

Chemical and Biological Interactions during Nitrate and Goethite Reduction by *Shewanella putrefaciens* 200

D. Craig Cooper,^{1*} Flynn W. Picardal,¹ Arndt Schimmelmann,² and Aaron J. Coby¹

Environmental Science Research Center, School of Public and Environmental Affairs,¹ and Department of Geological Sciences,² Indiana University, Bloomington, Indiana 47405-1405

Received 15 August 2002/Accepted 5 March 2003

Although previous research has demonstrated that NO_3^- inhibits microbial Fe(III) reduction in laboratory cultures and natural sediments, the mechanisms of this inhibition have not been fully studied in an environmentally relevant medium that utilizes solid-phase, iron oxide minerals as a Fe(III) source. To study the dynamics of Fe and NO_3^- biogeochemistry when ferric (hydr)oxides are used as the Fe(III) source, *Shewanella putrefaciens* 200 was incubated under anoxic conditions in a low-ionic-strength, artificial groundwater medium with various amounts of NO_3^- and synthetic, high-surface-area goethite. Results showed that the presence of NO_3^- inhibited microbial goethite reduction more severely than it inhibited microbial reduction of the aqueous or microcrystalline sources of Fe(III) used in other studies. More interestingly, the presence of goethite also resulted in a twofold decrease in the rate of NO_3^- reduction, a 10-fold decrease in the rate of NO_2^- reduction, and a 20-fold increase in the amounts of N_2O produced. Nitrogen stable isotope experiments that utilized $\delta^{15}\text{N}$ values of N_2O to distinguish between chemical and biological reduction of NO_2^- revealed that the N_2O produced during NO_2^- or NO_3^- reduction in the presence of goethite was primarily of abiotic origin. These results indicate that concomitant microbial Fe(III) and NO_3^- reduction produces NO_2^- and Fe(II), which then abiotically react to reduce NO_2^- to N_2O with the subsequent oxidation of Fe(II) to Fe(III).

In recent years, dissimilatory reduction of ferric (hydr)oxide minerals has been documented for a large number of microorganisms in a wide range of environments (31, 37) and has become recognized as an important constituent of the global carbon and iron cycles (31, 32). This process has a profound effect on groundwater geochemistry and places a key control on the fate of contaminant metals (12, 13, 19, 30, 60) and organic compounds (24, 42) in anoxic groundwater. As a consequence, processes that affect the rate and extent of microbial iron reduction in natural systems are of great interest. NO_3^- is a common groundwater contaminant in the United States (38) and may interfere with bioremediation schemes that seek to use microbial iron reduction to engender reductive immobilization of metal contaminants.

Shewanella putrefaciens 200 is a facultative anaerobe capable of utilizing O_2 , NO_3^- , NO_2^- , Fe(III), Mn(IV), and a number of other compounds as terminal electron acceptors for carbon metabolism (14, 40, 41). Obuekwe and Westlake (40, 41) reported that NO_3^- , NO_2^- , and a number of other terminal electron acceptors can inhibit reduction of Fe(III)-phosphate by *S. putrefaciens* 200, and subsequent work (1, 51) has demonstrated that the presence of NO_3^- can inhibit microbial Fe^{2+} production during sediment incubations. Additional studies with *S. putrefaciens* and other bacteria (14) have also observed that NO_3^- or NO_2^- inhibit microbial iron reduction. Studies with *S. putrefaciens* 200 suggest that this inhibition may result from kinetically favorable transport of electrons to NO_3^- or NO_2^- , a mechanism suggested by Arnold et al. to

explain inhibition of Fe(III) reduction by oxygen (3–5, 14). Although the respiratory electron transport chain of *S. putrefaciens* is known to include a variety of *b*- and *c*-type cytochromes and quinones (36, 42, 55), the sequential arrangement of electron carriers has not been elucidated, and terminal NO_3^- , NO_2^- , or Fe(III) reductases have not been fully purified. While the NO_3^- and NO_2^- reductases for *S. putrefaciens* MR-1 have been partially purified by Krause and Nealson (27), MR-1 is a denitrifier, whereas the *S. putrefaciens* 200 used in our studies primarily reduces NO_3^- and NO_2^- to ammonia (Cooper, Coby, and Picardal, unpublished data). It is therefore likely that strains 200 and MR-1 have substantial enzymatic differences.

Although illuminating, previous studies provide only limited insight into the dynamics of interactions between dissimilatory iron and NO_2^- or NO_3^- reduction (NO_x^- reduction) when solid-phase, ferric (hydr)oxide minerals (e.g., ferrihydrite, goethite, lepidocrocite) are the Fe(III) source. The chemistry of ferric (hydr)oxide minerals (FeOOH) is fundamentally different from that of aqueous or microcrystalline Fe(III) used by others in the previous studies. Not only does the FeOOH provide a textured surface that sorbs metal cations, but Fe^{2+} adsorption to a FeOOH surface forms highly reactive ferrous-ferric surface complexes that have distinctive chemical properties that are often similar to those of green rust (16, 20, 22, 35, 52). Biogeochemical interactions in systems containing NO_3^- and solid-phase Fe(III) therefore clearly may be different than those previously described in systems using aqueous Fe(III). The objective of this study is to determine the nature of the biological and geochemical interactions between microbial ferric (hydr)oxide and NO_3^- reduction using *S. putrefaciens* 200 as a model microorganism capable of reducing both

* Corresponding author. Present address: Geosciences Research, IF-IRC MS 2107, Idaho National Engineering and Environmental Laboratory, P.O. Box 1625, Idaho Falls, ID 83404-2107. Phone: (208) 526-5395. Fax: (208) 526-0875. E-mail: coopdc@inel.gov.

Fe(III) and NO_3^- and synthetic goethite as a model ferric (hydr)oxide mineral.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *S. putrefaciens* is a gram-negative motile rod with an obligate respiratory metabolism (49). The strain used in these experiments, *S. putrefaciens* 200, was originally isolated from a Canadian oil pipeline by Obuekwe (39). The culture was maintained on a solid medium of nutrient agar containing 5 g of yeast extract/liter (Difco, Detroit, Mich.), as previously described (42). Liquid cultures were grown in a 2.5-liter Bioflow 3000 fermentor (New Brunswick Scientific Company, Edison, N.J.) in a medium that consisted (per liter) of 2.0 g of Na_2SO_4 , 0.5 g of K_2HPO_4 , 1.0 g of NH_4Cl , 0.198 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 19.35 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.5 g of yeast extract, and 3 ml of 60% (wt/vol) aqueous sodium lactate. Following an initial period of aerobic growth, anaerobic reductase activity was induced by reducing the airflow to approximately 100 ml min^{-1} and maintaining the cells under suboxic ($[\text{aqueous } \text{O}_2 \{ \text{O}_{2(\text{aq})} \}] < 2.5 \text{ } \mu\text{mol/liter}$) conditions for a period of 12 h. It has been previously demonstrated that this culture technique induces anaerobic reduction systems for a wide range of terminal electron acceptors, including nitrate, nitrite, and Fe(III) (14). Cells were subsequently harvested by centrifugation and resuspended to a target optical density (A_{600} , ~ 1.2) in low-ionic-strength, artificial groundwater medium (AGW) (12).

A nitrate-reducing enrichment culture was developed from nitrate-contaminated sandy sediments obtained from a Uranium Mill Tailings Remedial Action (UMTRA) site in Shiprock, New Mexico, in October 1999 during sediment collection by the U.S. Department of Energy. Sediments were collected with a backhoe, placed in whirl-pack bags, flushed with argon, placed in sealed argon-flushed bottles, and stored at 4°C aphotically for 1 month prior to use. Enrichment of nitrate-reducing cultures was done with a sulfate-free AGW medium with 10 mM lactate as the electron donor and 2.5 mM nitrate as the electron acceptor. The sulfate-free medium was created by substituting chloride salts for sulfate salts in the AGW medium previously described (12). To establish enrichments, sterile sulfate-free AGW medium was degassed with nitrogen and dispensed (50 ml) into 120-ml serum bottles in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.). After the addition of approximately 10 g of sediment, the bottles were crimp sealed with butyl rubber stoppers and statically incubated in the dark at room temperature. Transfers (10% volume) were done every 7 to 10 days into identical medium in anoxic serum bottles or tubes. After eight transfers, an enrichment culture was developed that was capable of complete utilization of 2.5 mM nitrate in 1 to 3 days. Subsequent experiments revealed that the enrichment culture was also capable of slow reduction of solid-phase Fe(III) oxides.

Fe(III) reduction and NO_3^- reduction experiments. The AGW medium and goethite slurries used in these experiments have been described previously (12). When required, 1.0 ml of sterile NO_3^- or NO_2^- stock solutions of NaNO_3 and NaNO_2 (respectively) dissolved in Milli-Q water were added to the AGW medium (goethite free) or AGW + goethite slurry ($\text{NO}_x^- + \text{goethite}$). Although the general methodology employed in the Fe(III) and NO_3^- reduction experiments has been previously described (12), a summary of the procedure is presented here. Experiments were initiated by inoculating slurries with 2 ml of *S. putrefaciens* (or UMTRA enrichment culture) suspension under anoxic conditions (ca. 2×10^6 cells ml^{-1}). For each experiment, a series of 150-ml (nominal volume) glass serum bottles were crimp sealed with butyl rubber stoppers and incubated horizontally on a shaker table at room temperature (95% N_2 -5% H_2 headspace). Initial samples (t_0) were taken prior to inoculation, and subsequent samples were taken at regular intervals thereafter. During sampling, bottles were transferred to the anoxic chamber, shaken vigorously to resuspend any settled solids, and then immediately sampled with a 3-ml syringe (18-gauge needle). One 1.5-ml aliquot was extracted for 2 h in 2 M KCl for total ammonium-nitrogen ($\text{NH}_3\text{-N}$) measurements. The second 1.5-ml aliquot of slurry was placed into a separate microcentrifuge tube, and particles were separated by centrifugation (25 min at $14,900 \times g$). The supernatant was immediately sampled for pH, $\text{Fe}^{2+}_{(\text{aq})}$, $\text{NH}_3\text{-N}_{(\text{aq})}$, NO_3^- , and NO_2^- . Aqueous Fe^{2+} was immediately analyzed via a modification of the ferrozine method (26, 53). Samples for $\text{NH}_3\text{-N}$, NO_3^- , and NO_2^- were diluted $2 \times$ to $5 \times$ in Milli-Q water, frozen, and stored for later analysis. NO_3^- and NO_2^- were analyzed via ion chromatography (Dionex Series 4500i; column type, IonPac AS17 [4 by 250 mm]; eluent, 50 mM NaOH and Milli-Q H_2O gradient), and aqueous $\text{NH}_3\text{-N}$ was analyzed colorimetrically via the phenate method (21). Extraneous supernatant was then discarded, and the pellet was resuspended and extracted for 2 h in 0.5 N HCl. Both slurries (KCl and HCl) were separated via centrifugation (10 min at $14,900 \times g$). The HCl supernatant was immediately analyzed for bound Fe^{2+} via the ferrozine method, and

the KCl supernatant was diluted $2 \times$ in Milli-Q water, frozen, and stored for later analysis via the phenate method. Total 0.5 N HCl-extractable Fe^{2+} ($\text{Fe}^{2+}\text{-HCl}$) was defined as the sum of aqueous and bound Fe^{2+} , and the precision of the phenate analysis was monitored by comparing total and aqueous $\text{NH}_3\text{-N}$ in the goethite-free bottles.

For N_2O analyses, 25.0 μl of headspace gas was withdrawn with a Hamilton gas-tight syringe and analyzed via gas chromatography with a Hewlett Packard 5890 Series II gas chromatograph equipped with a GS-Q megabore capillary column (conditions: oven temperature, 75°C ; detector temperature, 275°C ; pressure, 130 kPa) and electron capture detector. The instrument was calibrated with both laboratory and commercial N_2O standards (Scotty I analyzed gases), and gas concentration was defined as the average of data for three injections. The concentration of aqueous N_2O was calculated from temperature and headspace N_2O by Henry's law (59), and total N_2O was defined as the sum of headspace and aqueous N_2O . For N_2 analyses, 3.0-ml aliquots of headspace were withdrawn with a syringe and immediately injected into a Shimadzu model 14A gas chromatograph equipped with a CR501 Chromatopac, 1.0-ml sample loop, 3.0 m MS-5A 60/80, and 1.0 m porapak R 80/100 columns (2.5 min run time; isothermal at 75°C) and thermal conductivity detector. To guard against contamination from atmospheric N_2 , the syringe was slowly compressed during injection, and excess volume acted as a purge for the sample loop. Check standards and He blanks were also analyzed at regular intervals. The instrument was calibrated with laboratory N_2 standards, and gas concentration was defined as the average of data for two injections. With the sole exception of the stable isotope studies (described below), the exact same methodology was used for all experiments with *S. putrefaciens* 200 and the UMTRA enrichment cultures. All experiments were conducted with the necessary controls.

Stable isotope experiments. With noted exceptions, stable isotope experiments were conducted as described previously. Experiments were conducted under a He atmosphere in specially fabricated Pyrex media bottles (~ 500 ml) equipped with a vacuum stopcock and crimp-sealed sampling port. After preparation of the experimental medium (100 ml) and replacement of the headspace with He, the bottles were allowed to equilibrate for 48 h, and t_0 samples were taken from all bottles prior to inoculation (biological and combined experiments) or NO_2^- addition (chemical experiments). Microbially reduced goethite (MrG) of the chemical experiment was harvested from previous NO_3^- -free experiments, sterilized via pasteurization (25 min at 95°C), and washed three times with sterile, anoxic, lactate-free AGW medium prior use in isotope experiments.

Isotopic analytical requirements dictated that the entire ~ 400 -ml headspace be collected. Thus, four bottles (two inoculated and two uninoculated) were continuously monitored for aqueous parameters and headspace N_2O , while six isotope bottles were continuously monitored for headspace N_2O but sampled for aqueous parameters only at the initial and final times. Two isotope bottles were sacrificed at each sampling point, and procedural standards were randomly extracted to monitor the overall precision of the extraction process.

The isotope extraction was conducted in two stages. During the first stage, aqueous slurries were frozen immediately after wet chemical sampling was complete (2 to 4 h; standard commercial freezer) and the headspace- N_2O was subsequently removed and stored for isotope analysis. The headspace separation was achieved by cryogenically trapping the condensable gasses (N_2O , CO_2 , H_2O) in a liquid- N_2 cold trap and slowly removing the incondensable gas (vacuum pumping) while cryogenically trapping any remaining headspace condensable gas. Next, the condensable gas was warmed and cryogenically transferred to a vacuum-tight storage vessel containing a few grains of degassed coconut charcoal. During the second stage, the condensable gas in the storage vessel was further purified on a more advanced vacuum line by cryogenically separating water and traces of incondensable gas from N_2O and CO_2 . The purified N_2O and CO_2 was then cryogenically transferred to a glass ampoule containing excess granular Cu^0 (ca. 2 g), sealed under vacuum, heated overnight at 500°C to reduce the N_2O to N_2 , and stored for subsequent determination of $\delta^{15}\text{N}$ values. Since the biological experiments did not produce the requisite $5 \text{ } \mu\text{mol}$ total N_2O needed for accurate $\delta^{15}\text{N}$ analysis, $5 \text{ } \mu\text{mol}$ of standard N_2O was injected into the sampling port of the isotope bottle during the initial trapping of condensable gasses.

The values for $\delta^{15}\text{N}$ of N_2O ($\delta^{15}\text{N}\text{-N}_2\text{O}$) for the biological experiment are based on dilution calculations utilizing the $\delta^{15}\text{N}\text{-N}_2\text{O}$ value for the standard, the $\delta^{15}\text{N}\text{-N}_2\text{O}$ value for a mixture of the sample and standard, and the mass ratio of sample to standard N_2O . The N_2O standard for $\delta^{15}\text{N}\text{-N}_2\text{O}$ analyses was a 2.0-liter aliquot of medical-grade N_2O gas (UN1070, Air Products Corp.) stored at 3,000 kPa to prevent fractionation effects from N_2O liquid-gas phase changes at high pressure. Two different aliquots of standard gas were needed for these studies, and these standards had $\delta^{15}\text{N}\text{-N}_2\text{O}$ values of $0.02 \pm 0.17\text{‰}$ ($n = 9$) (biological experiments) and $-2.12 \pm 0.40\text{‰}$ ($n = 9$) (chemical and combined

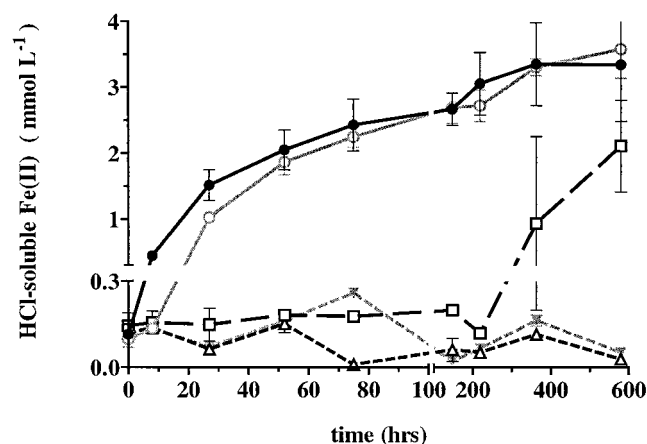


FIG. 1. Effect of NO_3^- on Fe(II) production (sum of aqueous and bound Fe) via dissimilatory reduction of synthetic goethite by *S. putrefaciens* 200. Data from experiments using no NO_3^- (●), 1.0 mM initial NO_3^- (○), 2.5 mM initial NO_3^- (□), 5.0 mM initial NO_3^- (△), and uninoculated controls (▼) are plotted versus time. Solid lines connect the points for experiments with no NO_3^- and 1 mM NO_3^- , and dotted lines connect all other points. Data represent average values of three replicates, and the error bars reflect standard deviation. Note the split axes.

experiments). The fractionation effect associated with the previously described method for extracting headspace N_2O in equilibrium with AGW medium was calculated to be $-2.8 \pm 1.0\text{‰}$. All reported $\delta^{15}\text{N}$ - N_2O values account for this extraction bias. $\delta^{15}\text{N}$ values of elemental nitrogen samples were determined off-line, manually, in dual-inlet mode with a Finnigan MAT 252 mass spectrometer with a cryogenic trap at the inlet. We used the ammonium sulfate nitrogen stable isotope standards NBS-N-1 and NBS-N-2 as internal standards for calibration and report our results relative to air nitrogen (air nitrogen $\delta^{15}\text{N} = 0\text{‰}$), with a mass-spectrometric precision of $\pm 0.2\text{‰}$.

RESULTS

NO_3^- inhibition of goethite reduction. Our batch experiments with goethite as the Fe(III) source in the absence of NO_3^- (data not shown) yielded results similar to those of previous researchers who have investigated microbial reduction of iron (hydr)oxide minerals. Fe^{2+} production rates are initially rapid and then slow with time, possibly as a result of passivation of the cell and iron oxide surfaces by adsorbed Fe(II) (44, 45). It is important to note that approximately 50% of the microbially produced Fe^{2+} is associated with the mineral or cellular surface (precipitated, adsorbed, or a reactive surface complex). Data in Fig. 1 illustrate the effect of NO_3^- on the rate and extent of Fe^{2+} production during goethite reduction by *S. putrefaciens* 200. All slurries reduced NO_3^- (data not shown), but only the slurries containing initial concentrations of 0.0, 1.0, and 2.5 mM NO_3^- produced Fe^{2+} at concentrations significantly above those in the uninoculated controls. The presence of 1.0 mM NO_3^- resulted in a 50% reduction in the initial rate (~ 28 h) of Fe^{2+} production (ca. $2\times$ slower) and prevented significant Fe^{2+} production for a period of 8 h. The presence of 2.5 mM initial NO_3^- prevented significant Fe^{2+} production for over 200 h, and no Fe^{2+} production was observed in the 5 mM NO_3^- treatment. No measurable effect on the ultimate extent of iron reduction was observed for the 1 mM NO_3^- treatment.

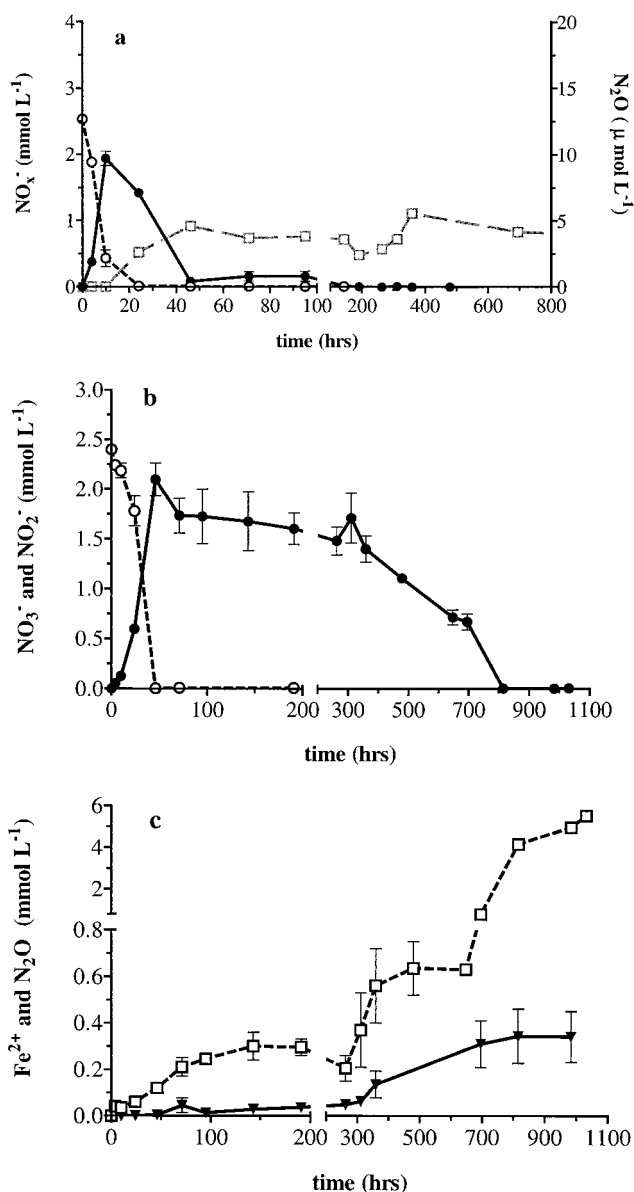


FIG. 2. NO_3^- reduction by *S. putrefaciens* 200 in the presence and absence of goethite. (a) Experiment with goethite absent, showing data for NO_3^- (○), NO_2^- (●), and N_2O (□). (b) Experiment with goethite present, showing data for NO_3^- (○) and NO_2^- (●). (c) Experiment with goethite present, showing data for N_2O (▼) and Fe(II)-HCl (□). Data represent average values of three replicates, and the error bars reflect standard deviation. Note the split axes and the dual y axes in Fig. 2a.

Subsequent experiments were conducted with goethite plus one concentration of NO_3^- (2.5 mM initial NO_3^-). This decision was made because the large sampling matrix prevented experimentation at multiple NO_3^- concentrations, and 2.5 mM represented a good midpoint between the 0.0 mM NO_3^- and 5 mM NO_3^- end members. These experiments show typical patterns of NO_2^- and N_2O production by *S. putrefaciens* 200 during NO_3^- reduction in the absence (Fig. 2a) and presence (Fig. 2b and c) of goethite and reveal that a very small amount of surface-associated Fe^{2+} (~ 0.1 mM) is produced

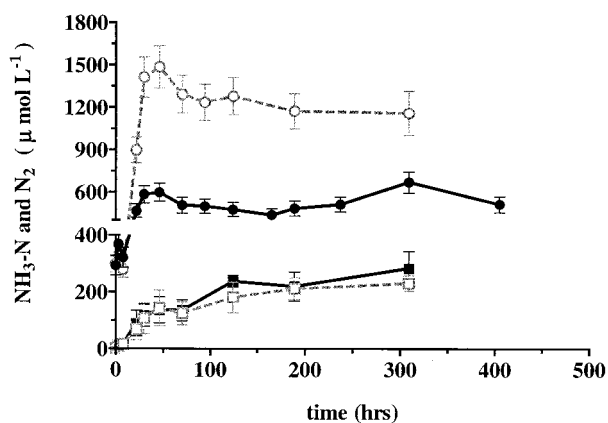


FIG. 3. $\text{NH}_3\text{-N}$ (circles) and N_2 (squares) production during 2.5 mM NO_3^- reduction by *S. putrefaciens* 200 in the presence (solid symbols) or absence (open symbols) of goethite. Whereas NO_3^- and biogenic NO_2^- were completely consumed in bottles lacking goethite, a mean NO_2^- concentration of 1.0 mM remained in bottles containing goethite at the conclusion of the experiment. Data represent average values of three replicates, and the error bars reflect standard deviation. Note the split y axis.

while NO_3^- is still present even though Fe^{2+} production rates are minimal until after the NO_2^- supply has been exhausted (Fig. 2b and c). More than 70% of the added NO_3^- was recovered as NO_3^- , NO_2^- , N_2 , N_2O , or $\text{NH}_3\text{-N}$ in all experiments with NO_3^- and goethite (data not shown). In these experiments, the proportion of nitrogen recovered was initially 100% and then decreased as experiments progressed and cell growth occurred. Since the modest amount of cell growth could not account for the unrecovered N, it is likely that products or complexes were formed that were not included in the N species quantified. Ammonia was the dominant end product of NO_x^- reduction in our experiments, and only minimal amounts of N_2 were produced (Fig. 3). Since the $\text{Fe}^{2+}\text{-Fe}^{3+}$ redox transition ($E_{\text{H}}^0 \cong 0.8$ V) is dominant in our system, this observation is consistent with a report by Samuelsson that ammonia is the dominant end product (and that N_2O is a minor by-product) of NO_3^- reduction by a different strain of *S. putrefaciens* at redox potentials greater than or equal to ~ 0 mV (46).

Goethite inhibition of NO_3^- and NO_2^- reduction. Results from NO_3^- reduction experiments conducted with 2.5 mM NO_3^- in the absence of goethite are summarized in Fig. 2a. Most NO_2^- was reduced within 48 h, but low levels persisted through the first 100 h. Observed trends in mass balance were statistically indistinguishable from experiments conducted in the presence of goethite, and trends in $\text{NH}_3\text{-N}$ and $\text{N}_2\text{-N}$ product distribution were similar as well. Examination of these data and comparison with results summarized in Fig. 2 reveal three

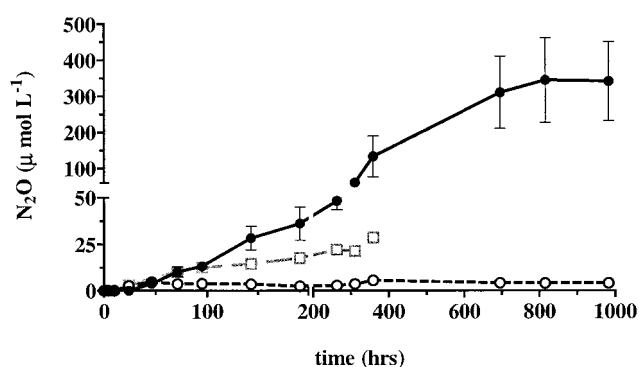


FIG. 4. Effect of goethite on N_2O production rate with 5 mM initial NO_3^- without goethite (\square), 2.5 mM initial NO_3^- without goethite (\circ), and 2.5 mM initial NO_3^- with goethite (\bullet). Data represent average values of three replicates, and the error bars reflect standard deviation. Note the split x and y axes.

important observations, namely (i) significantly more N_2O was produced when NO_3^- was reduced in the presence of goethite (Fig. 2c) than in the absence of goethite (Fig. 2a); (ii) in both systems, N_2O production was not observed until after all NO_3^- had been consumed (Fig. 2a to c); and (iii) the rates of NO_3^- and NO_2^- reduction were notably higher in the absence of goethite (Fig. 2a) than in the presence of goethite (Fig. 2b).

Table 1 provides summary data showing that the presence of goethite can inhibit the rate of dissimilatory NO_3^- and NO_2^- reduction by *S. putrefaciens*. This table summarizes results from experiments that compare the initial rates of Fe(III) and NO_x^- reduction across various experimental matrices. The presence of NO_3^- resulted in an ~ 20 -fold decrease in the rate of microbial goethite reduction, and the presence of goethite resulted in an ~ 10 -fold decrease in the rate of NO_2^- reduction and an ~ 2 -fold decrease in the rate of NO_3^- reduction. In addition, data presented in Fig. 4 indicate that the presence of goethite has a marked effect on the observed degree of N_2O production in slurries containing 2.5 mM NO_3^- . Indeed, the N_2O produced in slurries containing goethite and 2.5 mM NO_3^- was even substantially higher than in incubations containing 5.0 mM NO_3^- lacking goethite.

In order to determine if the observed mutual inhibition was due to a process unique to *S. putrefaciens* 200, similar experiments were also conducted with enrichment cultures. Data from these experiments (Fig. 5) indicate that the presence of goethite can also inhibit dissimilatory reduction of NO_3^- and NO_2^- by a natural enrichment culture under conditions that are analogous to those used in experiments with *S. putrefaciens*. These cultures produced 200 μM HCl-soluble Fe(II) and virtually no N_2O over the course of the experiments (data not shown). Although these experiments yielded different trends in

TABLE 1. Comparison of initial rates of NO_3^- reduction, NO_2^- reduction, and Fe^{2+} production across various experimental matrices

Matrix	NO_3^- reduction ($\mu\text{mol/liter/h}$)	NO_2^- reduction ($\mu\text{mol/liter/h}$)	Fe(II) production ($\mu\text{mol/liter/h}$)
Goethite only	NA ^a	NA	43.0 ± 24.0 ($n = 6$)
NO_3^- only	168 ± 72.5 ($n = 6$)	19.2 ± 2.1 ($n = 6$)	NA
Goethite and NO_3^-	72.7 ± 29.2 ($n = 8$)	2.5 ± 0.5 ($n = 6$)	2.3 ± 0.3 ($n = 4$)

^a NA, not applicable.

first two sampling periods. This observation would, on the surface, seem contrary to the results of DiChristina (14) and Obuekwe and Westlake (40, 41). DiChristina's studies of competitive usage of NO_3^- and Fe(III) by *S. putrefaciens* 200 suggested only partial inhibition of Fe(II) production by 15 mM NO_3^- . His studies were done with either ferric citrate or ferric chloride as the Fe(III) source. Ferric citrate is an aqueous complex, whereas an acidic solution of ferric chloride would immediately precipitate a ferric oxyhydroxides mineral at circumneutral pH. Thus, the experiments with ferric chloride were, in effect, done with an amorphous or microcrystalline Fe(III) oxyhydroxides, while the experiments with ferric citrate were done with a true aqueous Fe(III) source. Interestingly, his work showed that NO_3^- inhibition of Fe(II) production by microaerobically grown cells was 3- to 23-fold greater in the presence of this solid-phase Fe(III) (FeCl_3) than in the presence of Fe(III) citrate. Obuekwe's work, which also showed simultaneous NO_3^- reduction and Fe(II) production, utilized Fe(III) phosphate in which the Fe(III) is typically chelated by additional citrate. It would therefore appear that significant Fe(II) production occurs simultaneously with NO_3^- reduction when the Fe(III) is supplied in a chelated form. When Fe(III) is presented in the form of a solid-phase oxyhydroxide that would allow sorption of biogenic Fe(II), inhibition of Fe(II) production by NO_3^- is more substantial.

With respect to the N speciation and N balance in our system, our results are comparable to those of other researchers who have also reported incomplete recovery of N species during nitrate reduction to ammonia. Samuelsson and Rönner (47) reported that only 25 to 75% of NO_3^- added to isolates from the Baltic Sea was recovered as ammonia. In experiments with a different strain of *S. putrefaciens* (formerly *Pseudomonas putrefaciens*), Samuelsson was unable to account for 6 to 91% of the NO_3^- consumed. In those experiments, only NH_4^+ , N_2O , and NO_2^- were measured, and the missing N was attributed to N_2 or some other unmeasured N species (46). Using *S. putrefaciens* strain MR-1, Krause and Nealson, however, found little or no ammonia production and suggested that the carbon/ NO_3^- ratio may also play a role in determining whether *S. putrefaciens* produces gaseous compounds (N_2 or N_2O) or ammonia during dissimilatory NO_3^- reduction (27).

It is generally accepted that under most conditions, microorganisms can generate more energy from dissimilatory NO_3^- reduction than from dissimilatory Fe(III) reduction. Consequently, NO_3^- inhibition of microbial iron (hydr)oxide reduction may result from differences in the rate of electron transport to NO_x^- and Fe(III) that have evolved to allow preferential use of the most favorable oxidant (1, 14, 41, 50, 51). Alternate explanations, however, are also possible. Since a small amount of Fe(II) was produced in our goethite-containing bottles as NO_3^- reduction proceeded (Fig. 2c), NO_3^- , NO_2^- , and Fe^{2+} were simultaneously present in the same system. Under suitable conditions, Fe^{2+} can chemically reduce both NO_3^- to NO_2^- (11) and NO_2^- to N_2O and/or ammonia (10, 40, 57). Thus, concomitant reduction of NO_3^- and Fe(III) may create an apparent inhibition of microbial iron reduction by oxidizing Fe^{2+} to Fe(III) via chemical NO_x^- reduction. Results from previous studies testing this possibility are somewhat contradictory but do reveal two general themes. First, the chemical rate of NO_2^- reduction via oxidation of aqueous

Fe^{2+} (produced via the microbial reduction of ferric chloride or ferric citrate) is too slow to account for a significant degree of NO_x^- inhibition of microbial Fe(III) reduction (14). Second, the chemical rate of NO_2^- reduction via oxidation of surface-associated Fe^{2+} [produced via the microbial reduction of iron (hydr)oxide minerals] is notably faster than the rate of NO_2^- reduction by aqueous Fe^{2+} (57). This finding that Fe^{2+} -Fe(III) moieties (e.g., Fe^{2+} adsorbed to FeOOH minerals, green complexes, and/or ferrous precipitates) can reduce NO_2^- more rapidly than aqueous Fe^{2+} is supported by previous geochemical research into the catalytic effect of these moieties on the rate of NO_x^- reduction to N_2O and/or ammonium (22, 23, 52). This information, coupled with the observation that goethite reduction in the presence of NO_3^- produced notably more N_2O than NO_3^- reduction alone (Fig. 4), suggests that concomitant NO_3^- and goethite reduction in our systems does result in chemical reoxidation of Fe^{2+} coupled to NO_2^- reduction to N_2O . However, the extremely small amount of N_2O produced by this reaction indicates that most NO_2^- in our system is reduced enzymatically.

Goethite inhibition of NO_3^- and NO_2^- reduction. While our work agrees with previous studies with *S. putrefaciens* 200 which indicate that the presence of NO_3^- will substantially inhibit microbial Fe(III) reduction (14), the observation that goethite inhibits NO_3^- and NO_2^- reduction is unprecedented and differs from the results of DiChristina (14) and Lee et al. (28), who reported that the presence of Fe(III)-chloride and/or Fe(III)-citrate had no notable effect on the observed rates of NO_3^- and NO_2^- reduction by *S. putrefaciens*. This disparity likely arises from significant differences in the nature of the Fe(III) source used in these experiments and the time scale of the experiments described by DiChristina (minutes to hours) and by the present work (hours to weeks). In addition, the observation that natural enrichment cultures can also show goethite inhibition of NO_3^- reduction (Fig. 5) indicates that this process may be important in natural systems with high iron content and low flow rates and thus merits significant further investigation.

Several mechanisms can potentially explain the goethite inhibition of NO_x^- reduction in both *S. putrefaciens* 200 and the UMTRA enrichment culture.

(i) The presence of goethite particles might have a toxic effect. Direct goethite toxicity is probably not a factor, since goethite is not known to be toxic, especially to microorganisms capable of dissimilatory iron reduction. To examine the prospect that goethite particles could have abraded the cells and either increased cell mortality or made cells more susceptible to other toxic effects, cells were incubated (5 days at 30°C with no carbon substrate and in the same experimental medium) in the presence and absence of goethite and a series of other sediments. In all cases, cells incubated in the presence of a solid surface yielded a higher final cell number than cells incubated in the absence of a solid surface (data not shown). Thus, cell abrasion probably does not contribute significantly to the observed goethite inhibition of microbial NO_x^- reduction.

(ii) If all NO_x^- -reducing cells are coated by a dense layer of goethite crystals, such a coating might reduce the diffusive flux of NO_x^- to the cell surface. There is currently no evidence,

however, that such dense coatings are formed or that they would limit NO_x^- diffusion to the cell surface.

(iii) If rates of electron transport to NO_x^- and Fe(III) are similar, goethite inhibition of NO_x^- reduction could result from simple kinetic competition between the two electron acceptors, a mechanism previously suggested to explain inhibition of Fe(III) reduction by oxygen (4). However, given the very small amount of Fe(III) reduction observed in the presence of NO_x^- , the extent of inhibition shown in Table 1 probably cannot be explained via a competitive mechanism alone.

(iv) Prolonged exposure to elevated concentrations of NO_2^- may be toxic (2). This mechanism, however, would not explain why NO_2^- reduction proceeded much more slowly in cultures containing goethite than in cultures absent goethite. Oxygen uptake studies on cells that had been cultured aerobically in the presence of NO_2^- indicated that NO_2^- showed little toxicity at concentrations up to 5 mmol/liter (data not shown). When combined with the observation that the NO_2^- reduction rate in the absence of goethite and presence of high (~2 mM) NO_2^- concentrations (Fig. 2a) was markedly greater than the NO_2^- reduction rate in the presence of goethite and similar NO_2^- concentrations (Fig. 2b), these experiments indicate that NO_2^- toxicity could not explain the observed goethite inhibition of NO_x^- reduction.

(v) Chemical oxidation of biogenic Fe^{2+} by NO_2^- results in the nucleation of ferric (hydr)oxide minerals on the surface of a cell, forming coatings which impede transport of NO_x^- into the cell. Such a hypothetical mechanism would proceed via three steps. Firstly, *S. putrefaciens* concomitantly reduces NO_3^- and, to a lesser extent, goethite, producing NO_2^- and small amounts of aqueous Fe^{2+} . Some of the microbially produced Fe^{2+} then sorbs to the cellular surface, forming a reactive complex that can catalyze the oxidation of Fe^{2+} to Fe(III) via the chemical reduction of NO_2^- to N_2O . Most of the NO_2^- produced is reduced biologically to ammonia and/or N_2 , with N_2O as a minor by-product. Lastly, the Fe(III) precipitates as an iron oxyhydroxide mineral. This mineral can nucleate on the cell surface and thereby inhibit transport of NO_3^- and NO_2^- to NO_x^- reductases inside the cell. Since chemical NO_2^- reduction via Fe^{2+} oxidation is known to produce N_2O (22, 23, 52, 57), this mechanism is supported by the increased N_2O production observed to occur during concomitant NO_x^- and goethite reduction (Fig. 4). In addition, the data presented in Fig. 6 provide compelling evidence that N_2O is being produced via chemical NO_2^- reduction via Fe^{2+} oxidation. An inhibitory mechanism involving the presence of Fe oxyhydroxide coatings on the cell surface is supported by the recent work of Liu et al. (29), who observed the formation of Fe mineral coatings on *S. putrefaciens* after exposure of cells to Fe^{2+} . These coatings coincided with a lag phase during which reduction of Fe(III) citrate was inhibited. The mineral coatings disappeared as cells recovered from the lag phase, and the authors suggested that the cells had developed a mechanism for coating removal. Formation and subsequent removal of such coatings in our experiments would explain the long lag period seen in Fig. 2b and c (between ~50 and 300 h), during which very little NO_2^- or Fe(III) was reduced.

At the current time, however, a definitive mechanism of NO_x^- reduction inhibition by goethite cannot be completely substantiated by the data generated in these experiments.

Since all cultures in our experiments were grown identically prior to resuspension in slurries containing or lacking goethite, levels of nitrate or nitrite reductase were initially induced equally in all reactors, regardless of whether goethite was present or absent.

$\delta^{15}\text{N}_2\text{O}$ studies of interactions between microbial NO_x^- and goethite reduction. We are not aware of any reported values for the isotopic fractionation between NO_2^- and N_2O for the chemical reduction of NO_2^- by Fe^{2+} . However, it is possible to compare our values for biological NO_2^- reduction to N_2O with values reported in the literature. Because the actual $\delta^{15}\text{N}\text{-N}_2\text{O}$ value is somewhat dependent on the $\delta^{15}\text{N}$ of the starting NO_x^- species, these comparisons were made on the basis of the difference in $\delta^{15}\text{N}$ between reactant and product ($\Delta^{15}\text{N} = [\delta^{15}\text{N}\text{-NO}_x^- \text{ species}] - [\delta^{15}\text{N}\text{-N}_2\text{O}]$). The key difference between our microbial studies and previously reported $\Delta^{15}\text{N}$ values for N_2O formation during denitrification and NH_4^+ oxidation (7, 33, 34, 56, 58) is that we report a negative $\Delta^{15}\text{N}$ value (enrichment in ^{15}N with respect to the source), whereas previous studies reported positive $\Delta^{15}\text{N}$ values (depletion in ^{15}N with respect to the source). Considering these reports, it should be noted that the N_2O produced during denitrification is produced as an intermediate or final product, whereas the N_2O produced during nitrate reduction to ammonia is thought to be produced as a minor side reaction (6, 54). As these processes are quite different, it is not surprising that our $\Delta^{15}\text{N}$ values display a trend not reported in the literature. The production of ^{15}N -enriched N_2O during reduction of NO_x^- by *S. putrefaciens* 200 can easily be explained if (i) active biological NO_2^- reduction to ammonia by *S. putrefaciens* favors ^{14}N over ^{15}N and creates a pool of ^{15}N -enriched NO_2^- and (ii) the N_2O -producing side reaction utilizes this remnant pool of ^{15}N -enriched NO_2^- to produce N_2O that is depleted in ^{15}N relative to the remnant pool but that is still enriched in ^{15}N relative to the original NO_2^- .

Conclusion. This work has demonstrated not only that NO_3^- inhibits microbial reduction of crystalline Fe^{3+} sources more strongly than that of aqueous Fe^{3+} sources but also that the presence of crystalline Fe^{3+} (e.g., goethite) inhibits microbial NO_3^- and NO_2^- reduction. Increased N_2O production in the presence of goethite and stable isotopic evidence both indicate that abiotic NO_2^- reduction to N_2O coupled to Fe^{2+} oxidation is occurring concomitantly with microbial NO_x^- and goethite reduction.

The implications of this process can be dramatic in a number of ways. Most relevantly, it provides for a series of geochemical reactions that can potentially inhibit a microbiological process by nucleating minerals on an active cellular surface. While novel, the idea that the formation of minerals on a cell membrane can inhibit intracellular processes is not without precedent. Both gram-negative and gram-positive bacterial cell membranes are documented to be ideal sites for mineral nucleation (8, 9, 15, 17, 18, 25, 48), and previous researchers have demonstrated that the production of Fe^{2+} and ferrous minerals may be responsible for the cell passivation commonly observed during batch mode iron reduction experiments (29, 43). Further studies of the metabolic and environmental implications of abiotic Fe^{2+} oxidation occurring concomitantly with microbial goethite and NO_x^- reduction are needed.

ACKNOWLEDGMENTS

We acknowledge funding by the Natural and Accelerated Bioremediation Research (NABIR) program, Biological and Environmental Research (BER), U.S. Department of Energy (grant no. DE-FG02-97ER62482).

We also thank Phil Long for assistance in collection of sediments from the DOE UMTRA site in Shiprock, N.Mex., Eric Roden for invaluable discussions, and the anonymous reviewers for useful suggestions for improving the manuscript.

REFERENCES

- Acht nich, C. A., F. Bak, and R. Conrad. 1995. Competition for electron donors among nitrate reducers, ferric iron reducers, sulfate reducers, and methanogens in anoxic paddy soil. *Biol. Fertil. Soils* **19**:65–72.
- Almeida, J. S., S. M. Julio, M. A. M. Reis, and M. J. T. Carrondo. 1995. Nitrite inhibition of denitrification by *Pseudomonas fluorescens*. *Biotechnol. Bioeng.* **46**:194–201.
- Arnold, R. G., T. J. DiChristina, and M. R. Hoffman. 1986. Inhibitor studies of dissimilative Fe(III) reduction by *Pseudomonas* sp. strain 200 ("Pseudomonas ferrireductans"). *Appl. Environ. Microbiol.* **52**:281–289.
- Arnold, R. G., M. R. Hoffman, T. J. DiChristina, and F. W. Picardal. 1990. Regulation of dissimilative Fe(III) reduction activity in *Shewanella putrefaciens*. *Appl. Environ. Microbiol.* **56**:2811–2817.
- Arnold, R. G., T. M. Olson, and M. R. Hoffman. 1986. Kinetics and mechanism of dissimilative Fe(III) reduction by *Pseudomonas* sp. 200. *Biotechnol. Bioeng.* **28**:1657–1671.
- Averill, B. A., and J. M. Tiedje. 1982. The chemical mechanism of microbial denitrification. *FEBS Lett.* **138**:8–12.
- Barford, C. C., J. P. Montoya, M. A. Altabet, and R. Mitchell. 1999. Steady-state nitrogen isotope effects of N_2 and N_2O production in *Paracoccus denitrificans*. *Appl. Environ. Microbiol.* **65**:989–994.
- Beveridge, T. J., and W. S. Fyfe. 1985. Metal fixation by bacterial cell walls. *Can. J. Earth Sci.* **22**:1893–1898.
- Beveridge, T. J., J. D. Meloche, W. S. Fyfe, and R. G. E. Murray. 1983. Diagenesis of metals chemically complexed to bacteria: laboratory formation of metal phosphates, sulfides, and organic condensates in artificial sediments. *Appl. Environ. Microbiol.* **45**:1094–1108.
- Brons, H. J., W. R. Hagen, and A. J. B. Zehnder. 1991. Ferrous iron dependent nitric oxide production in nitrate reducing cultures of *Escherichia coli*. *Arch. Microbiol.* **155**:341–347.
- Buresh, R. J., and J. T. Moraghan. 1976. Chemical reduction of nitrate by ferrous iron. *J. Environ. Qual.* **5**:320–325.
- Cooper, D. C., F. Picardal, J. Rivera, and C. Talbot. 2000. Zinc immobilization and magnetite formation via ferric oxide reduction by *Shewanella putrefaciens* 200. *Environ. Sci. Technol.* **34**:100–106.
- Cummings, D. E., F. Caccavo, S. Fendorf, and R. F. Rosenzweig. 1999. Arsenic mobilization by the dissimilatory Fe(III)-reducing bacterium *Shewanella alga* BrY. *Environ. Sci. Technol.* **33**:723–729.
- DiChristina, T. J. 1992. Effects of nitrate and nitrite on dissimilatory iron reduction by *Shewanella putrefaciens* 200. *J. Bacteriol.* **174**:1891–1896.
- Douglas, S., and T. J. Beveridge. 1998. Mineral formation by bacteria in natural microbial communities. *FEMS Microbiol. Ecol.* **26**:79–88.
- Erbs, M., H. C. B. Hansen, and C. E. Olsen. 1999. Reductive dechlorination of carbon tetrachloride using iron(II) iron(III) hydroxide sulfate (green rust). *Environ. Sci. Technol.* **33**:307–311.
- Fein, J. B., C. J. Daughney, N. Yee, and T. A. Davis. 1997. A chemical equilibrium model for metal adsorption onto bacterial surfaces. *Geochim. Cosmochim. Acta* **61**:3319–3328.
- Fortin, D., F. G. Ferris, and T. J. Beveridge. 1997. Surface-mediated mineral development by bacteria., p. 161–181. *In* J. F. Banfield and K. H. Nealson (ed.), *Geomicrobiology*, vol. 35. Mineralogical Society of America, Washington, D.C.
- Fredrickson, J. K., J. M. Zachara, D. W. Kennedy, M. C. Duff, Y. A. Gorby, S.-M. Li, and K. M. Krupka. 2000. Reduction of U(VI) in goethite (α -FeOOH) suspensions by a dissimilatory metal-reducing bacterium. *Geochim. Cosmochim. Acta* **64**:3085–3098.
- Génin, J.-M. R., G. Bourrié, F. Trolard, M. Abdelmoula, A. Jaffrezic, P. Refait, V. Maitre, B. Humbert, and A. Herbillon. 1998. Thermodynamic equilibria in aqueous suspensions of synthetic and natural Fe(II)-Fe(III) green rusts: occurrences of the mineral in hydromorphic soils. *Environ. Sci. Technol.* **32**:1058–1068.
- Greenberg, A. E., L. S. Clesceri, and A. D. Eaton (ed.). 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington D.C.
- Hansen, H. C. B., O. K. Borggaard, and J. Sørensen. 1994. Evaluation of the free energy of formation of Fe(II)-Fe(III) hydroxide-sulfate (green rust) and its reduction of nitrite. *Geochim. Cosmochim. Acta* **58**:2599–2608.
- Hansen, H. C. B., C. B. Koch, H. Nancke-Krogh, O. K. Borggaard, and J. Sørensen. 1996. Abiotic nitrate reduction to ammonium: key role of green rust. *Environ. Sci. Technol.* **30**:2053–2056.
- Kim, S., and F. W. Picardal. 1999. Enhanced anaerobic biotransformation of carbon tetrachloride in the presence of reduced iron oxides. *Environ. Toxicol. Chem.* **18**:2142–2150.
- Konhauser, K. O., W. S. Fyfe, F. G. Ferris, and T. J. Beveridge. 1993. Metal sorption and mineral precipitation by bacteria in two Amazonian river systems: Rio Solimões and Rio Negro, Brazil. *Geology* **21**:1103–1106.
- Kostka, J. E., and G. W. Luther. 1994. Partitioning and speciation of solid phase iron in saltmarsh sediments. *Geochim. Cosmochim. Acta* **58**:1701–1710.
- Krause, B., and K. H. Nealson. 1997. Physiology and enzymology involved in denitrification by *Shewanella putrefaciens*. *Appl. Environ. Microbiol.* **63**:2613–2618.
- Lee, I.-G., S.-J. Kim, and T.-Y. Ahn. 2000. Inhibitory effect of nitrate on Fe(III) and humic acid reduction in *Shewanella putrefaciens* DK-1. *J. Microbiol.* **38**:180–182.
- Liu, C., J. M. Zachara, Y. A. Gorby, J. E. Szecsoy, and C. F. Brown. 2001. Microbial reduction of Fe(III) and sorption/precipitation of Fe(II) on *Shewanella putrefaciens* strain CN32. *Environ. Sci. Technol.* **35**:1385–1393.
- Lloyd, J. R., P. Yong, and L. E. Macaskie. 2000. Biological reduction and removal of Np(V) by two microorganisms. *Environ. Sci. Technol.* **34**:1297–1301.
- Lovely, D. R. 1991. Dissimilatory Fe(III) and Mn(IV) reduction. *Microbiol. Rev.* **55**:259–287.
- Lovely, D. R., and E. J. P. Phillips. 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Appl. Environ. Microbiol.* **51**:683–689.
- Mandernack, K. W., T. Rahn, C. Kinney, and M. Wahlen. 2000. The biogeochemical controls of the $\delta^{15}N$ and $\delta^{18}O$ of N_2O produced in landfill cover soils. *J. Geophys. Res.* **105**:709–720.
- Mariotti, A., J. C. Germon, and A. Leclerc. 1982. Nitrogen isotope fractionation associated with the $NO_2^- \rightarrow N_2O$ step of denitrification in soils. *Can. J. Soil Sci.* **62**:227–241.
- Misawa, T., K. Hashimoto, and S. Shimodaira. 1974. The mechanism of formation of iron oxide and oxyhydroxides in aqueous solutions at room temperature. *Corr. Sci.* **14**:131–149.
- Myers, C. R., and J. M. Myers. 1993. Role of menaquinone in the reduction of fumarate, nitrate, iron(III), and manganese(IV) by *Shewanella putrefaciens* MR-1. *FEMS Microbiol. Lett.* **114**:215–222.
- Myers, C. R., and K. H. Nealson. 1990. Iron mineralization by bacteria: metabolic coupling of iron reduction to cell metabolism in *Aeromonas putrefaciens* strain MR-1, p. 131–149. *In* R. B. Frankel and R. P. Blakemore (ed.), *Iron biominerals*. Plenum Press, New York, N.Y.
- Nolan, B. T., and J. D. Stoner. 2000. Nutrients in groundwaters of the conterminous United States, 1992–1995. *Environ. Sci. Technol.* **34**:1156–1165.
- Obuekwe, C. O. 1980. Microbial corrosion of a crude oil pipeline. Ph.D. thesis, University of Alberta, Edmonton, Alberta, Canada.
- Obuekwe, C. O., and D. W. S. Westlake. 1981. Effect of nitrate on reduction of ferric iron by a bacterium isolated from crude oil. *Can. J. Microbiol.* **27**:692–697.
- Obuekwe, C. O., and D. W. S. Westlake. 1982. Effect of reducible compounds (potential electron acceptors) on reduction of ferric iron by *Pseudomonas* species. *Microbiol. Lett.* **19**:57–62.
- Picardal, F. W., R. G. Arnold, H. Couch, A. M. Little, and M. E. Smith. 1993. Involvement of cytochromes in the anaerobic biotransformation of tetrachloromethane by *Shewanella putrefaciens* 200. *Appl. Environ. Microbiol.* **59**:3763–3770.
- Roden, E. E., and M. M. Urrutia. 1999. Ferrous iron removal promotes microbial reduction of crystalline iron(III) oxides. *Environ. Sci. Technol.* **33**:1847–1853.
- Roden, E. E., M. M. Urrutia, and C. J. Mann. 2000. Bacterial reductive dissolution of crystalline Fe(III) oxide in continuous-flow column reactors. *Appl. Environ. Microbiol.* **66**:1062–1065.
- Roden, E. E., and J. M. Zachara. 1996. Microbial reduction of crystalline iron(III) oxides: influence of oxide surface area and potential for cell growth. *Environ. Sci. Technol.* **30**:1618–1628.
- Samuelsson, M.-O. 1985. Dissimilatory nitrate reduction to nitrite, nitrous oxide, and ammonium by *Pseudomonas putrefaciens*. *Appl. Environ. Microbiol.* **50**:812–815.
- Samuelsson, M.-O., and U. Rønner. 1982. Ammonium production by dissimilatory nitrate reducers isolated from Baltic Sea water, as indicated by ^{15}N study. *Appl. Environ. Microbiol.* **44**:1241–1243.
- Schultze-Lam, S., D. Fortin, and T. J. Beveridge. 1996. Mineralization of bacterial surfaces. *Chem. Geol.* **132**:171–181.
- Semple, K. M., and D. W. S. Westlake. 1987. Characterization of iron-reducing *Aeromonas putrefaciens* strains from oil-field fluids. *Can. J. Microbiol.* **33**:366–371.
- Sørensen, J. 1987. Nitrate reduction in marine sediment: Pathways and interactions with iron and sulfur cycling. *Geomicrobiol. J.* **5**:401–421.
- Sørensen, J. 1982. Reduction of ferric iron in anaerobic, marine sediment and interaction with reduction of nitrate and sulfate. *Appl. Environ. Microbiol.* **43**:319–324.
- Sørensen, J., and L. Thorling. 1991. Stimulation by lepidocrocite (γ -FeOOH) of Fe(II)-dependent nitrite reduction. *Geochim. Cosmochim. Acta* **55**:1289–1294.

53. **Stookey, L. L.** 1970. Ferrozine—a new spectrophotometric reagent for iron. *Anal. Chem.* **42**:779–781.
54. **Stouthamer, A. H.** 1988. Dissimilatory reduction of oxidized nitrogen compounds, p. 245–303. *In* A. J. B. Zehnder (ed.), *Biology of anaerobic microorganisms*. John Wiley & Sons, New York, N.Y.
55. **Tsapin, A., K. Nealson, T. Meyers, M. A. Cusanovich, J. Van Beuumen, L. D. Crosby, B. A. Feinberg, and C. Zhang.** 1996. Purification and properties of a low-redox-potential tetraheme cytochrome *c*₃ from *Shewanella putrefaciens*. *J. Bacteriol.* **178**:6386–6388.
56. **Ueda, S., N. Ogura, and E. Wada.** 1991. Nitrogen stable isotope ratio of groundwater N₂O. *Geophys. Res. Lett.* **18**:1449–1452.
57. **Weber, K. A., F. W. Picardal, and E. E. Roden.** 2001. Microbially-catalyzed nitrate-dependent oxidation of biogenic solid-phase Fe(II) compounds. *Environ. Sci. Technol.* **35**:1644–1650.
58. **Webster, E. A., and D. W. Hopkins.** 1996. Nitrogen and oxygen isotope ratios of nitrous oxide emitted from soil and produced by nitrifying and denitrifying bacteria. *Biol. Fertil. Soils* **22**:326–330.
59. **Wilhelm, E., R. Battino, and R. J. Wilcock.** 1977. Low-pressure solubility of gases in liquid water. *Chem. Rev.* **77**:219–262.
60. **Zachara, J. M., J. K. Fredrickson, S. C. Smith, and P. L. Gassman.** 2001. Solubilization of Fe(III) oxide-bound trace metals by a dissimilatory Fe(III) reducing bacterium. *Geochim. Cosmochim. Acta* **65**:75–93.