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Tugel, J.B., M.E. Hines, and G.E. Jones. 1986. Microbial Iron Reduction by Enrichment Cultures Isolated from Estuarine Sediments. Applied and Environmental Microbiology 52:1167-1172.

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Applied and Environmental Microbiology, Nov. 1986, p. 1167–1172
0099-2240/86/111167-06$02.00/0
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Vol. 52, No. 5

Microbial Iron Reduction by Enrichment Cultures Isolated from Estuarine Sediments†

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Received 9 June 1986/Accepted 18 August 1986

Microbial Fe reduction in acetate- and succinate-containing enrichment cultures initiated with an estuarine sediment inoculum was studied. Fe reduction was unaffected when SO_4^{2-} reduction was inhibited by MoO_4^{2-}, indicating that both processes could occur independently. Bacterially produced sulfide precipitated as FeS but was not completely responsible for Fe reduction. The separation of oxidized Fe particles from bacteria by dialysis tubing demonstrated that direct bacterial contact was necessary for Fe reduction. Fe reduction in cultures amended with NO_3^- was delayed until NO_3^- and NO_2^- were removed. However, bacterial attachment to oxidized Fe particles in NO_3^--amended cultures occurred early during growth in a manner similar to NO_3^--free cultures. During late stages of growth, bacteria not attached to Fe particles became pale and swollen, while attached cells remained bright blue when examined by 4′,6-diamidine-2-phenylindole epifluorescence microscopy. The presence of added oxidized Mn had no effect on Fe reduction. The results suggested that enzymatic Fe reduction was responsible for reducing Fe in these cultures even in the presence of sulfide and that cells incapable of Fe reduction became unhealthy when Fe(III) was the only available electron acceptor.

Terminal decomposition processes in marine sedimentary environments have received considerable attention recently (2, 7, 11, 12, 15, 20, 24–27, 29). In particular, SO_4^{2-} reduction, methanogenesis, and the reduction of inorganic nitrogen are important anaerobic processes in sediments. They vary in their magnitude, depending on the availability of electron acceptors and organic matter (1, 3, 12, 14). Geochemical studies have demonstrated that the vertical zonation of biogeochemical processes in sediments includes regions of Mn and Fe reduction (4, 24). Sufficient free energy may be generated from these reductions to support microbial growth (4). Although the delineation of microbial and chemical reduction of Fe is difficult in some instances, sufficient evidence exists which suggests that microbial Fe reduction occurs in nature and that it is an important component of metabolism in freshwater (9, 10, 13) and marine (13, 28) sediments and in waterlogged soils (16, 18, 22). The bacteria responsible for Fe reduction appear to be facultative anaerobes with the ability to utilize O_2, NO_3^-, oxidized Mn, or a combination of these as electron acceptors (8–10, 18, 28).

Previous work in our laboratory demonstrated that Fe cycling was extremely rapid in estuarine sediments, particularly when O_2 was introduced by the activities of mobile infauna (6, 7). The aim of the present study was to determine the presence of Fe-reducing bacteria in these sediments and to study the characteristics of reduction in relation to alternate electron acceptors. This goal was accomplished by using enrichment cultures to eliminate interferences caused by sediment particles. Experiments involving the separation of bacteria from Fe(III) and the microscopic examination of cell attachment to Fe(III) particles demonstrated that attachment was required before Fe reduction could proceed.

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MATERIALS AND METHODS

Growth medium. The basal medium consisted of the following in artificial seawater adjusted to a salinity of 26 ppt (g liter^{-1}): 1.9 mM NH_4Cl, 100 mg of yeast extract liter^{-1}, and 735 μM sodium acetate · $3H_2O$ or 370 μM sodium succinate · $6H_2O$ or both. Acetate, which cannot be fermented except during methanogenesis, was selected to avoid Fe(III) reduction because of the production of reduced organic end products. Succinate can be fermented to propionate. In some instances, acetate and succinate were replaced with 1.1 mM glucose. In other instances, yeast extract served as the only organic substrate. Portions (50 ml) of the medium were autoclaved in 100-ml serum vials stoppered with cotton. After the vials were cooled to 25°C, 1 ml of a filter-sterilized solution containing 8.3 mM K_2HPO_4 and 6.6 mM KH_2PO_4 was added. The vials were rendered anoxic overnight with an anaerobic jar (Gas Pak, BBL Microbiology Systems, Cockeysville, Md.). The jar was opened in a N_2-flushed glove bag, and 0.1 ml of an autoclaved solution containing 200 mM $FeCl_3 \cdot 6H_2O$ (pH adjusted to 7.0 with 6 N NaOH) was added, resulting in a final concentration of 400 μM Fe(III). The Fe(III) was prepared under oxic conditions, and iron oxyhydroxide precipitated immediately upon the addition of NaOH. The vials were sealed with serum stoppers.

Fe reduction assay. Reduced Fe was measured spectrophotometrically by a modification of the FerroZine extraction technique developed by Sørensen (28). With a sterile N_2-flushed 1-ml syringe, 0.5 ml of a culture was withdrawn and mixed with 50 μl of 1 N HCl for 15 s to release Fe^{2+} associated with acid-soluble material such as FeS. This concentration of acid was sufficient to dissolve all precipitated Fe^{2+} and was the optimum for the maximum response of FerroZine. A 1.5-ml solution of 0.1% (wt/vol) FerroZine in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH adjusted to 7.0 with 6 N NaOH) was added. After 1 min, the solution was filtered (0.4-μm-

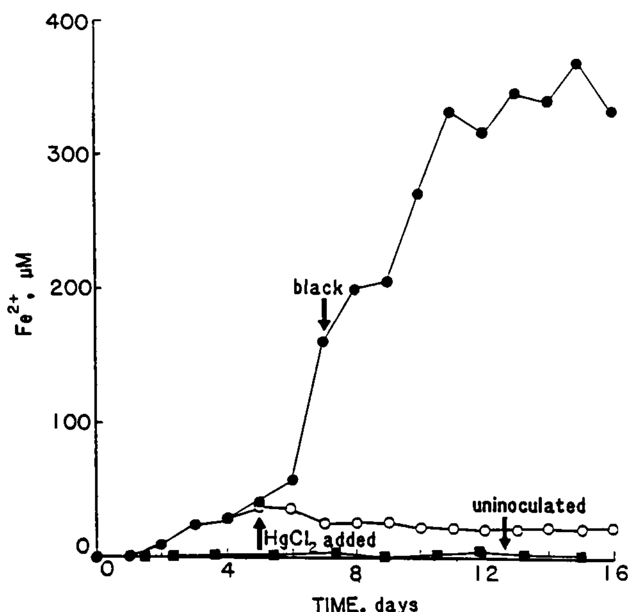

FIG. 1. Time course of Fe reduction in inoculated and uninoculated enrichment cultures and effect of $HgCl_2$ (0.1 mg ml^{-1}) added on day 5. The uninhibited culture turned black on day 7, indicating the formation of FeS.

pore-size filters; Nuclepore Corp., Pleasanton, Calif.) and the concentration of Fe^{2+} was determined by a comparison of A_{562} of the extract with those of a series of Fe standards. This technique measures reduced Fe only. Fe standards were measured after the addition of the reducing agent, hydroxylamine (30).

Experimental treatments. Surficial sediments were collected from a shallow-water site in Great Bay, N.H. (6). Subsamples (0.1 ml) were added to the medium, and cultures were transferred three times before experimentation. In all experiments, the production of Fe^{2+} was monitored over time and compared with Fe^{2+} concentrations in uninoculated controls. In some instances, a 0.1-ml solution of 50 mg of $HgCl_2$ ml^{-1} (pH 7.0) was added during growth to arrest microbial activity. Anaerobic conditions were maintained throughout all experiments, and vessels were held at 20°C without agitation. Redox potential (E_h) was measured with a combination platinum electrode (Orion Research, Inc., Cambridge, Mass.) standardized with a redox buffer (31).

The effect of bacterial SO_4^{2-} reduction on Fe reduction was examined by a comparison of Fe^{2+} accumulation in the above-mentioned medium with accumulations in cultures containing SO_4^{2-}-free artificial seawater or cultures amended with the SO_4^{2-} reduction inhibitor Na_2MoO_4 (20 mM final concentration). The concentration of MoO_4^{2-} used here has been shown to effectively inhibit SO_4^{2-} reduction (20, 26). The influence of NO_3^- on Fe reduction was examined by amending cultures with 200 μM $NaNO_3$ and measuring Fe^{2+}, NO_3^-, and NO_2^- concentrations over time. Inorganic nitrogen compounds were measured with an Autoanalyzer (Technicon Instruments Corp., Tarrytown, N.Y.) (5). The influence of Mn(IV) on Fe reduction was examined by amending cultures with 200 μM MnO_2 powder (Alpha Products, Danvers, Mass.) and measuring Fe^{2+} accumulation over time. Methane was measured with a Sigma 300 gas chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.), a flame ionization detector, and a 2-m stainless steel column packed with Poropak Q.

Dialysis experiments. To determine if bacterial attachment was a prerequisite for Fe reduction in enrichment cultures, we sealed oxidized Fe inside 1.6-cm-diameter dialysis tubing (A. H. Thomas Co.), which retained molecules of molecular weight greater than 12,000. The tubing was autoclaved with the basal medium, and phosphate was added as described above. After inoculation, Fe^{2+} production in the medium surrounding the dialysis tubing was measured over time and compared with results obtained with cultures in which Fe(III) was not held within dialysis tubing. Experiments were conducted with and without the addition of MoO_4^{2-}. At the end of the experiments, the dialysis tubing was removed and the Fe^{2+} concentration within the tubing was determined.

Microscopy. To observe microbial growth and attachment to Fe(III) particles, we withdrew 0.5-ml subsamples daily and preserved them in 4.5 ml of 4% formaldehyde. After appropriate dilution, 2.0-ml samples were stained in the dark for 10 min with 0.2 ml of a 10-μg-ml^{-1} solution of 4',6-diamidine-2-phenylindole (23). Samples were collected on Irgalan Black-stained, 25-mm-diameter, 0.2-μm-pore Nuclepore filters. Bacteria and Fe(III) particles were examined by phase-contrast and 4',6-diamidine-2-phenylindole epifluorescence with a 100× lens objective and Zeiss standard microscope. During enumerations, 10 microscopic fields for counting were selected randomly and not all fields contained oxidized Fe particles. Therefore, the precision of counts used for a comparison of the percentages of total bacteria which were attached to particles was poor (coefficient of variation = 20 to 80%). In addition, some bacteria originally attached to particles may have been removed during sample processing.

RESULTS

The majority of data reported here were obtained from enrichments containing both acetate and succinate. However, cultures grown with acetate or succinate alone behaved the same as when grown on the mixture of the substrates. The onset of Fe reduction in enrichment cultures occurred after 1 to 4 days, and the average maximum rate of reduction for all uninhibited cultures, including data not presented here, was 39 μmol $liter^{-1}$ day^{-1} (Fig. 1). Little to no Fe reduction occurred in uninoculated cultures, and the addition of $HgCl_2$ during growth arrested Fe reduction immediately (Fig. 1). Methane was not detected. A 10-fold decrease in the yeast extract concentration did not affect the rate of Fe reduction or the yield of Fe^{2+}. Bacterial growth and Fe reduction occurred in cultures devoid of yeast extract. However, without yeast extract, Fe reduction decreased in magnitude with each subsequent transfer into fresh medium. In addition, Fe reduction occurred in yeast extract-containing media which did not contain any additional organic substrate.

A black precipitate, indicative of FeS formation, was observed after 5 to 8 days of growth. No black precipitate (FeS or HgS) formed in cultures amended with $HgCl_2$. Acid treatment before FerroZine extraction resulted in a fourfold increase in the recovery of Fe^{2+} compared with extraction without acid. The black FeS precipitate which formed during incubation dissolved immediately upon acidification. When cultures amended with the SO_4^{2-} reduction inhibitor MoO_4^{2-} were subjected to acidification, the Fe^{2+} yield was identical to that in nonacidified samples, indicating that undissolved Fe^{2+} was precipitated as FeS.

The inhibition of SO_4^{2-} reduction by the addition of MoO_4^{2-} to SO_4^{2-}-containing cultures did not affect Fe reduction (Fig. 2). Black FeS formation was never observed in MoO_4^{2-}-containing cultures. Fe reduction in SO_4^{2-}-free media occurred more slowly than in SO_4^{2-}-containing cultures and never produced a black FeS precipitate (Fig. 3). After 3 weeks of incubation, the Fe^{2+} concentration in the SO_4^{2-}-free culture in Fig. 3 remained 125 μM less than in the SO_4^{2-}-containing culture (305 versus 430 μM).

Fe reduction did not occur when the Fe(III) oxyhydroxide particles were held within dialysis tubing and when SO_4^{2-} reduction was inhibited by MoO_4^{2-} (Fig. 4). When SO_4^{2-} reduction was not inhibited, FeS precipitation was observed within the dialysis tubing. However, Fe^{2+} was not detected outside of the tubing, indicating that sulfide-mediated Fe reduction and precipitation were confined within the tubing and that Fe^{2+} did not diffuse into the surrounding medium.

Microscopic examination of SO_4^{2-}-containing cultures revealed a mixed bacterial population made up of motile and nonmotile spirilla, slender and large rods, and cocci. A gradual attachment of bacteria to Fe particles developed (Fig. 5). During the first 24 h of incubation, little association between the bacteria and Fe particles was seen. After 72 h, any Fe particles observed by phase-contrast microscopy were covered by fluorescent blue bacteria when 4′,6-diamidine-2-phenylindole epifluorescence techniques were used. After 7 days, any cells that were not attached to Fe particles were pale yellow and swollen grossly, whereas the attached cells remained bright blue. Even though the bacterial population multiplied rapidly during the first 24 h of incubation, Fe^{2+} did not begin accumulating until bacteria became attached to Fe particles (Fig. 5).

The addition of NO_3^- to enrichment cultures delayed the onset of rapid Fe reduction and decreased the total amount of Fe^{2+} produced (Fig. 6). However, the addition of reagent-grade MnO_2 had no apparent effect on Fe reduction. NO_3^- was removed from cultures in less than 48 h, since it was reduced stoichiometrically to NO_2^- (Fig. 7A). NO_2^- reduction proceeded more slowly than NO_3^- reduction and was complete after 6 days. Fe reduction did not increase significantly until 2 to 3 days after NO_2^- removal. The redox potential (E_h) in cultures with and without added NO_3^- ranged from −150 to −200 mV throughout a 13-day incubation. Bacterial growth during the first few days of incubation was more rapid in NO_3^--containing cultures (Fig. 7B) than in the absence of NO_3^- (Fig. 5). The attachment of bacteria to Fe(III) particles in NO_3^--containing media occurred in a manner similar to the attachment in NO_3^--free media, even though Fe reduction in the former did not commence until several days later. However, maximum attachment was noted on days 9 and 10, when Fe reduction was observed.

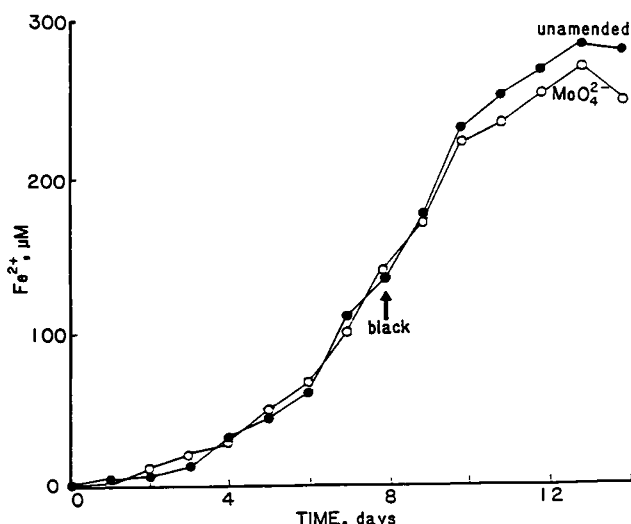

FIG. 2. Fe reduction in the presence and absence of the SO_4^{2-} reduction inhibitor MoO_4^{2-} (20 mM) added at the start of the experiment. Only the unamended culture turned black on day 8.

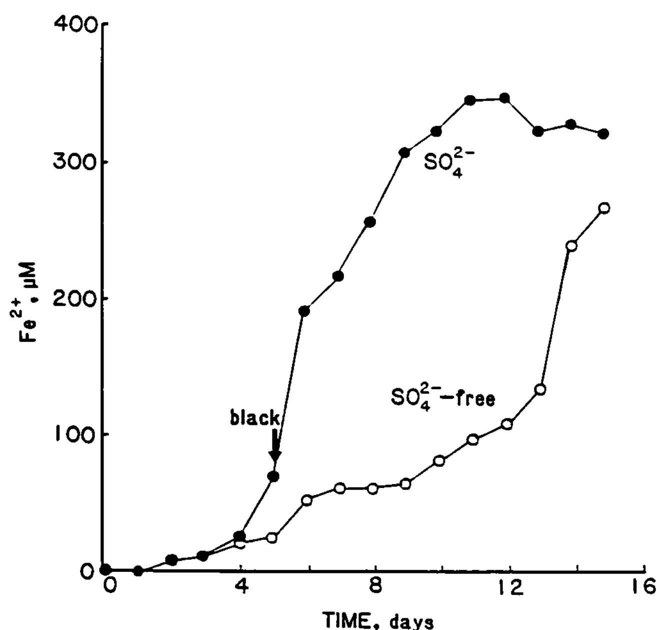

FIG. 3. Fe reduction in growth media prepared with or without SO_4^{2-}.

When acetate and succinate were replaced with glucose, Fe reduction increased to an average rate of 160 μmol liter^{-1} day^{-1} (data not shown). The presence of glucose did not alter the effects of SO_4^{2-}, NO_3^-, or MnO_2 on Fe reduction (J. B. Tugel, M.S. thesis, University of New Hampshire, Durham, 1985). Furthermore, when Fe(III) was held within dialysis tubing and glucose was used as substrate, no Fe reduction occurred, indicating that metabolites potentially liberated during glucose metabolism did not chemically reduce Fe.

DISCUSSION

The use of enrichment cultures allowed us to study Fe reduction without interference from sediment particles. Sørensen (28) and Lovley and Phillips (13) studied Fe reduction in sediment slurries. During preliminary experimentation, we were able to demonstrate Fe reduction in slurry systems as well (Tugel, M.S. thesis). However, we obtained erratic results when we amended slurries with inhibitors or alternate electron acceptors such as MoO_4^{2-} or NO_3^-, respectively.

Acid treatment of subsamples of our enrichment cultures produced Fe^{2+} recoveries which were four times greater than those without acid. Except for the recent study by Lovley and Phillips (13), this procedure was not used by others who have employed Fe measurement techniques specific for reduced Fe (16–18, 28). One cannot use acid

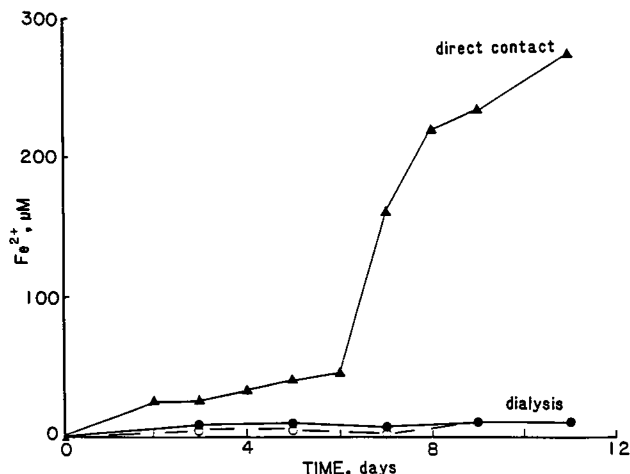

FIG. 4. Inhibition of Fe reduction by the separation of bacteria from oxidized Fe by dialysis tubing. SO_4^{2-} reduction was inhibited by MoO_4^{2-} (20 mM). ○, Uninoculated medium.

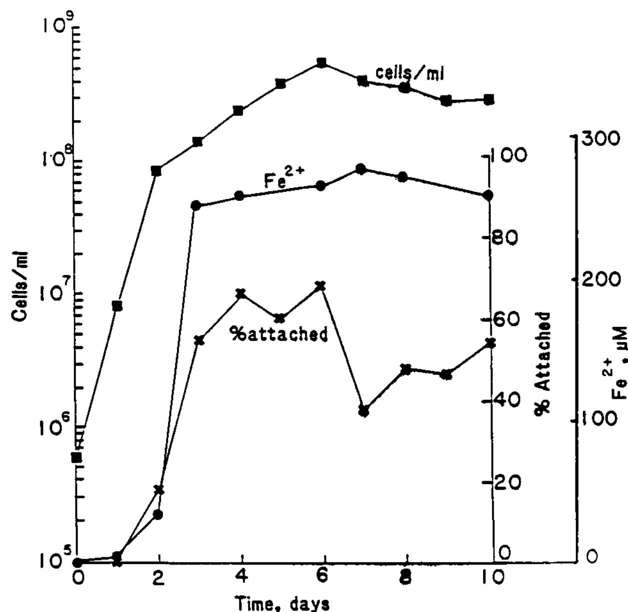

FIG. 5. Bacterial growth, percentage of cells attached to oxidized Fe particles, and Fe reduction in an enrichment culture.

treatment for measurements of Fe^{2+} accumulation in sediment slurries unless the quantity of Fe^{2+} produced during incubation is large enough not to be masked by the dissolution of endogenous FeS. However, acid treatment is recommended for studies such as ours involving cultures with the capability for sulfide production.

The results from enrichment cultures were indicative of a direct microbial involvement in Fe reduction rather than indirect chemical reduction by microbially generated reduced end products. First, $HgCl_2$ arrested Fe reduction immediately (Fig. 1). Second, the utilization of acetate did not allow for the production of reducing organic products capable of Fe reduction. Neither acetate nor succinate reduced Fe in the absence of a microbial inoculum. Third, separation of Fe(III) from microorganisms by dialysis tubing prevented Fe reduction. The initial rapid growth of bacteria before Fe reduction (Fig. 5) probably was caused by either the utilization of yeast extract or SO_4^{2-} reduction, since acetate metabolism requires an exogeneous electron acceptor in the absence of methanogenesis. However, because Fe reduction did not occur in the dialysis experiment when SO_4^{2-} reduction was inhibited (Fig. 4), we can conclude that the utilization of yeast extract and glucose did not result in the production of sufficient quantities of soluble end products to reduce Fe in the dialysis tubing. Fourth, the finding that the inhibition of SO_4^{2-} reduction by MoO_4^{2-} had no effect on Fe^{2+} accumulation demonstrated that Fe reduction was not caused solely by a chemical reduction by sulfide.

The occurrence of Fe reduction in enrichment cultures containing acetate, MoO_4^{2-}, and little or no yeast extract suggested that Fe(III) acted as a terminal electron acceptor. Munch and Ottow (18) reported that Fe appears to serve as a terminal electron acceptor in waterlogged soils, and Lovley and Phillips (13) found that Fe(III) could be a major sink for electrons during organic matter decomposition in anaerobic sediments. However, the present results were not unequivocal, since mixed cultures were used.

The finding that Fe reduction was not affected when SO_4^{2-} reduction was inhibited suggested that these two processes proceeded independently. Sørensen (28) reported the cooccurrence of Fe reduction and SO_4^{2-} reduction in slurries of marine sediment. However, sedimentary pore water data reveal an accumulation of Fe^{2+} before a depletion in SO_4^{2-} (4), suggesting that Fe reduction can outcompete SO_4^{2-} reduction. Lovley and Phillips (13) demonstrated that Fe reduction systems can surpass methanogenic food chains in the utilization of organic matter. Since the concentrations of organic matter added to our enrichment cultures were higher than those normally encountered in nature, it was likely that any competition which might have existed between these groups of bacteria was relieved by the excess substrate. Therefore, additional studies are needed to determine the nature of competition between Fe reduction and SO_4^{2-} reduction.

If microbial sulfide production was responsible for part or all of the Fe reduction in our enrichment cultures, then Fe

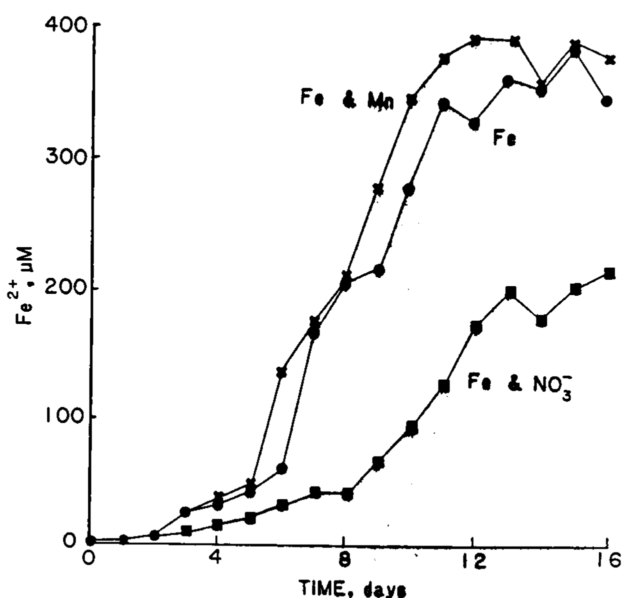

FIG. 6. Fe reduction in media supplemented with Mn (IV) (200 µM) or NO_3^- (200 µM) compared with that in unamended medium.

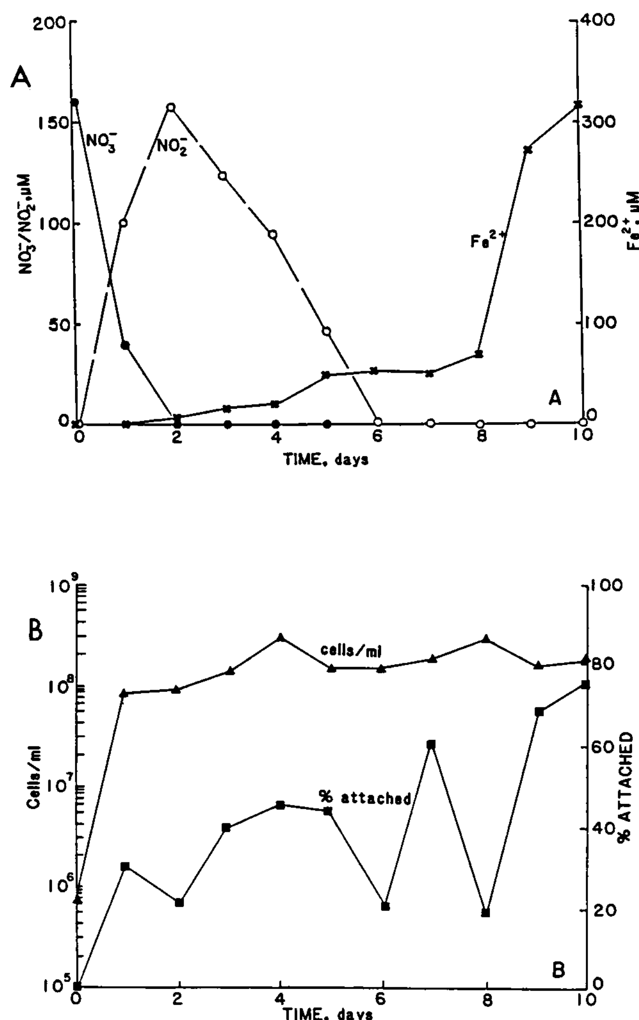

FIG. 7. Effect of NO_3^- (200 µM) on Fe reduction. (A) NO_3^-, NO_2^-, and Fe^{2+} concentrations; (B) bacterial growth and attachment.

reduction would have decreased or ceased in cultures amended with MoO_4^{2-}. The omission of SO_4^{2-} from cultures did cause a decrease in the quantity and rate of Fe reduction (Fig. 3). Therefore, it was not clear to what extent sulfide-Fe interaction influenced Fe reduction in cultures. However, it was clear that Fe reduction occurred in the absence of sulfide production and that at least a portion of Fe reduced in sulfide-producing cultures was due to a direct microbial reduction in lieu of an indirect reduction by sulfide. It is possible that bacterial metabolism was S-limited in SO_4^{2-}-free media.

Cell attachment to oxidized Fe particles was a prerequisite for Fe reduction (Fig. 5). Munch and Ottow (17) reported a similar result when oxidized Fe was separated from cultures of soil bacteria by dialysis tubing. Cell attachment in enrichments occurred primarily within the first 2 to 4 days of growth, even when Fe reduction was inhibited by NO_3^- and NO_2^- reduction (Fig. 7). Therefore, cell attachment to Fe particles occurred regardless of the necessity for Fe reduction. Cells which were not attached to Fe(III) particles became swollen and lost their ability to be stained bright blue by 4',6-diamidine-2-phenylindole. It appeared that once the yeast extract supplement was near exhaustion, cells which were unable to reduce Fe(III) deteriorated, while Fe(III)-reducing bacteria remained healthy. It is also possible that organic substrates adsorbed by Fe particles provided additional substrates for particle-associated bacteria. Since the vast majority of Fe in sediments is particulate, the process of bacterial attachment to Fe(III) particles may be important in regulating sedimentary Fe transformations.

The inhibition of Fe reduction by NO_3^- was not surprising, since previous reports have shown this effect on Fe and SO_4^{2-} reduction (19, 28). Although NO_2^- is capable of reoxidizing Fe^{2+} to Fe^{3+} (19), we were not able to determine whether inhibition or reoxidation prevented the accumulation of Fe^{2+} in NO_3^--containing cultures. The finding that the E_h did not vary throughout incubation with oxidized nitrogen compounds supported the former mechanism. The addition of MnO_2 to media had no effect on Fe reduction. Ottow (21) reported that Mn reduction occurred before Fe reduction in pure cultures of soil bacteria. Since crystalline forms of oxidized metals are more difficult to reduce microbially than amorphous forms (13, 16), it was likely that the reagent-grade MnO_2 used during the present study was not capable of competing with amorphous Fe as an oxidant.

In conclusion, bacterial populations isolated from estuarine sediments possessed the ability to reduce Fe. Fe in sediments under the appropriate geochemical conditions may act as an important oxidant for organic matter utilization. The significance of cell attachment to Fe(III) particles and the possible biogeochemical interactions between Fe reduction and SO_4^{2-} reduction processes deserve further attention.

ACKNOWLEDGMENTS

We thank R. P. Blakemore, D. A. Bazylinski, and W. B. Lyons for advice. We also thank D. J. Burdige and two anonymous reviewers for critically reading the manuscript. We especially appreciate the discussions with D. J. Burdige. T. C. Loder conducted the nitrate and nitrite analyses. We thank Conrad Fischer for technical assistance.

This work was supported by National Science Foundation grant OCE82-14863 and by the University of New Hampshire Ocean Process Analysis Laboratory.

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