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# Iron reduction and alteration of nontronite NAu-2 by a sulfate-reducing bacterium

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**Abstract**—Iron-rich clay minerals are abundant in the natural environment and are an important source of iron for microbial metabolism. The objective of this study was to understand the mechanism(s) of enhanced reduction of Fe(III) in iron-rich 2:1 clay minerals under sulfate-reducing conditions. In particular, biogenic reduction of structural Fe(III) in nontronite NAu-2, an Fe-rich smectite-group mineral, was studied using a *Desulfovibrio* spp. strain G-11 with or without amended sulfate. The microbial production of Fe(II) from NAu-2 is about 10% of total structural Fe(III) (30 mM) when Fe(III) is available as the sole electron acceptor. The measured production of Fe(II), however, can reach 29% of the total structural Fe(III) during sulfate reduction by G-11 when sulfate (50 mM) is concurrently added with NAu-2. In contrast, abiotic production of Fe(II) from the reaction of NAu-2 with $Na_2S$ (50 mM) is only ca. 7.5% of the total structural Fe(III). The enhanced reduction of structural Fe(III) by G-11, particularly in the presence of sulfate, is closely related to the growth rate and metabolic activities of the bacteria. Analyses by X-ray diffraction, transmission electron microscopy, and energy dispersive spectroscopy reveal significant changes in the structure and composition of NAu-2 during its alteration by bacterial sulfate reduction. G-11 can also derive nutrients from NAu-2 to support its growth in the absence of amended minerals and vitamins. Results of this study suggest that sulfate-reducing bacteria may play a more significant role than previously recognized in the cycling of Fe, S, and other elements during alteration of Fe-rich 2:1 clay minerals and other silicate minerals. 

## 1. INTRODUCTION

Sulfate-reducing bacteria (SRB) play an important role in the cycling of Fe and S in a variety of natural environments (Tuttle and Goldhaber, 1993; Bechtel et al., 1996). Coleman et al. (1993) reported that Fe reduction and formation of siderite in a salt marsh are due to the metabolism of SRB. Fortin et al. (2000) demonstrated that the seasonal cycling of Fe in a constructed wetland is due to the activity of SRB. White et al. (1998) and McKinley et al. (2000) observed that SRB may be involved in the reduction of Fe(III) from aluminosilicates.

Laboratory studies have demonstrated that some known SRB are able to reduce soluble ferric iron or solid iron oxides; in some cases, this may be an energy-conserving process. For example, Lovley et al. (1993) showed that various strains of SRB can reduce amorphous Fe-oxides in pure cultures. Tebo and Obraztsova (1998) showed that *Desulfotomaculum reducens* can gain energy by reducing Fe(III) and other metals. Neal et al. (2001) observed that hematite is dissolved in the presence of *Desulfovibrio* spp, which is potentially caused by a combination of enzymatic reduction of hematite and oxidation of hydrogen sulfide by the iron oxide.

A variety of clay minerals contain high concentrations of structural ferric iron (Köster et al., 1999; Manceau et al., 2000; Gates et al., 2002), which may be used as an electron acceptor for Fe-reducing bacteria. Kostka et al. (1996, 1999a, 2002) showed that *Shewanella oneidensis* strain MR-1 can gain energy by respiring structural Fe(III) in Fe-rich smectite-group minerals. This and other Fe-reducing bacteria can reduce up to 40% of the total structural Fe(III) and significantly change the structural and compositional properties of Fe-rich clay minerals (Gates et al., 1993; Ernstsen et al., 1998; Gates et al., 1998; Kostka et al., 1999b).

The capability of sulfate-reducing bacteria in reducing structure Fe(III) from iron-rich clay minerals has not been demonstrated. In this study, we show that reduction of structural Fe(III) in a nontronite (see below) is several times higher in a sulfate-reducing bacterial culture than abiotic reduction by $Na_2S$. The results of this study suggest that SRB can significantly enhance the alteration of Fe-rich clay minerals via biogenic sulfide during sulfate reduction. Release of K, Ca, Mg, and adsorbed P from the clay may support the growth of SRB in the absence of amended trace minerals and vitamins.

## 2. MATERIAL AND METHODS

### 2.1. Description of Nontronite NAu-2 and Sulfate-Reducing Bacterium G-11

Nontronite is a dioctahedral smectite-group mineral and represents the ferric end member of the nontronite-beidellite series (Brindley, 1980). Nontronite NAu-2 is a reference nontronite from the Source Clay Repository of the Clay Minerals Society. It contains trace amounts of submicron carbonate and iron oxyhydroxides (Keeling et al., 2000). While most of the Fe in NAu-2 is present as Fe(III) in octahedral coordination, some Fe is also in tetrahedral coordination as indicated by infrared and Mössbauer data (Keeling et al., 2000; Gates et al., 2002).

The sulfate-reducing bacterium used in this study is a mesophilic, *Desulfovibrio* strain G-11, provided by Dr. Lee Kromble at the University of Oklahoma. G-11 was isolated from an enrichment of rumen

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Table 1. Experimental conditions for dissolution of nontronite NAu-2 in bacterial cultures (Exp. I-III) and abiotic control (Exp. IV). Exp. I and II used fresh NAu-2; Exp. III and IV used NAu-2 washed with a solution of NaCl and HCl at pH 3.0. The check-marks indicate amendments (lactate, sulfate, NAu-2, and $Na_2S$) or chemical analyses (pH, AODC, Fe (II) and other elements) for an experiment.

| | | Amendments | | | | Measuring Parameters | | |
|---|---|---|---|---|---|---|---|---|
| Exp. | Medium | Lactate 50 mM | $SO_4^-$ 50 mM | Nau-2 30 mM $Fe^{3+}$ | $Na_2S$ 50 mM | pH | AODC | Fe(II), Si, K, Mg, Ca, P |
| I-a | Standard | √ | | √ | | √ | √ | √ |
| I-b | Standard | √ | √ | √ | | √ | √ | √ |
| II-a | $H_2O$ + $NH_4Cl$ | √ | | √ | | √ | √ | √ |
| II-b | $H_2O$ + $NH_4Cl$ | √ | √ | √ | | √ | √ | √ |
| III-a | $H_2O$ + $NH_4Cl$ | √ | | √ | | √ | √ | √ |
| III-b | $H_2O$ + $NH_4Cl$ | √ | √ | √ | | √ | √ | √ |
| IV | $H_2O$ + $NH_4Cl$ | | | √ | √ | √ | | √ |

fluid (McInerney et al., 1979). It uses lactate, ethanol, or $H_2$ as an energy source in the presence, but not absence, of sulfate, acetate, propionate, butyrate, or glucose. G-11 represents one of the most versatile groups of sulfate-reducing bacteria, so our results may have general implications of iron cycling coupled to sulfate reduction in the natural environments.

### 2.2. Experimental Design

Bulk NAu-2 was ground with a pestle and mortar. The powder of NAu-2 was then dispersed in distilled $H_2O$. The $<3.5$-$\mu$m size fractions were selected to investigate the biologic alteration of nontronite by G-11 and abiotic alteration by $Na_2S$. The standard medium for growing G-11 contained (in 1-L double-distilled water): 0.1-g KCl, 0.05-g $CaCl_2 \cdot 2H_2O$, 1.5-g $NH_4Cl$, 0.1-g NaCl, 0.1-g $MgCl_2 \cdot 6H_2O$, 1-g yeast extract, 1-mL vitamin stock solution, and 10-mL trace mineral solution (Phelps et al., 1989). Piperazine-1,4-bis(2-ethanesulfonic Acid) [PIPES] ($C_8H_{16}N_2O_6S_2Na_2$) (10 g/L) was used as the buffer. Sodium lactate (50-mM final concentration) was used as the electron donor and nontronite NAu-2 (~30-mM Fe(III), final concentration) as the electron acceptor for Fe reduction by G-11 (Exp. I-a, Table 1). Sodium sulfate (50-mM final concentration) was used as an additional electron acceptor for sulfate reduction by G-11 (Exp. I-b, Table 1). Resazurin (0.1%) was used as a redox indicator during heat sterilization of the media.

Nontronite may contain exchangeable cations, such as K, Mg, and Ca in the interlayers, or adsorbed elements such as P and Na on the outer surfaces. As these elements can serve as nutrients for bacterial growth, we also examined the growth of G-11 on NAu-2 in double-distilled water without adding vitamins, trace minerals, and yeast extract (Exp. II-a and II-b, Table 1). In addition, a set of experiments (Exp. III-a and III-b, Table 1) was conducted using NAu-2 treated with a 1-mol/L NaCl/HCl solution (pH 3.0) following the procedure described by Schroth and Sposito (1997). Basically, the clay particles were dispersed in the solution for 3 d, filtered, and air-dried. This treatment removes carbonate and amorphous Fe-oxide phases as well as all exchangeable cations. A previous study demonstrates that the crystal structure of nontronite remains intact after this type of treatment (Maurice et al., 2001). In this study, however, chemical analyses were not conducted to verify the chemical composition of this material.

In Exp. II-a and II-b and Exp. III-a and III-b, $NH_4Cl$ (1.5 g/L) was added as the nitrogen source, lactate as the electron donor, and structural Fe(III) in NAu-2 as the electron acceptor with or without sulfate (Table 1). The experiment involving the abiotic reduction of structural Fe(III) was carried out using $Na_2S$ (50 mM) as the reductant in the $H_2O$ + $NH_4Cl$ medium (Exp. IV, Table 1).

Inocula were prepared by growing fresh G-11 in standard medium using sulfate as the electron acceptor. The biomass was collected at the end of the log-phase by centrifuging the culture at 10000 g. The collected pellets were washed in an anaerobic chamber using sterile and anoxic double-distilled water to remove residual sulfate and reduced sulfur species. The pellets were resuspended in standard medium or oxygen-free water containing $NH_4Cl$ for inoculation of corresponding experiments (Table 1).

Each experiment consisted of eight or nine time points and every time point contained duplicated tubes. Along with each experiment were two control tubes with the same medium, but no bacterial cells were added. The incubation temperature was 30°C for all experiments. During the time-course experiments, two tubes were taken out of the incubator every 72 h, except the second time point when the tubes were taken out after 24 h of incubation.

### 2.3. Bacterial Cell Counts

Subsamples for cell counts were taken from the sacrificed tubes and fixed with 2% glutaraldehyde. Cell numbers were determined by acridine-orange direct counts (AODC) (Zhang et al., 1996). In this procedure, the preserved sample was homogenized on a touch mixer for 1 min and diluted 10 to 1000 times using a filter-sterilized phosphate buffer (pH 7.2). One mL of the diluted sample was mixed with 0.5-mL filter-sterilized, particle-free solution of acridine-orange (0.1-g acridine orange in 1-L phosphate buffer) for 2 min. This solution was filtered onto a black Nuclepore filter (0.2-$\mu$m pore diameter), that was then mounted onto a slide glass and viewed under an epifluorescence microscope for cell counting. At least 10 fields were counted for calculation of cell numbers.

### 2.4. Analysis of Fluid Composition

Analysis of fluid chemistry included pH and concentrations of Fe(II), Si, Ca, Mg, K and P. The pH was measured in the oxygen-free glove bag to avoid artifacts resulting from the reoxidization of sulfides to sulfate.

Fe(II) was measured using the Ferrozine methods (Stookey, 1970; Lovley and Phillips, 1986; Zhang et al., 1996). No reducing agent was added during the assay. In this procedure, a 0.2-mL subsample was transferred to 0.5-mol/L HCl solution free of oxygen and allowed to react for one h. A known amount of the solution was filtered through a 0.22-$\mu$m filter and added to the ferrozine solution to develop color for spectrophotometer measurement (Zhang et al., 1996).

Subsamples of solution for elemental analysis were prepared by filtering the liquid sample through a 0.22-$\mu$m filter. Each filtered sample (0.5 mL) was mixed with double distilled $H_2O$ and HPLC-grade $HNO_3$ (final concentration 10%) before being analyzed with an inductively coupled plasma mass spectrometer (ICP-MS).

### 2.5. X-Ray Diffraction, Conventional and High-Resolution Transmission Electron Microscopy

To evaluate the morphologic and structural changes of NAu-2 during reduction of the structural Fe(III), samples were examined by X-ray powder diffraction (XRD), conventional transmission electron microscopy (CTEM), and high-resolution TEM (HRTEM). Energy dispersive

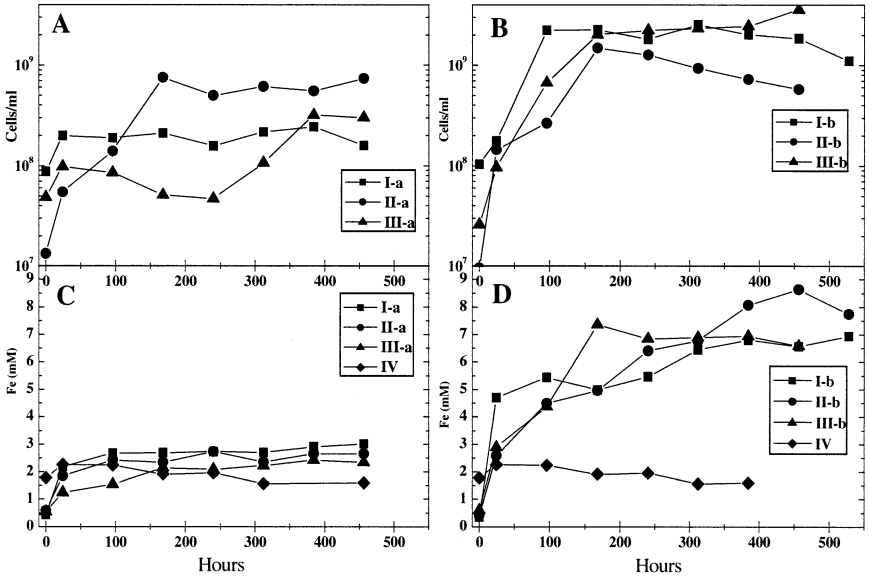


Fig. 1. Comparison of changes in cell numbers (A, B) and Fe(II) (C, D) in experiments using structural Fe(III) of NAu-2 as the sole electron acceptor (I-a, II-a, and III-a), and experiments using NAu-2 plus sulfate as the electron acceptors (I-b, II-b, and III-b). Also shown are changes in Fe(II) (C, D) during abitoic dissolution of NAu-2 by $Na_2S$ in Experiment IV.

spectroscopy (EDS) was also performed during TEM analysis to evaluate changes in elemental compositions of the clay mineral.

Samples for analysis by XRD were prepared from specimens that were dispersed and washed in 95% ethyl alcohol six to eight times to remove growth medium and salts. The treated sample was dispersed in ethyl alcohol, pipetted onto circular glass slides, and dried in air at room temperature. X-ray diffraction was performed on the dried sample using a Rigaku D/Max 2400 automated diffractometer equipped with a 12-kW rotating anode, a graphite diffracted-beam monochromator (Cu K$\alpha$ radiation), and a $\theta$-compensating slit assembly. The analytical conditions were: operating voltage of 40 kV, beam current of 160 mA, receiving slit of 0.15 mm, step-size of 0.04° 2$\theta$, counting time of 1 s per step, scan speed of 2.4° 2$\theta$, and a scanning range of 0.1 to 65° 2$\theta$.

For CTEM and HRTEM, the samples were washed with a 1:1 solution of 95% ethyl alcohol and deionized water, dehydrated with 95% ethyl alcohol, and embedded in a low-viscosity, thermally curing Epon epoxy resin. Ultrathin sections (70–100 nm) were cut from the resin blocks with Reichert Ultracut AV ultramicrotome equipped with a Diatome diamond knife. The ultrathin sections were transferred onto 300-mesh formvar-coated Cu grids and imaged in bright-field illumination under conditions of Scherzer defocus with a JEOL JEM-2000FX TEM at an accelerating voltage of 80 kV. The TEM was equipped with Gatan 792 Bioscan 1k x 1k Wide Angle Multiscan CCD camera and a PGT Prism EDS.

## 3. RESULTS AND DISCUSSION

### 3.1. Bacterial Growth During Alteration of NAu-2 in the Absence or Presence of Sulfate

Samples were only available for a single measurement at each time point. The AODC data, however, collectively show consistently lower cell numbers in cultures containing NAu-2 in the absence of sulfate (Fig. 1A) than in cultures containing both NAu-2 and sulfate (Fig. 1B). In Exp. I, for example, the maximum cell number is about tenfold lower ($2.4 \times 10^8$ cells/mL; 384 h) when structural Fe(III) is available as the sole electron acceptor in comparison to cultures containing both Fe(III) and sulfate as the electron acceptors ($2.5 \times 10^9$ cells/mL; 312 h) (Table 2 and Figs. 1A and 1B). In Exp. II, G-11 is grown in a medium containing NAu-2 in double-distilled water without added vitamins, trace minerals, and yeast extract; the maximum cell number is $7.5 \times 10^8$ cells/mL (168 h) when the structural Fe(III) of nontronite is available as the sole electron acceptor. In comparison, the cultures containing both sulfate and the structural Fe(III) has twofold higher cell numbers ($1.5 \times 10^9$ cells/mL; 168 h). In cultures containing NAu-2 treated with NaCl/HCl (Exp. III), the difference is about elevenfold with a maximum cell number of $3.2 \times 10^8$ cells/mL (384 h) in the absence of sulfate, and $3.6 \times 10^9$ cells/mL (456 h) in the presence of both sulfate and NAu-2 (Table 2). The growth rates in the log phases are also consistently slower in cultures lacking sulfate than in cultures containing sulfate (Table 2).

The results above suggest that sulfate is a preferred electron acceptor for the growth of G-11 in the presence of structural Fe(III) in the nontronite. While the structural Fe(III) may serve as the electron acceptor for bacterial growth in the absence of sulfate, it may have been out-competed by sulfate when both are present. This is partially supported by the lack of a slower growth rate than expected when using Fe(III) as the sole electron acceptor (Fig. 1A), which should be followed by a faster growth rate of sulfate reduction. Thus, we hypothesize

Table 2. Comparison of maximum cell numbers (AODC) and growth rates between experiments using structural Fe(III) as sole electron acceptor (I-a, II-a, III-a), and those using both Fe(III) and sulfate (I-b, II-b, III-b) as electron acceptors.

| Exp. | Max AODC, cells/mL | b/a | Rate, cells/mL/h | b/a |
|---|---|---|---|---|
| I-a | 2.4E8 (384 h) | 10 | 4.6E6 (0-24 h) | 4.8 |
| I-b | 2.5E9 (312 h) | | 2.2E7 (0-96 h) | |
| II-a | 7.5E8 (168 h) | 2 | 4.4E6 (0-168 h) | 2.0 |
| II-b | 1.5E9 (168 h) | | 8.8E6 (0-168 h) | |
| III-a | 3.2E8 (384 h) | 11 | 2.1E6 (0-24 h) | 5.7 |
| III-b | 3.6E9 (456 h) | | 1.2E7 (0-168 h) | |

Table 3. Comparison of Fe(II) production in G-11 cultures using structural Fe(III) as the sole electron acceptor (I-a, II-a, III-a), and using Fe(III) and sulfate as electron acceptors (I-b, II-b, III-b). The concentration of initial structural Fe(III) was 30 mM in all experiments.

| Exp. | Max Fe(II)[†], mM | b/a | Initial Fe(II) rate, mM/h | b/a | Final Fe(II), mM | Final Cntrl[‡] Fe(II), mM | Final Fe(II)/Cntrl Fe(II) |
|---|---|---|---|---|---|---|---|
| I-a | 3.01 (456 h) | 2.3 | 0.07 (0-24 h) | 2 | 3.01 | 0.60 | 5 |
| I-b | 6.94 (528 h) | | 0.18 (0-24 h) | | 6.94 | 0.65 | 11 |
| II-a | 2.65 (384-456 h) | 3.3 | 0.05 (0-24 h) | 2 | 2.65 | 0.54 | 5 |
| II-b | 8.64 (456 h) | | 0.09 (0-24 h) | | 7.74 | 0.21 | 37 |
| III-a | 2.42 (384 h) | 3.0 | 0.03 (0-24 h) | 3 | 2.35 | 0.36 | 7 |
| III-b | 7.36 (168 h) | | 0.10 (0-24 h) | | 6.58 | 0.49 | 13 |

[†] The maximum Fe(II) accounts for up to 10% of total Fe(III) (30 mM) when using structural Fe(III) as the sole electron acceptor (Exp. I-a), and up to 29% of total Fe(III) when both sulfate and structural Fe(III) were used (Exp. II-b).
[‡] The control Fe(II) for all experiments I-a through III-b was not higher than 2% of total Fe(III).

that the release of Fe(III) from the clay mineral is a limiting step for bacterial growth; and when abundant sulfate is available the bacterium will switch to sulfate reduction, leading to the production of $H_2S$ that may react with Fe(III), either in solution or as structural iron. This will make Fe(III) even less available as an energy source for bacterial growth. In a separate study, the growth rate of a similar sulfate-reducing bacterium (*Desulfovibrio* strain G-20) was investigated by comparing the results of experiments using both Fe(III) and sulfate as electron acceptors with those using sulfate alone (Li et al., unpublished data); cell numbers in cultures containing both Fe(III) and sulfate are not significantly different from those using sulfate as the sole electron acceptor, suggesting that sulfate reduction bypasses iron reduction as an energy-yielding process.

Growth rates of bacteria were also studied by comparing cultures in the presence (Exp. I) or absence (Exp. II and III) of the added trace elements and vitamins (Table 2). The results suggest that bacteria grow faster in the cultures containing the untreated NAu-2 and the amendments, than cultures containing purified NAu-2 (treated with NaCl/HCl) without trace elements and vitamins. The maximum cell numbers, however, are not consistent. For example, the largest value occurs in Exp. II-a ($7.6 \times 10^8$ cells/mL) where Fe(III) is available as the sole electron acceptor and in III-b ($3.6 \times 10^9$ cells/mL) where both Fe(III) and sulfate are available as electron acceptors (Table 2).

Nevertheless, these experiments suggest that the sulfate-reducing bacterium G-11 can utilize structural Fe(III) as the sole electron acceptor for growth when sulfate is not available; however, growth is significantly enhanced when sulfate is available. Recent studies have shown that the structural Fe(III) of Fe-rich smectite-group minerals can support the growth of Fe-reducing bacteria (Kostka et al., 1999b; Kostka et al., 2002; Kim et al., 2004). As Fe(III)-rich 2:1 clay minerals are abundant in the natural environment (Köster et al., 1999; Keeling et al., 2000; Manceau et al., 2000), they may be a potential electron acceptor which can be utilized by either Fe-reducing or SRB present in the environments. The capability of SRB to reduce Fe(III) has been demonstrated in the sulfide-rich environment of a salt-marsh sediment where siderite is formed (Coleman et al., 1993).

### 3.2. Fe(II) Production During Alteration of Nontronite NAu-2

The production of Fe(II) by G-11 is significantly different when structural Fe(III) (Fig. 1C) or sulfate plus Fe(III) (Fig. 1D) are used as the electron acceptors. In the former case, the maximum Fe(II) is 3.01 mM (456 h) in Exp. I-a, 2.65 mM (384–456 h) in Exp. II-a, and 2.42 mM (384 h) in Exp. III-a (Fig. 1C and Table 3). In the latter case, the maximum Fe(II) is two- to three-times higher with values of 6.94 mM (528 h) in Exp. I-b, 8.64 mM (456 h) in Exp. II-b, and 7.36 mM (168 h) in Exp. III-b (Fig. 1D and Table 3). These measured values represent up to 10% of the total structural Fe(III) in the absence of sulfate and up to 29% of the total structural Fe(III) in the presence of sulfate.

In the first 24 h of incubation, the initial rate of Fe (II) production is also consistently lower when structural Fe(III) is available as the electron acceptor, than both sulfate and structure Fe(III) are available. In the former case, the rate is 0.07 mM/h in Exp. I-a, 0.05 mM/h in Exp. II-a, and 0.03 mM/h in Exp. III-a (Table 3). In the latter case, the rate of Fe(II) production is about two- to three-times faster with values of 0.18 mM/h in Exp. I-b, 0.09 mM/h in Exp. II-b, and 0.10 mM/h in Exp. III-b (Table 3). These rates also show a consistent pattern: higher rates of production of Fe(II) occur when G-11 is grown in cultures containing the untreated nontronite and added nutrients (Exp. I-a and I-b); lower rates occur when G-11 is grown without the added nutrients or vitamins (Exp. II-a and II-b and Exp. III-a and III-b) (Table 3). These results are in agreement with rates of bacterial growth in Exp. I, Exp. II and III (Table 2). Rates of Fe reduction in controls of Exp. I, II, or III were not determined. The final concentrations of Fe(II) in these controls, however, are five- to thirty-seven times lower than the final Fe(II) concentrations in cultures containing G-11.

The results above indicate that Fe reduction is significantly enhanced in the presence of bacterial cells compared to that in abiotic controls. The enhanced reduction of structural Fe(III) is likely due to the enzymatic activity of bacterial cells. When using structural Fe(III) as the sole electron acceptor, cells may boost Fe reduction by direct contact with the clay mineral or by releasing organic acids or chelators that facilitate solubilization of the structural Fe(III) (Lovley, 1997; Fredrickson et al., 1998; Lovley et al., 1998; Nevin and Lovley, 2002; Kostka et al., 2002). The mechanism(s) of Fe reduction is more complex when sulfate is used as the electron acceptor. Here, the direct role of G-11 is to reduce sulfate to $H_2S$, which then reduces Fe(III) to Fe(II), and forms iron sulfides. The subsequent Fe reduction is theoretically a pure chemical process. Based on Exp. IV, however, this may not be the case in these experiments. For example, Fe(II) in the abiotic system (50 mM $Na_2S$)

never exceeds 2.3 mM, and the final concentration (1.6 mM) is five to sixfold lower than the final concentrations in G-11 cultures containing sulfate (Exp. I-b, II-b, III-b). It should be noted that the solution pH in the abiotic experiment is significantly higher (>8.0) than in the bacterial cultures (<7.5). When the solution is adjusted to pH 7.5, the capability of $Na_2S$ to reduce Fe(III) may be less because the relative proportion of $S^{2-}$ is less than that at pH 8.5. These results provide convincing evidence that the enhanced reduction of structural Fe(III) during sulfate reduction cannot be exclusively attributed to the inorganic reaction and may be related to the enzymatic activity of the bacteria (Neal et al., 2001).

It is important to emphasize that the concentration of Fe(II) in the solution does not necessarily reflect the rate of reduction of Fe (III) in the octahedral sites of nontronite. As nontronite is a dioctahedral 2:1 clay mineral, Fe (III) occupies two of the three octahedral sites in the octahedral sheet. Under reducing conditions, some of the Fe(III) is reduced to Fe(II), which would occupy the empty octahedral site and create a partial trioctahedral structure.

Reduction of structural Fe(III), particularly through sulfate reduction, leads to the release of elements, such as Mg, Ca, K, and adsorbed P, that are associated with nontronite. Such elements may be available to support bacterial growth in the absence of added nutrients and vitamins.

### 3.3. Changes in Composition and Structure of NAu-2 After Alteration by G-11

The mineralogical composition and morphologic characteristics of the solid phases, as well as the effects of the bacteria and their by-products on the nontronite, were investigated by XRD (Fig. 2), TEM (Figs. 3 and 4), and EDS (Fig. 5). The XRD pattern of fresh nontronite has two major peaks at 6° and 29° 2Θ, respectively, which are characteristic of the NAu-2 clay mineral (Fig. 2A). The nontronite also contains quartz (Fig. 2A).

In bacterial cultures having sulfate as the major electron acceptor, the NAu-2 peaks are reduced considerably at the end of experiment (Fig. 2B), suggesting extensive destruction of the crystal structure of nontronite by microbial activity. On the other hand, a considerable amount of calcite is formed (Fig. 2B). The formation of calcite is puzzling considering the potentially abundant Fe(II) in solution during iron dissolution, which should yield Fe-rich carbonate before calcium carbonate precipitates (see Kim et al., 2004). One explanation may be that the produced Fe(II) quickly reacts with $H_2S$ to form iron sulfides, making Fe(II) unavailable for carbonate precipitation.

Conventional TEM images show the presence of large aggregates of both altered and nonaltered crystals of nontronite collected from different cultures (Figs. 3A and 3B). Aggregation is likely due to the interaction of the charged surfaces of individual crystals of nontronite and the organic and inorganic ions present in the growth medium. Adsorption of ions and molecules by nontronite has implications for the kinetics of reaction, and accessibility to the nutrients by the bacteria. In addition, “flocs” of nontronite, which are clumps of primary particles with a face to face (FF) structure, are relatively dense (Vali and Bachman, 1988, Vali and Hesse, 1992). Thus, only crystals of nontronite within the flocs that have exposed surfaces are accessible to chemical reaction with the growth medium. As a result of flocculation, all the solid phases, including the bacteria and their by-products, are deposited at bottom of the test tube during the experiments. It is possible that the chemical composition of the growth medium within the microenvironment of the deposited material is different from the composition of the overlying growth medium.

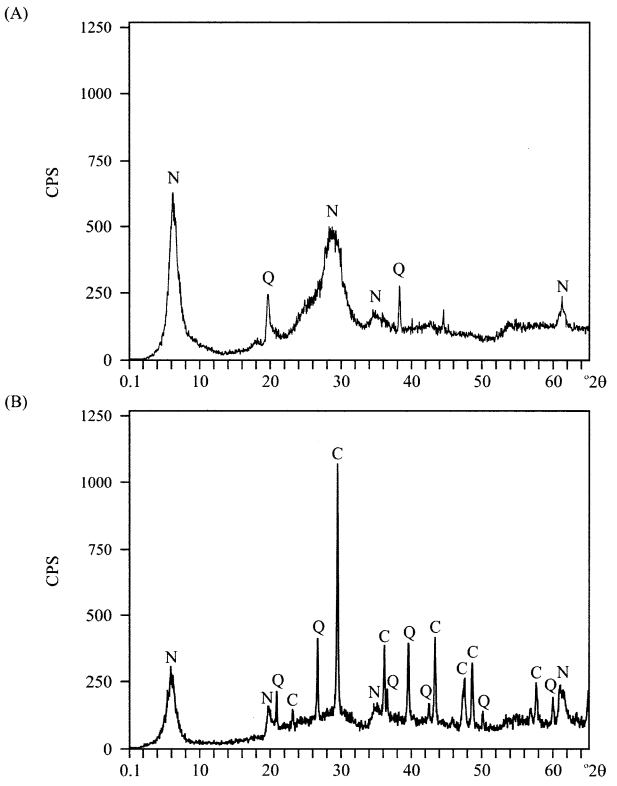


Fig. 2. X-ray diffraction patterns of nontronite (A) and altered nontronite in a culture containing G-11 with the addition of sulfate as an electron acceptor (B). N = nontronite, C = calcium carbonate, Q = $SiO_2$.

Despite flocculation, significant structural differences are observed between the original and altered crystals of nontronite (Figs. 3A–3D). In cultures containing G-11 and nontronite only, nontronite appears to maintain intact structures without any obvious alteration (Fig. 3A). For example, no differences are detected in the stacking order of individual 2:1 layers between the original untreated nontronite and the nontronite during reduction of structure Fe(III) in the absence of sulfate (data not shown). The resolution of the TEM, however, is insufficient to detect ionic changes in the octahedral and tetrahedral sites within individual 2:1 layers.

In contrast, the nontronite exposed to both G-11 and sulfate shows extensive textural and structural alterations (Fig. 3B–3D). These alterations, however, are nonhomogenous: while some nontronite crystals remain intact (Fig. 3B), others are severely degraded (Figs. 3C and 3D). High-resolution TEM images provide further evidence at the nm-scale for heterogeneous degradation of the nontronite exposed to G-11 and sulfate (Fig. 4). The results of this study are in agreement with other studies that document the alteration and collapse of the

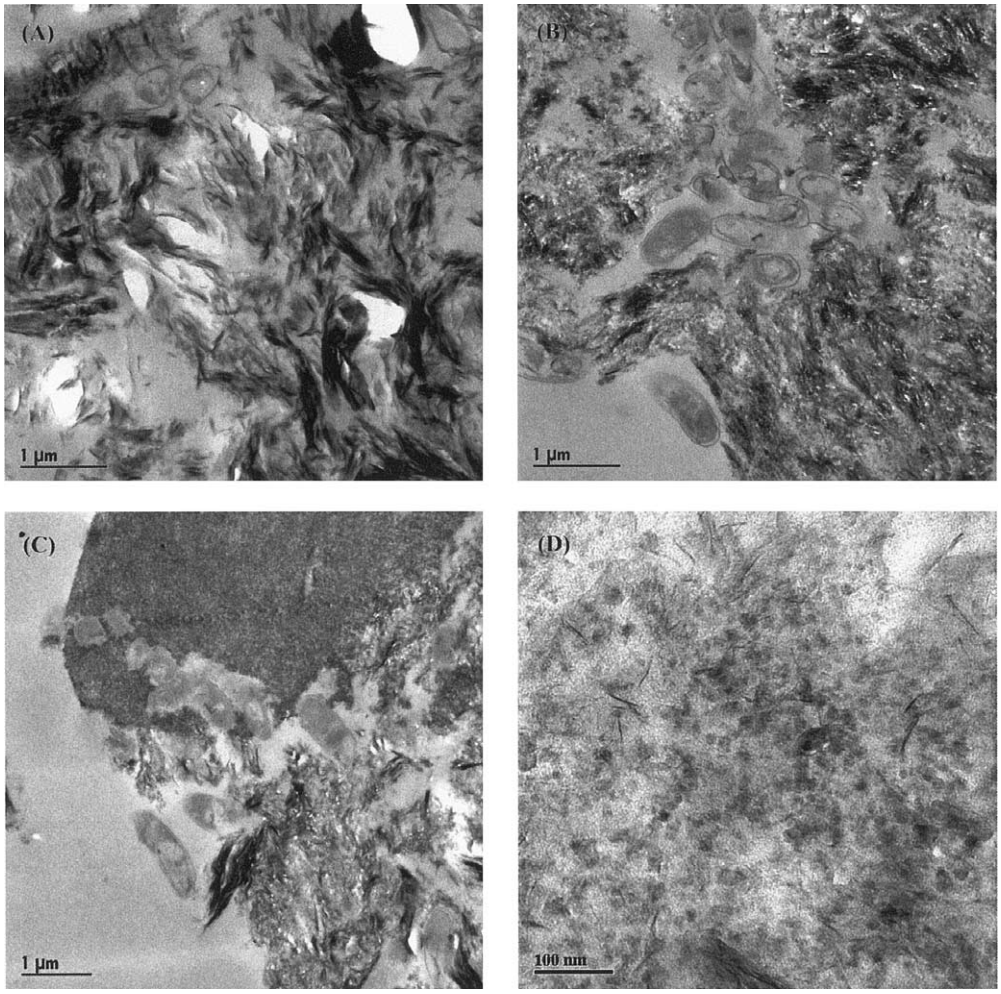


Fig. 3. Conventional TEM images of ultrathin sections of: (A) nontronite in a culture containing G-11 showing aggregates of relatively intact nontronite with a few bacteria included in the voids (top center); (B) Nontronite in a culture containing G-11 with the addition of sulfate showing aggregates of altered and nonaltered nontronite and abundant bacteria in the void spaces; (C) Same as (B) showing the presence of amorphous silica (upper area) embedded with bacteria; (D) Same as (B) showing completely altered containing nano-sized particles composed of Fe-sulfides.

crystal structure of Fe-rich clay minerals by Fe-reducing bacteria (Gates et al., 1998; Kostka et al., 1999b; Kim et al., 2004).

Most aggregates consisted of both crystalline and amorphous material surrounded by a biomass (Figs. 3A–3C). The biomass consisted of well-preserved bacteria as well as cell debris and other metabolic by-products present in the voids between flocs (e.g., Fig. 3B). There is no evidence, however, for the presence of the bacteria or cell debris within the flocs of nontronite, suggesting the spontaneous flocculation of nontronite crystals, as described above. In contrast, intact bacteria are present within aggregates of amorphous Silica that is precipitated as a secondary phase as a result of alteration of the nontronite (Fig. 3C). Direct contact between the cell walls of bacteria and the crystals of nontronite could not be observed.

Collectively, it is estimated that approximately 50% of the nontronite crystals may be destroyed due to reaction of structural Fe(III) with biogenic $H_2S$, which may be enhanced by enzymatic activity of the bacteria (Neal et al., 2001). However, most bacterial cells are present in voids between nontronite particles (Figs. 3B–3C); thus it is unknown whether this enhancement will require direct contact between the cell walls of bacteria and the crystals of nontronite.

Energy-dispersive spectroscopic analysis reveals changes in elemental compositions of nontronite experiencing different experimental conditions (Fig. 5). In experiments without added sulfate, no sulfur and calcium are detected (Fig. 5B). In experiments with added sulfate, the altered nontronite revealed high concentration of elemental S (Fig. 5C) which is associated with noncrystalline nano-sized particles (Fig. 3D). It is not possible, however, to identify any specific FeS phase in the samples because of the nano-sizes of particles.

### 3.4. Implications for the Natural Environment

Microorganisms often play an important role in the formation and alteration of clay minerals in natural environments. Aerobic microorganisms have most often been studied and are known to facilitate weathering of silicate minerals (Barker et al., 1997; Maurice et al., 2001; Santelli et al., 2001; Welch and Banfield, 2002), likely through the

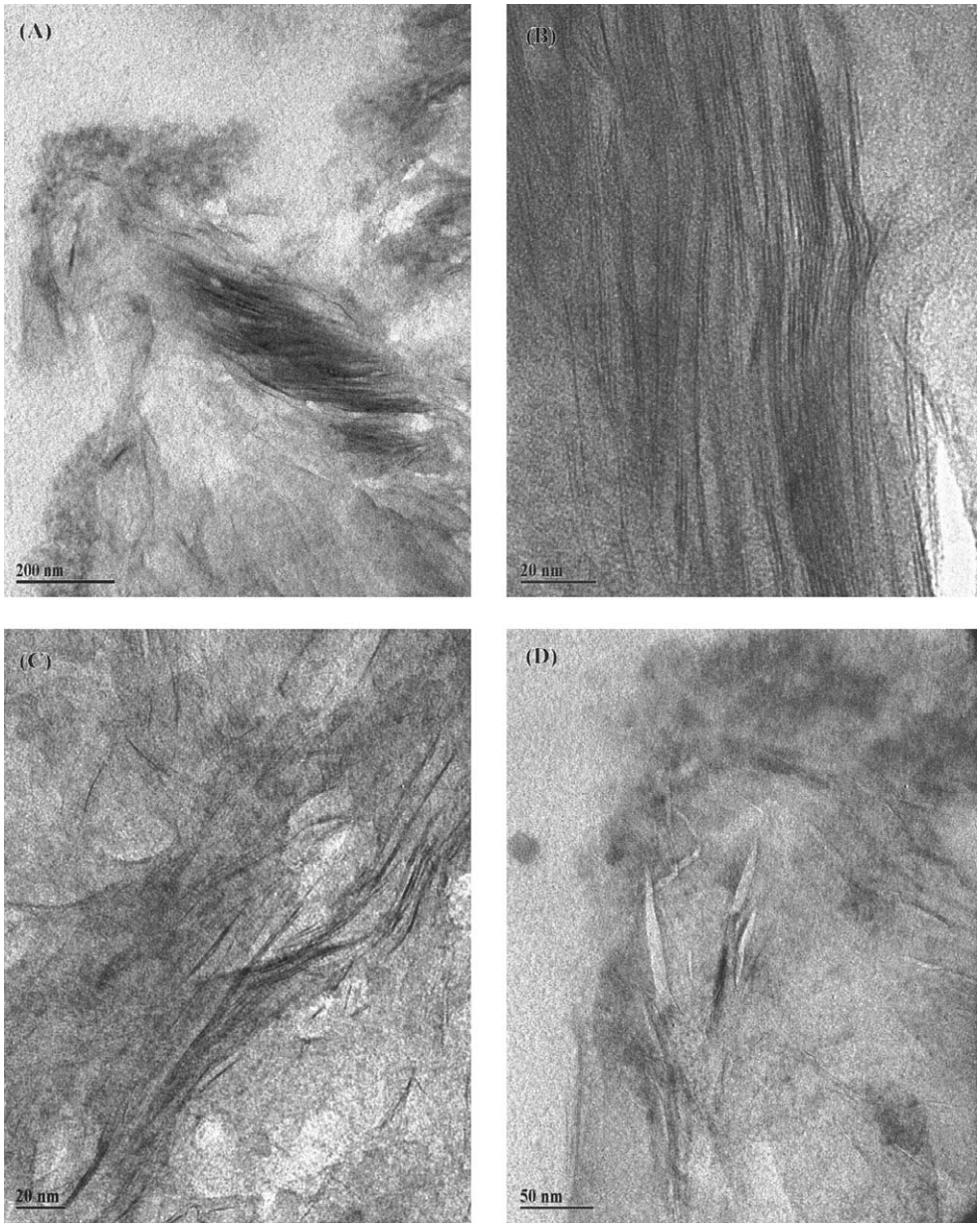


Fig. 4. High-resolution TEM images of ultrathin sections of nontronite in a culture containing G-11 with the addition of sulfate as an electron acceptor. (A) Aggregate of nontronite consisting of a core of intact nontronite surrounded by completely altered nontronite; (B) Close-up of the core of the intact nontronite in (A) consisting of a stack of packets of individual 2:1 layers of nontronite ($\delta_{(001)}$ = ~1.3 nm); (C) Altered crystals of nontronite showing extensive dissolution; (D) Close up of the altered region in (A) showing amorphous material including nano-sized particles of Fe-sulfide.

reaction of mineral surfaces with organic acids or siderophores produced by bacterial activity (Ullman et al., 1996; Kalinowski et al., 2000; Brantley et al., 2001). Iron-rich clay minerals are often the by-product of this weathering process; for example, nontronite forms by the alteration of amphibole during biologic weathering (Barker and Banfield, 1996). In addition, Fe(III)-rich clay minerals, including nontronite, are formed in soils and sediments through the nucleation and precipitation of a precursor ferric hydroxide phase on bacterial cell surfaces and subsequent reaction with dissolved silica and aluminum (Konhauser and Urrutia, 1999). The inevitable consequence of the weathering or authigenic products of Fe(III)-rich clay minerals have important implications for early diagenesis of these minerals in freshwater and marine systems. Furthermore, Kim et al. (2004)recently demonstrated that an Fe-reducing bacterium can promote the smectite-to-illite (*S*-I) reaction at room temperature and atmospheric pressure, which challenges the conventional concept of abiotic *S*-I reaction that occurs only at high temperature (300–350°C) and high pressure (100 megapascals).

While it is clearly shown that Fe-reducing bacteria are ca-

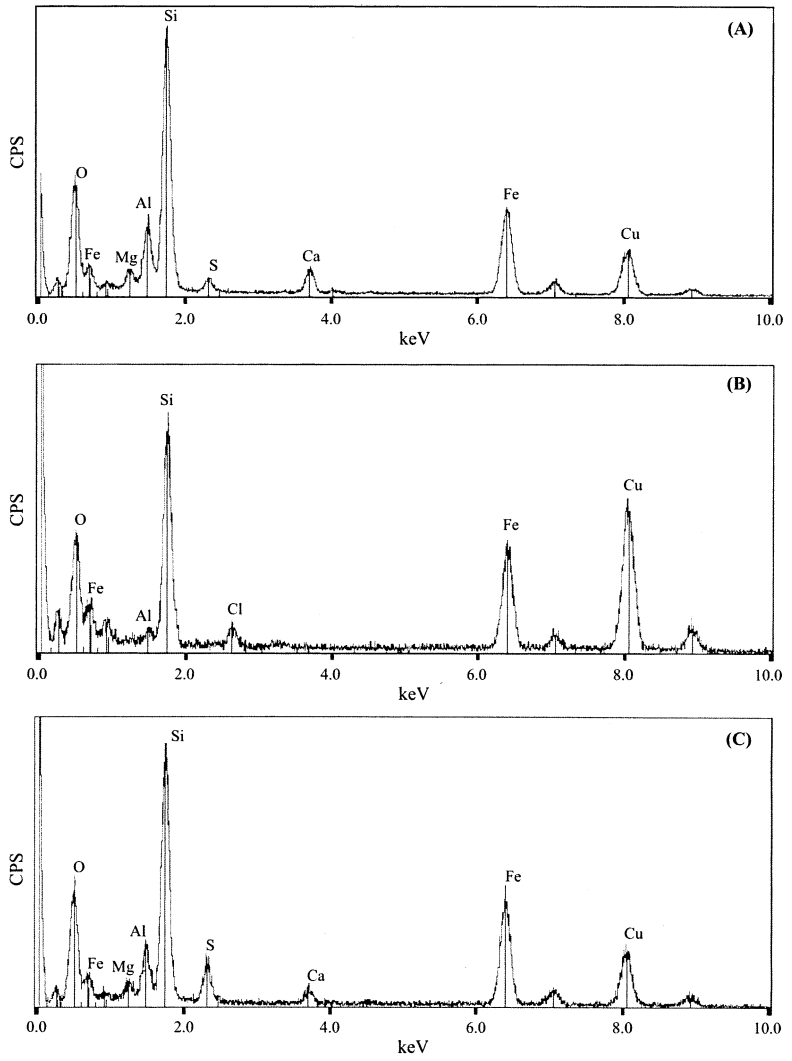


Fig. 5. Energy dispersive spectra showing: (A) untreated nontronite; (B) nontronite in a culture containing G-11; (C) nontronite in a culture containing G-11with the addition of sulfate as an electron acceptor. Note that the sulfur in (C) is associated with the particles of Fe-sulfide shown in Figures 3D and 4D.

pable of metabolizing Fe(III) associated with 2:1 clay minerals in soils and sediments (Gates et al., 1993; Kostka and Luther, 1994; Kostka et al., 1996; Kostka et al., 1999b; Kostka et al., 2002; Kim et al., 2004), the role of SRB in the cycling of Fe present in the structure of clay minerals has not been well understood, particularly in the marine environment. For example, reactivity of Fe to sulfide is an important limiting factor in the formation of pyrite in marine sediments (Berner, 1984; Raiswell and Berner, 1985). It has been shown that increased pyrite sulfur is associated with clay minerals, which contain the largest pool of unpyritized Fe in an anoxic mud (Canfield et al., 1992).

Sulfate-reducing bacteria may also play important roles in dissolution or alteration of other silicate minerals. One relevant environment will be midoceanic ridges, where bioalteration of basaltic glass has been well documented (Thorseth et al., 1995; Fisk et al., 1998; Furnes and Staudigel, 1999; Furnes et al., 2001). Studies using transmission electron microscopy and analytical electron microscopy have shown that losses of Fe and Mn from altered glass are primarily due to exploitation by microbes and that the primary products of basalt-alteration are Fe-rich clay minerals (Alt and Mata, 2000; Zhou et al., 2001). Pyrite is also typically associated with altered glass and smectite-group minerals, which suggests microbial pyrite formation during basalt diagenesis by SRB (Alt et al., 1989; Alt and Shanks, 1998).

## 4. CONCLUSIONS

The sulfate-reducing bacterium G-11 can reduce structural Fe(III) in nontronite NAu-2 either in the absence or presence of sulfate. When using structural Fe(III) as the sole electron acceptor, G-11 produces Fe(II) up to 10% of total Fe(III), whereas in the abiotic control (sulfate plus NAu-2 but without G-11), the production of Fe(II) is no more than 2% of total Fe(III). When sulfate is used as the electron acceptor in the presence of NAu-2, Fe(II) production can reach 29% of total Fe(III). This far exceeds Fe(II) production in the abiotic control

and exceeds Fe (II) production by inorganic $Na_2S$ (<8% of total Fe(III)). The comparison of abiotic and bacteria-induced reactions suggests that the enhanced production of Fe(II) during bacterial sulfate reduction is influenced by metabolic activity of the bacterium, and not merely a passive reaction of the nontronite with $H_2S$. Reduction of structural Fe(III) through biogenic $H_2S$ results in destruction of up to 50% of nontronite crystals. The structural damage consequently results in release of interlayer K, Mg, Ca, and adsorbed P, which appear to support the growth of G-11 in the absence of added nutrients. This study indicates that sulfate-reducing bacteria may play a more important role than previously recognized in the cycling of Fe and S in natural environments where Fe-rich clay minerals coexist with sulfates.

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