of this slurry were dispensed with methane pressure into methane-flushed quart Mason jars whose lids were equipped with gas inlet and outlet ports and a large-bore dispensing tube. These jars contained inhibitor solutions or the NaCl-Na_2SO_4 solution. One jar containing no inhibitors was autoclaved; the remaining jars were gently agitated and maintained at constant temperature in a water bath for 12 h. The final concentration of BES and molybdate in the slurries was 20 mM. The Mason jars were flushed with methane prior to dispensing into the syringes. The syringes were set to contain 6.4 ml of slurry; two to four replicate samples were dispensed with methane pressure by overflowing the slurry into upright syringes. The gentle rotation (8 rpm) of the incubator maintained slurry homogeneity in these experiments. Tracer addition and incubation were the same as above. This treatment ensured anoxic conditions and maintained sulfate and methane at uniform, near in situ concentrations.

Intact sediment. The syringes were used as piston corers in the intact sediment experiments. These experiments were performed in triplicate on 5-cm segments (25 to 30 cm) of subcores from the same box core. Constant sample volumes were ensured by positioning an O ring on the syringe barrel and using it as a mark for piston withdrawal.

Stock concentrations of the inhibitors used in the intact sediment experiment were adjusted so that the same volume of inhibitor solution (200 μl) was injected into each sample syringe. The uninhibited samples were injected with 200 μl of the anoxic inhibitor-free NaCl-Na_2SO_4 solution. The inhibitors were not mixed in the sediment but were allowed to diffuse from the point of addition. Uniform distribution of the inhibitors would result in concentrations of 50 mM for

BES and molybdate and 30 mM for fluoroacetate. Since the inhibitors and tracers were injected into the same location in the syringe, these inhibitor concentrations are minima. The control and inhibited intact sediment syringes were acclimated at in situ temperature for 15 h prior to injection of the tracers. Tracer injection and incubation were as above.

Analyses. The samples from this study were analyzed by methods similar to those reported previously (13, 21). Modifications included a glass-polypropylene stripping line with a small-(125-ml) volume stripping vessel and high-efficiency Harvey Biological Material oxidizer traps (R. J. Harvey Instrument Co., Hillsdale, N.J.). Samples were introduced to the stripping vessels as frozen plugs, which minimized gas loss.

Methane oxidation rate samples were introduced to stripping vessels containing 0.1 N NaOH; after collection of all methane, they were acidified with 7.2 N H_2SO_4 to release the CO_2. The methane was collected in a trap containing molecular sieve 5A maintained at −60°C. The methane was released from the trap by heating and quantified with the flame ionization detector of a HP 5710A gas chromatograph. Methane was quantitatively oxidized in the flame ionization detector and collected as CO_2 in 20 ml of Woeller solution (27). An aliquot was counted with a shipboard Beckman LS-100 scintillation counter; quench corrections were determined with external standard ratios. Agreement between the methane depth distributions and the methane recovered during stripping indicated quantitative recovery of CH_4 and $^{14}CH_4$. The acid-released CO_2 was also collected in Harvey traps containing Woeller solution and counted without dilution.

The sulfate reduction rate samples were introduced to stripping vessels containing oxygen-free distilled water and subsequently acidified with 3.6 N HCl. The H_2S released was collected as ZnS in Harvey traps containing 10% (wt/vol) zinc acetate solution. An aliquot was counted in a gel formed with Aquasol II (New England Nuclear Corp., Boston, Mass.).

Methane oxidation rate samples were stripped with He for 20 min at 80 ml min^{-1}; sulfate reduction rate samples were stripped for 30 min at 120 ml min^{-1} with Hungate furnace-treated N_2. Methane pool sizes were measured in the incubation syringes as well as in parallel cores. Methane was measured in the incubation syringe samples after release from the molecular sieve trap; a headspace method was used on syringe subcore samples from a parallel core (1). Sulfate samples were collected by centrifuging sediment from parallel cores. The samples were acidified (HCl), stripped of volatile S compounds with a stream of He, and analyzed with a Dionex 2000i ion chromatograph. The $TiCl_3$ (2) added to molybdate-inhibited samples to permit release of reduced sulfur compounds precluded analysis of SO_4^{2-} and counting of $^{35}SO_4^{2-}$.

Killed control experiments were performed by autoclaving the slurried sediment or sediment-filled syringes prior to tracer injection. Blanks were determined by freezing syringes within 30 s after tracer addition. Rates of methane oxidation and sulfate reduction were calculated by multiplying the interstitial water concentration and the fractional turnover rate constant.

Precautions were taken to remove and exclude oxygen during the manipulations, so the methane oxidation observed in this study occurs under anaerobic conditions. The sediments also contained millimolar concentrations of sulfide and thus had sufficient reducing capacity to rapidly consume added oxygen.

RESULTS

Depth distributions of methane and sulfate concentrations and methane oxidation and sulfate reduction rates are shown in Fig. 2. The distributions are similar to those from previous work in Skan Bay (1, 21) and other anoxic environments (10, 12a, 14). The methane distribution (Fig. 2a) is concave upward, indicating net methane oxidation (14, 21). The integrated methane oxidation rate approximately equals the calculated upward diffusive flux of methane. A secondary maximum in the sulfate reduction rate is evident (Fig. 2c and d) in the zone of methane oxidation (20 to 35 cm).

The results of the inhibition experiments on sediment slurries and intact sediments are shown in Fig. 3 and 4. Specific inhibitors were added to the sediments in amounts equal to or in excess of levels reported to cause complete inhibition. Natural variability and decreased rates caused by manipulation make consideration of partial inhibition uncertain, so we consider a process to be inhibited only if it has rates equivalent to those in the autoclaved treatment. These experiments show that anaerobic methane oxidation in Skan Bay sediments is not inhibited by molybdate, which caused complete inhibition of sulfate reduction in parallel experiments. Methane oxidation is not inhibited by BES, which has been shown to inhibit both methanogenesis and methane oxidation by methanogenic bacteria (28). Fluoroacetate, which inhibits acetate utilization (2), also did not inhibit anaerobic methane oxidation.

Methane oxidation and sulfate reduction rates were significantly slower in the slurried sediment inhibition experiment than in the intact sediment inhibition experiment. Slurrying the sediment with an equal volume of anoxic saline solution decreased sulfate reduction rates by a factor of 5 to 10; methane oxidation rates were decreased by as much as two orders of magnitude.

The methane oxidation and sulfate reduction rates are more reproducible in the slurried sediment experiment (Fig. 3) than in the intact sediment experiment (Fig. 4). We interpret variability in the intact sediment experiment to be the result of natural heterogeneity in the sediment. Scatter in the methane oxidation and sulfate reduction rates in the zone of methane oxidation is also evident in the depth distributions (Figs. 2c and d).

A very small amount of $^{14}C_2H_6$ (0.3 nCi) was introduced to each sample with the $^{14}CH_4$ tracer. This amount of $^{14}C_2H_6$ was too small to account for the $^{14}CO_2$ in the methane oxidation rate depth distributions and intact sediment inhibition experiment and could not have biased these results. The $^{14}C_2H_6$ contamination could have biased the results of the slurried sediment inhibition experiment because of the large decrease in the methane oxidation rate caused by slurrying. Since there is no direct information on ethane oxidation in anoxic sediments, we used the ethane depth distribution from the methane oxidizing zone of Big Soda Lake sediments (16) to obtain a model estimate of ethane reaction rates. This steady-state model is similar to those used to estimate methane oxidation rates (1) and balances ethane consumption with diffusion. The model indicates that ethane oxidation is 1,000 times too slow for the amount of $^{14}C_2H_6$ present in the $^{14}CH_4$ to affect the slurried sediment inhibition experiment results.

DISCUSSION

Anaerobic methane oxidation in sediment samples from the methane oxidizing zone of Skan Bay sediments is not inhibited by molybdate, BES, or fluoroacetate. Consistent

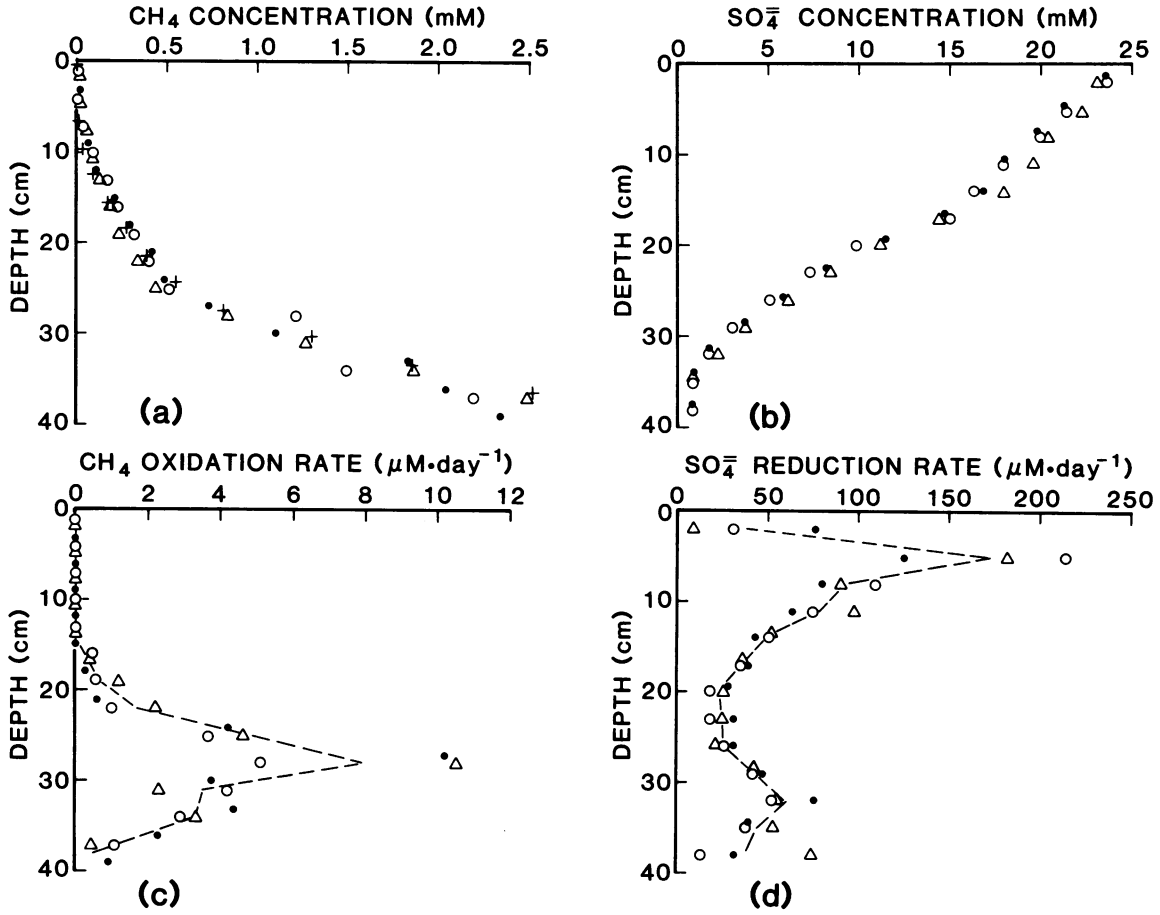

FIG. 2. Depth distributions in Skan Bay sediments. (a) Methane; (b) sulfate; (c) methane oxidation rate; (d) sulfate reduction rate. ●, ○, and △ represent triplicate subcores from the same box core on which both concentration and rate measurements were performed. In panel a, + is the average methane concentration from three subcores analyzed with a headspace technique. The dashed lines in panels c and d are through the mean of observations at a given depth.

results were obtained for all inhibitors, although the rates for intact sediment experiment varied between subcores, and the slurried sediment experiments gave greatly reduced rates. These results provide information on the possible organisms, electron acceptors, and substrates involved in anaerobic methane oxidation. Based on our inhibition experiment results, we consider four possibilities: that anaerobic methane oxidation in Skan Bay sediments is mediated by methanogenic bacteria, sulfate-reducing bacteria, an unknown organism, or a consortium involving sulfate reducers.

Methanogenic bacteria. Zehnder and Brock (29) hypothesized that a consortium involving methanogenic bacteria may be responsible for methane oxidation in anoxic environments. They have shown that methane oxidation is completely inhibited by BES concentrations of 10 mM in pure cultures of methanogens, digested sewage sludge, and lake sediment slurries. We used BES concentrations of 20 mM (slurried sediment experiment) and 50 mM (intact sediment experiment) and did not observe inhibition of methane oxidation. This result is contrary to the hypothesis of Zehnder and Brock and suggests that methanogens are not responsible for anaerobic methane oxidation in Skan Bay sediments.

Sulfate-reducing bacteria. Several lines of evidence suggest that sulfate-reducing bacteria play a role in anaerobic methane oxidation. Pure cultures of sulfate reducers are able to oxidize methane (9; N. Iversen, thesis), provided an additional electron acceptor is present. Anaerobic methane oxidation was stimulated by the addition of sulfate in enrichment cultures from the sediment surface of a stratified lake (17). Anaerobic methane oxidation is restricted to a thin

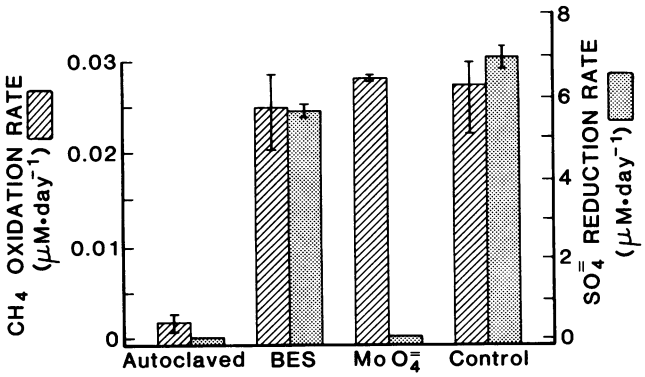

FIG. 3. Effect of inhibitors on methane oxidation and sulfate reduction rates in slurried sediment. The slurry was prepared with sediment from the 22- to 32-cm depth interval. The results reported are means of two to four replicates; the bars are ranges.

subsurface zone in which methane and sulfate are both present; methane and sulfate concentrations approach zero above and below the methane oxidation zone, respectively (11, 12, 12a, 14, 21). Sulfate appears to be one of the dominant electron acceptors in the zone of anaerobic methane oxidation from the standpoint of both energy yield and oxidizing capacity (23). A secondary maximum in sulfate reduction rate coincident with the maximum in methane oxidation rate has been observed in several sediment systems (11, 12a; Fig. 2c and d). Measured sulfate reduction rates in the methane oxidation zone of Skan Bay sediments are approximately 10 times higher than the methane oxidation rates. Differences of this magnitude have been observed elsewhere (11), but equal methane oxidation and sulfate reduction rates were observed in the methane oxidizing zone in sediments from the Kattegat and Skagerrak (12a). The differences between methane oxidation and sulfate reduction rates may reflect the difficulties encountered in measuring absolute in situ rates (10, 12a, 23).

Our observation that molybdate inhibited sulfate reduction without inhibiting methane oxidation (Fig. 3 and 4) suggests that sulfate-reducing bacteria are not directly responsible for the methane oxidation. Methane oxidation could be directly mediated by sulfate reducers only if the methane oxidizer was an atypical sulfate reducer that was not inhibited by molybdate. The methane oxidation rates in both inhibition experiments were one to two orders of magnitude slower than the sulfate reduction rates. Although molybdate inhibited 98 to 100% of the sulfate-reducing activity, the methane oxidation rates are approximately equal to the molybdate-inhibited sulfate reduction rates. Direct methane oxidation by a sulfate-reducing bacteria would require a sulfate reducer with a unique sulfate-activating enzyme capable of discriminating between the sulfate ion and its structural analog, molybdate.

Unknown organism. Anaerobic methane oxidation may be mediated by an organism that is distinct from methanogens and sulfate reducers. The secondary sulfate reduction rate maximum, which coincides with the methane oxidation rate maximum, was cited earlier as evidence of a link between the two processes. However, the secondary sulfate reduction rate maximum may result from processes other than anaerobic methane oxidation (C. Martens and P. Crill, Abstr. Winter Meet. Am. Geophys. Union, Eos **65**:905, 1984).

The electron acceptor used by the methane-oxidizing organism must be present in the zone of methane oxidation and must be capable of producing a negative free-energy change in a reaction with methane. Oxygen, nitrate, and nitrite are not present; bicarbonate and water are insufficiently strong oxidants. Possible electron acceptors include iron oxides, manganese oxides, and reduced sulfur [S(0) to S(IV)] compounds. The metal oxides are introduced to the sediments as oxidized particles and have high oxidizing capacity (23), but their persistence as oxides in anoxic systems and their role in microbial reactions are uncertain. Elemental sulfur has been shown to be an active component in anoxic marine sediments (26). Other reduced sulfur compounds, such as sulfite and thiosulfite, are intracellular metabolites in dissimilatory sulfate reduction (19) and may also be present in the zone of methane oxidation.

Bacterial consortium. A consortium in which sulfate-reducing bacteria are indirectly involved in methane oxidation may explain why sulfate reducers appear to be involved in methane oxidation, while methane oxidation appears to be unaffected by molybdate. An unknown organism capable of

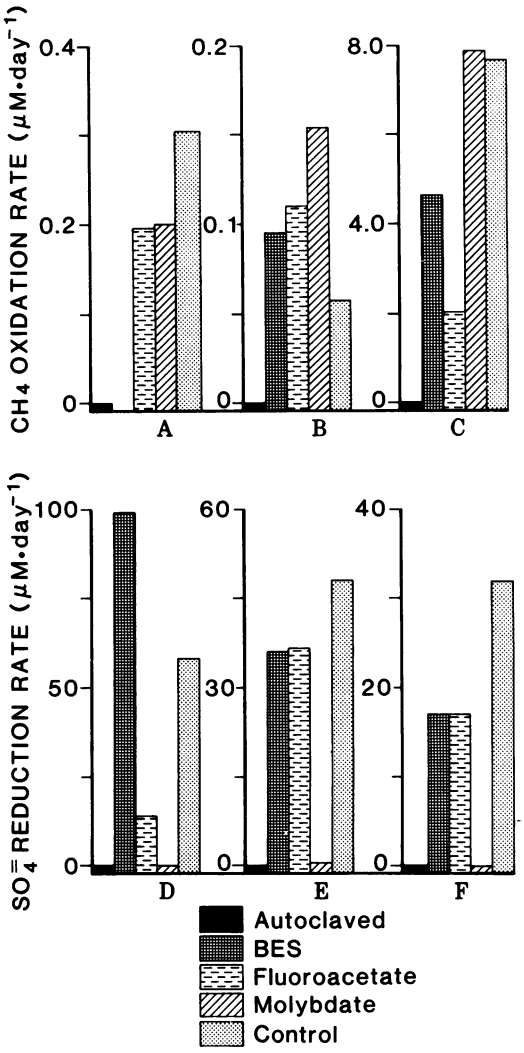

FIG. 4. Effect of inhibitors on methane oxidation and sulfate reduction rates in intact sediment. The experiments were performed on the 25- to 35-cm depth interval of subcores from the same box core. Each plot (A through F) is for samples taken from the same subcore. The BES treatment from subcore A was lost.

methane oxidation may convert methane to an intermediate substrate, which is then utilized by sulfate-reducing bacteria.

The compound responsible for coupling methane oxidation and sulfate reduction must be a substrate for sulfate-reducing bacteria and could be a low-molecular-weight fatty acid, alcohol, or hydrogen (19). If fatty acids or alcohols are intermediates in anaerobic methane oxidation, inhibition of sulfate-reducing bacteria should prevent the complete oxidation of methane to carbon dioxide. Our methane oxidation technique measures carbon dioxide resulting from the complete oxidation of methane; since we observed no inhibition of methane oxidation with molybdate, we conclude that fatty acids and alcohols are not the coupling substrates. Fluoroacetate, which inhibits acetate utilizers, was added in concentrations 1,000-fold greater than the natural acetate concentration (10 μM). Fluoroacetate had no effect on methane oxidation, confirming that acetate is not the coupling substrate. Hydrogen may be the intermediate compound that couples methane oxidation and sulfate reduction. The methane-oxidizing organism may oxidize methane to

carbon dioxide and release hydrogen, which serves as a substrate for sulfate-reducing bacteria. Since molybdate does not inhibit all hydrogen-consuming organisms, methane oxidation may proceed when sulfate reducers are inhibited. For example, Nedwell and Banat (15) have shown that hydrogen consumption by methanogens is stimulated by molybdate.

Inhibition experiments on anoxic marine sediments that were actively oxidizing methane indicate that anaerobic methane oxidation is not directly mediated by methanogenic or sulfate-reducing bacteria. Our results are consistent with two possibilities: anaerobic methane oxidation may be conducted by (i) an unknown organism using metal oxides or reduced sulfur compounds as an electron acceptor or (ii) a consortium involving an unknown organism and a sulfate-reducer which use hydrogen as the coupling substrate.

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