

Reduction of Ferric Iron in Anaerobic, Marine Sediment and Interaction with Reduction of Nitrate and Sulfate

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Received 29 June 1981/Accepted 5 October 1981

Studies were carried out to elucidate the nature and importance of Fe^{3+} reduction in anaerobic slurries of marine surface sediment. A constant accumulation of Fe^{2+} took place immediately after the endogenous NO_3^- was depleted. Pasteurized controls showed no activity of Fe^{3+} reduction. Additions of 0.2 mM NO_3^- and NO_2^- to the active slurries arrested the Fe^{3+} reduction, and the process was resumed only after a depletion of the added compounds. Extended, initial aeration of the sediment did not affect the capacity for reduction of NO_3^- and Fe^{3+} , but the treatments with NO_3^- increased the capacity for Fe^{3+} reduction. Addition of 20 mM MoO_4^{2-} completely inhibited the SO_4^{2-} reduction, but did not affect the reduction of Fe^{3+} . The process of Fe^{3+} reduction was most likely associated with the activity of facultative anaerobic, NO_3^- -reducing bacteria. In surface sediment, the bulk of the Fe^{3+} reduction may be microbial, and the process may be important for mineralization in situ if the availability of NO_3^- is low.

The origin of Fe^{3+} reduction in anaerobic environments has been a matter of controversy, since both a chemical and a biological source may exist. A chemical reduction of Fe^{3+} by organic acids (12) and notably by inorganic sulfide (1) could thus be responsible, though a bacterial origin seems possible as judged from the apparent role of Fe^{3+} as an alternative electron acceptor for NO_3^- -reducing bacteria (7, 9, 10).

A vertical stratification of O_2 -, NO_3^- -, and SO_4^{2-} -reducing activities has been found in the marine sediments (14), and in the pore waters, an accumulation of dissolved Fe^{2+} may be observed immediately below the NO_3^- -containing surface zone (4). Though all of these reductions may occur in close association in the sediment, the metabolic relationships between them have not yet been studied in detail. Thus, the nature of the Fe^{3+} reduction is totally unknown.

In this study, the aim was to demonstrate a significance of the microbial Fe^{3+} reduction in marine sediment and to study the interactions with the reduction of NO_3^- and SO_4^{2-} . The changes of NO_3^- , NO_2^- and Fe^{2+} were followed over time to measure the NO_3^- and Fe^{3+} reduction in suspended sediment, and radiotracer experiments with $^{35}\text{SO}_4^{2-}$ were performed to follow the reduction of SO_4^{2-} . The capacities for reduction of NO_3^- , Fe^{3+} and SO_4^{2-} were compared in sediments of different origin.

MATERIALS AND METHODS

Sample collection and preparation. A batch of the "oxidized" surface sediment (0- to 5-cm depth) was

collected in a shallow (0- to 1-m depth) coastal lagoon (Kysing Fjord). Sampling was done during spring 1981 when the in situ water temperature was 5 to 10°C. The sediment was sieved through a 1-mm screen and diluted with seawater to a water content of about 80% (wt/wt). Bottles with 1 liter of the slurry were then left overnight for equilibration in the dark and at room temperature. The bottles were open during the conditioning, but anaerobiosis was soon established under the stagnant water phase. The day after, magnetic stirring was applied in the open bottles for about 30 min to reduce the endogenous Fe^{2+} concentration. The bottles were then stoppered, and complete anaerobiosis was obtained as the gas phase was flushed with N_2 for 10 min. A slight but constant N_2 pressure was maintained through a pipette in the stopper. An outlet at the bottom of the bottles served for subsampling of the sediment, and the activities of NO_3^- , Fe^{3+} , and SO_4^{2-} reduction were determined by the techniques described below. Pasteurized bottles, in which the temperature was raised and kept at 80°C for 10 min before cooling, were included to determine any chemical transformations in the sediment.

Assay of NO_3^- reduction. The capacity for NO_3^- reduction was determined by injection of 2.5 ml of a 0.1 M NaNO_3 solution to 1 liter of sediment to give an initial concentration of about 0.2 mM NO_3^- in the interstitial water. The rate of the NO_3^- depletion was then measured after centrifugation of subsamples and measurement of NO_3^- in the supernatant by an automated Cd reduction procedure (Chemlab, Hornchurch, England).

Assay of Fe^{3+} reduction. A technique was developed to determine the reduction of Fe^{3+} to Fe^{2+} in the slurries. The assay was based on a short-term extraction of Fe^{2+} by ferrozine, a colorimetric reagent which forms a stable magenta complex with Fe^{2+} (11). A subsample of 0.3 g of sediment was pipetted into 3 ml

of a 0.1% (wt/wt) ferrozine solution in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH adjusted to 7.0 with NaOH). A rapid coloration took place, but the extraction time was standardized to 1 min under constant mixing in a Vortex mixer. The colored solution was then filtered (0.45 μm ; Millipore Corp.) and assayed by its absorbance at 562 nm. Longer extraction times (hours) gave higher absorbance in the filtrates, but preliminary experiments showed that 1 min was enough to extract the Fe^{2+} produced by Fe^{3+} reduction during the experiments. The exclusion of O_2 during the extraction was found to be unnecessary, apparently due to the rapid processing and the large excess of the ferrozine reagent.

Assay of SO_4^{2-} reduction. The SO_4^{2-} reduction rate was determined by ^{35}S tracer data and the SO_4^{2-} concentration in the sediment (5). The ^{35}S assay was initiated by injecting 2 μl of carrier-free $^{35}\text{SO}_4^{2-}$ (about 3×10^6 dpm) into the slurry. At regular intervals, subsamples of 5 g were then taken into 5 ml of a 2% zinc acetate solution to trap any ^{35}S -labeled sulfide produced. The acid-volatile sulfide was later released from the subsamples by addition of HCl under N_2 and transferred to other traps with 5 ml of zinc acetate solution (5). Five milliliters of Lumagel (Lumac) scintillation fluid was added before the samples were counted in a liquid scintillation counter (Intertechne SL 30). Other subsamples of the sediment were taken into zinc acetate for a measurement of the ^{35}S activity in the CS_2 -extractable fraction used for elemental sulfur (S^0) determinations (see below). This extraction was performed overnight with 5 ml of CS_2 in stoppered glass tubes. After centrifugation, the overlying water phase was discarded, and 1 ml of the CS_2 phase was pipetted into scintillation vials and evaporated in the hood. The precipitate was then dissolved in 5 ml of Lumagel scintillation fluid. Five milliliters of distilled water was added to the vials before they were counted in the liquid scintillation counter. The counting efficiency of these samples was 70 to 80% of that obtained in the [^{35}S] sulfide samples. Finally, the analysis of $^{35}\text{SO}_4^{2-}$ activity was performed on 1 ml of supernatant obtained by centrifugation of the acidified and sulfide-free sediment. The samples were then made up to 5 ml with distilled water, and 5 ml of Lumagel was added before counting.

In a separate bottle, the SO_4^{2-} reduction was inhibited by addition of 10 ml of a 2 M Na_2MoO_4 solution (pH adjusted to 7.2 with NaOH) to give a concentration of about 20 mM in the bottles. This was previously shown to give a complete inhibition of the SO_4^{2-} reduction in the sediments (13). In the MoO_4^{2-} -containing subsamples, the HCl treatment did not release the sulfide (13), but this problem was overcome by incorporating a strong reducing agent, TiCl_3 , in the HCl solution at a concentration of 1% (wt/wt).

The concentration of HCl-volatile sulfide was determined by the methylene blue assay (2) after a transfer of the sulfide to zinc acetate traps as described above. The S^0 concentration was determined in 1- to 3-ml portions of the CS_2 extract. After evaporation of the CS_2 , the S^0 -containing precipitate was dissolved in 5 ml of a cyanide reagent (1 g of NaCN per liter of acetone plus water, 19:1 by volume). After cyanolysis for 4 h at room temperature, 1.5 ml of the cyanolysate

was mixed with 1.5 ml of a ferric chloride reagent (5 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ per liter of acetone plus water, 19:1 by volume). A colored complex was formed, and the absorbance was read at 460 nm. Details of the S^0 procedure (3) were changed for assays in the sediments (H. Troelsen and B. B. Jørgensen, *Estuarine Coastal Shelf Sci.*, in press). A gravimetric assay (Ba precipitation) of the SO_4^{2-} was performed with filter-sterilized samples (0.45 μm ; Millipore Corp.) obtained by pressure filtration at 3 atm (300 kPa) (5).

Activities are given in micromoles per gram per hour (wet sediment), and concentrations are given in molarity or micromoles per gram (wet sediment).

RESULTS

The SO_4^{2-} concentration was about 10 mM in the preconditioned slurries, whereas the endogenous NO_3^- (0 to 20 μM) was depleted soon after a complete anoxia was established. At this time, the $^{35}\text{SO}_4^{2-}$ was added to initiate the experiments (zero time).

Interactions between Fe^{3+} and SO_4^{2-} reduction. Concurrent reduction of both Fe^{3+} and SO_4^{2-} took place initially in the slurries as shown by the accumulation of Fe^{2+} and ^{35}S -labeled sulfide (Fig. 1). The subsequent addition of 20 mM MoO_4^{2-} completely arrested the SO_4^{2-} -reducing activity (about 0.05 $\mu\text{mol g}^{-1} \text{h}^{-1}$); no further production of ^{35}S -labeled sulfide was observed. The accumulation of ^{35}S activity in the CS_2 extract also stopped after the addition of MoO_4^{2-} , and the activity remained constant throughout the experiment. This indicated that the CS_2 -extractable ^{35}S activity, which was about 10% of the ^{35}S -labeled sulfide, was present in the organic matter rather than in S^0 and thus represented $^{35}\text{SO}_4^{2-}$ assimilation rather than $^{35}\text{S}^0$ produced by oxidation of the ^{35}S -labeled sulfide. The measured concentrations of HCl-volatile sulfide and CS_2 -extractable S^0 were about 0.5 and 0.2 $\mu\text{mol g}^{-1}$, respectively, and remained constant in the presence of MoO_4^{2-} (data not shown). The reduction of Fe^{3+} was not affected by the presence of MoO_4^{2-} , however, and the accumulation of Fe^{2+} continued at a rate of 0.12 $\mu\text{mol g}^{-1} \text{h}^{-1}$, similar to the activity before the MoO_4^{2-} was added (Fig. 1). The apparent absence of sulfide-mediated Fe^{3+} reduction during this period was confirmed by the constant radioactivity and size of the sulfide pool.

A comparison may be made to a preliminary experiment in which a preselection against the SO_4^{2-} -reducing bacteria was performed by extending (overnight) the initial air exposure under rigid mixing. This resulted in a 10-fold lower level of SO_4^{2-} reduction, whereas the capacity for Fe^{3+} reduction as well as for NO_3^- reduction remained high (data not shown).

The absence of Fe^{2+} accumulation in the pasteurized slurry suggested that the reduction

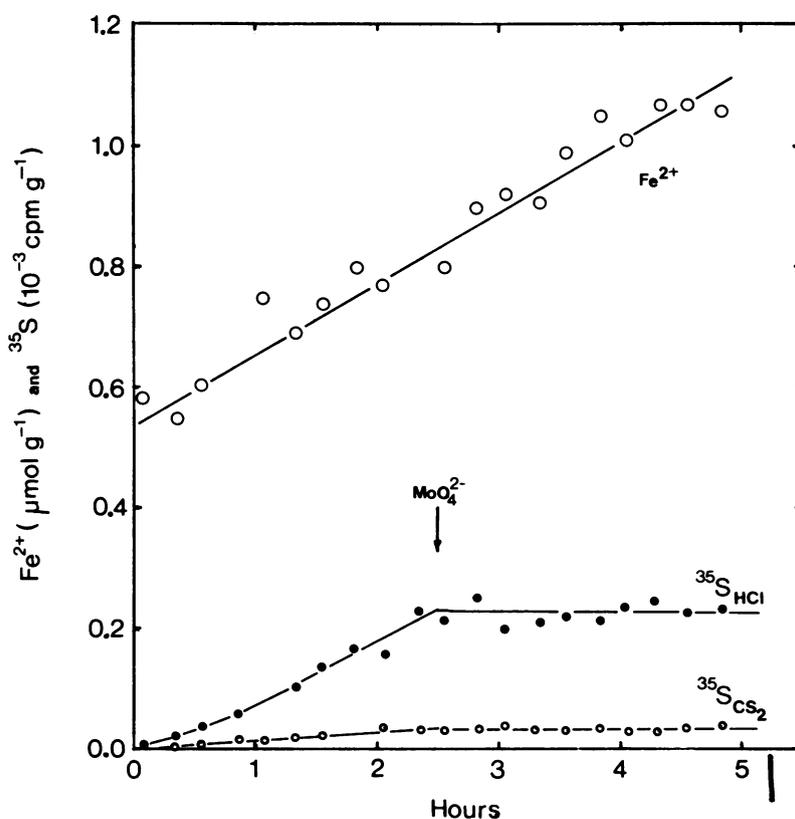


FIG. 1. Reduction of Fe^{3+} in presence of MoO_4^{2-} added to inhibit reduction of SO_4^{2-} . The $^{35}\text{SO}_4^{2-}$ was added at zero time, and the arrow indicates addition of 20 mM Na_2MoO_4 . Symbols: \circ , Fe^{2+} ; \bullet , HCl-volatile [^{35}S] sulfide; \circ , CS_2 -extractable [^{35}S] sulfur compounds.

of Fe^{3+} was directly associated with enzymatic activity or mediated by a production of bacterial metabolites (Fig. 2). The SO_4^{2-} reduction was also stopped by the heat treatment, and the constant sulfide pool in this experiment confirmed that sulfide did not interact chemically with Fe^{3+} in the slurries.

Interactions between Fe^{3+} and NO_3^- reduction. Addition of 0.2 mM NO_3^- to a slurry stopped the accumulation of both Fe^{2+} and ^{35}S -labeled sulfide, and their production was resumed only after the added NO_3^- was depleted (Fig. 3). Addition of 0.2 mM NO_2^- gave a similar effect (data not shown). A chemical oxidation of Fe^{2+} by NO_2^- has been reported (8), but no consumption of NO_2^- could be detected after 2 h when 0.2 mM NO_2^- was added to a pasteurized slurry (data not shown). The added NO_3^- gave rise to only a small, transient accumulation of NO_2^- of about 20 μM . The NO_3^- was reduced at a rate of about 0.20 $\mu\text{mol g}^{-1} \text{h}^{-1}$, and after its depletion, high rates of Fe^{3+} and SO_4^{2-} reduction were again observed. At this time, the Fe^{3+} reduction was actually stimulated, and a rate of about 0.25 $\mu\text{mol g}^{-1} \text{h}^{-1}$ was recorded.

Apparently, the application of NO_3^- had a stimulatory effect on the subsequent reduction of Fe^{3+} , and this observation was made consistently in the slurries.

Comparison of NO_3^- , Fe^{3+} , and SO_4^{2-} reduction. Slurries from three coastal localities, which were different in terms of water depth and salinity, were compared to illustrate the variation of NO_3^- , Fe^{3+} , and SO_4^{2-} reduction in the sediments (Table 1). Though the absolute values varied 10-fold, the capacities for reduction of NO_3^- and Fe^{3+} were comparable at the three localities.

DISCUSSION

Inorganic sulfide was a potential reductant for a chemical conversion of the Fe^{3+} , but evidence against significant oxidation of sulfide in the anaerobic sediment was provided by the constant specific activity of the ^{35}S -labeled sulfide pool in the pasteurized control and in the active slurry in which SO_4^{2-} reduction was stopped by MoO_4^{2-} . The unaffected reduction of Fe^{3+} in the absence of SO_4^{2-} reduction and the concurrent reduction of both Fe^{3+} and SO_4^{2-} in the

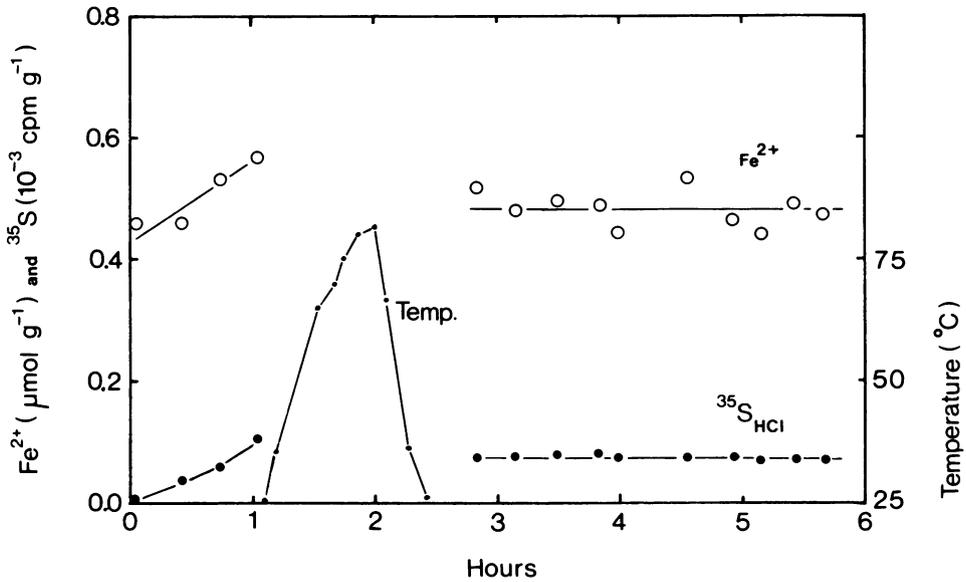


FIG. 2. Effect of pasteurization (80°C, 10 min) on reduction of Fe³⁺ and SO₄²⁻. The ³⁵SO₄²⁻ was added at zero time. Symbols: ○, Fe²⁺; ●, HCl-volatile [³⁵S] sulfide; ▴, temperature.

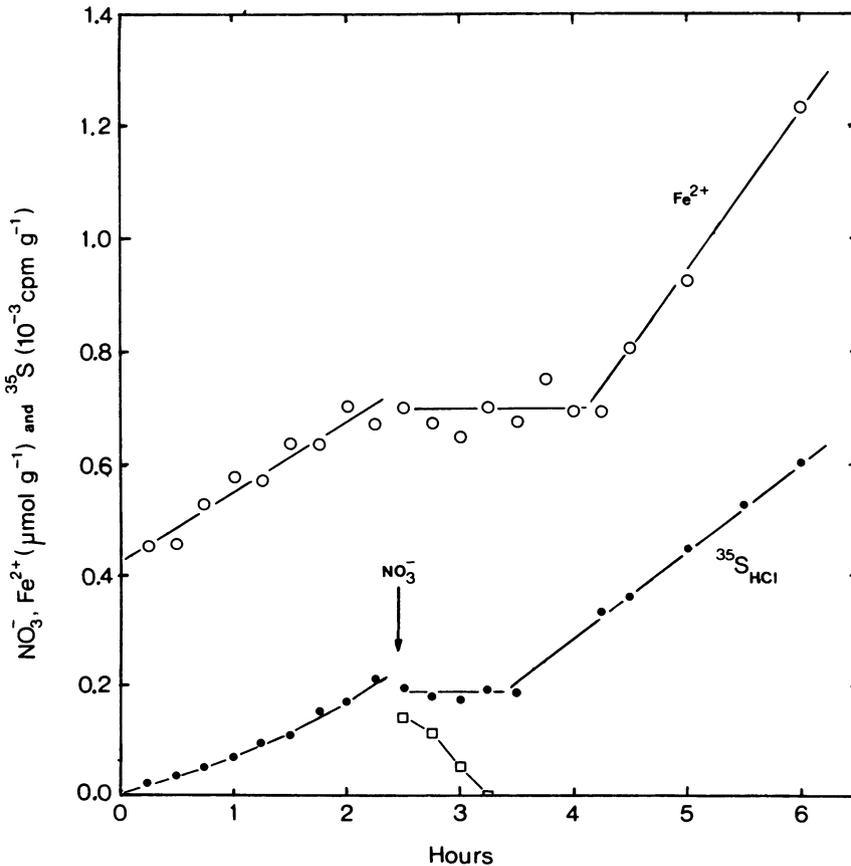


FIG. 3. Effect of NO₃⁻ on reduction of Fe³⁺ and SO₄²⁻. The ³⁵SO₄²⁻ was added at zero time, and the arrow indicates addition of 0.2 mM NaNO₃. Symbols: □, NO₃⁻; ○, Fe²⁺; ●, HCl-volatile [³⁵S] sulfide.

TABLE 1. Comparison of activities of NO_3^- , Fe^{3+} , and SO_4^{2-} reduction in anaerobic slurries of marine sediment

Locality	Depth (m)	Salinity range (‰)	Reduction activity ($\text{nmol g}^{-1} \text{h}^{-1}$)		
			NO_3^- ^a	Fe^{3+} ^b	SO_4^{2-} ^b
Randers Fjord	0-1	2-20	25	18	<5
Aarhus Bight	10	15-20	25	9	<5
Kysing Fjord	0-1	15-20	200	130	50

^a Maximal activity after addition of 0.2 mM NO_3^- .

^b Before addition of NO_3^- .

noninhibited sediment indicated a lack of interactions between the two processes.

The reduction of Fe^{3+} was stopped by the applications of NO_3^- and NO_2^- . Komatsu et al. (6) observed that the accumulation of Fe^{2+} in anaerobic soil was retarded while NO_2^- was being reduced, but this was interpreted as a stimulation of chemical Fe^{2+} oxidation by NO_2^- rather than an inhibition of the Fe^{3+} reduction while the NO_2^- was being reduced. In the present study, a chemical Fe^{2+} oxidation was not observed in the presence of NO_2^- in the pasteurized samples. The present data did not exclude, however, that biological Fe^{2+} oxidation may occur at the expense of NO_3^- and NO_2^- reduction, but the bacteria capable of this reaction are yet unknown.

There were several indications of a reduction of Fe^{3+} by NO_3^- -reducing bacteria. (i) A facultative anaerobic nature of the Fe^{3+} -reducing activity was shown by the nonaffected response to extended preaeration of the slurries. This treatment selected strongly against the SO_4^{2-} -reducing bacteria. (ii) The inhibition of the activity of Fe^{3+} reduction by NO_3^- and NO_2^- was in accordance with the apparent role of Fe^{3+} as an alternative electron acceptor in NO_3^- -reducing bacteria (9, 10). If these bacteria are involved, a preferential oxidation of the reduced components of the respiratory chain by NO_3^- and NO_2^- may take place before Fe^{3+} is reduced (7). (iii) The increased activity of Fe^{3+} reduction after the treatment with NO_3^- suggested a stimulatory effect of the NO_3^- on enzyme activation or growth of Fe^{3+} -reducing bacteria.

A comparison of the capacities for NO_3^- , Fe^{3+} , and SO_4^{2-} reduction in three different sediments showed that significant variation of the activities may be found in situ. Though the Fe^{3+} was abundant at all locations as judged from the brown coloration (ferric hydrous oxides) of the sediment, the capacity for Fe^{3+} reduction was clearly dependent on the origin of the samples. At each locality, the activity was comparable with that recorded for the reduction of NO_3^- . Though NO_3^- and SO_4^{2-} reductions are most efficient in terms of the number of

reducing equivalents transformed per unit of oxidant, the in situ conditions may be such that the reduction of Fe^{3+} is relatively more important than was revealed from the present assays. At a comparable temperature (18°C), the in situ activity of reduction of NO_3^- to N_2 (denitrification) was low, apparently a result of the low concentrations of NO_3^- in the sediments (20 μM or less) (14). The Fe^{3+} reduction may thus be significant in the sediments where the availability of NO_3^- is low. The present work suggests that the reduction of Fe^{3+} may constitute a significant contribution to the mineralization in sediments, but the in situ activity of the process needs to be determined.

ACKNOWLEDGMENTS

The skillful technical assistance of Hartwig Klapp and Preben Grann Sørensen is gratefully acknowledged.

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