

Bacterial Reductive Dissolution of Crystalline Fe(III) Oxide in Continuous-Flow Column Reactors

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Bacterial reductive dissolution of synthetic crystalline Fe(III) oxide-coated sand was studied in continuous-flow column reactors in comparison with parallel batch cultures. The cumulative amount of aqueous Fe(II) exported from the columns over a 6-month incubation period corresponded to (95.0 ± 3.7)% (*n* = 3) of their original Fe(III) content. Wet-chemical analysis revealed that only (6.5 ± 3.2)% of the initial Fe(III) content remained in the columns at the end of the experiment. The near-quantitative removal of Fe was visibly evidenced by extensive bleaching of color from the sand in the columns. In contrast to the column reactors, Fe(II) production quickly reached an asymptote in batch cultures, and only (13.0 ± 2.2)% (*n* = 3) of the Fe(III) oxide content was reduced. Sustained bacterial-cell growth occurred in the column reactors, leading to the production and export of a quantity of cells 100-fold greater than that added during inoculation. Indirect estimates of cell growth, based on the quantity of Fe(III) reduced, suggest that only an approximate doubling of initial cell abundance was likely to have occurred in the batch cultures. Our results indicate that removal of biogenic Fe(II) via aqueous-phase transport in the column reactors decreased the passivating influence of surface-bound Fe(II) on oxide reduction activity, thereby allowing a dramatic increase in the extent of Fe(III) oxide reduction and associated bacterial growth. These findings have important implications for understanding the fate of organic and inorganic contaminants whose geochemical behavior is linked to Fe(III) oxide reduction.

Microbial Fe(III) oxide reduction is a key biogeochemical process in anaerobic sedimentary environments (10, 19). Although crystalline minerals such as goethite and hematite are typically the dominant Fe(III) oxide phases in soils and sediments (23), the apparent resistance of such minerals to enzymatic reduction (14, 16) has led to the view that amorphous Fe(III) oxide is the main form of Fe(III) oxide available for microbial reduction (10). Laboratory studies of bacterial crystalline Fe(III) oxide reduction typically reveal only minor degrees of reduction (14, 16, 21), and crystalline Fe(III) oxides have been shown to persist with depth in aquatic sediments (14, 18). However, extensive reduction of crystalline Fe(III) oxides has recently been observed in aquifer sediments contaminated with landfill leachate (5).

In a recent series of studies (21, 26, 27), we have shown that the low microbial reducibility of crystalline Fe(III) oxides is caused by sorption (adsorption and/or surface precipitation [25] of biogenic Fe(II) on oxide and Fe(III)-reducing bacterial (FeRB) surfaces. This process deactivates enzymatic Fe(III) reduction, possibly through an electrochemical passivation effect analogous to how buildup of Fe(III) oxide surface precipitates inhibits anodic corrosion of iron metal (28). The passivating influence of Fe(II) sorption can be relieved by chemical removal of sorbed Fe(II) from the mineral surface (21), as well as by the presence in culture medium of aqueous and solid-phase Fe(II) complexants which delay or retard the accumulation of surface-bound Fe(II) and thereby extend the degree of crystalline Fe(III) oxide reduction (27). In addition, removal

of Fe(II) during aqueous-phase replacement in semicontinuous cultures stimulated crystalline Fe(III) oxide reduction, increasing the extent of oxide reduction two- to threefold relative to that observed in parallel batch cultures over a 2-month period (20). These results suggested the possibility that complete bacterial reductive dissolution of crystalline Fe(III) oxides could occur under conditions of sustained aqueous-phase flux. Such an effect would have important implications for the geochemistry of subsurface environments, in which Fe(III) oxides often constitute major phases for sorption of various organic and metal-radionuclide contaminants (9), as well as the dominant source of aquifer oxidation capacity (6). Complete microbial reduction of crystalline Fe(III) minerals has never been demonstrated experimentally; the observations of Heron and Christensen (5) in contaminated aquifer sediments provide the first indication of the possibility for quantitative removal of crystalline Fe(III) oxides through dissimilatory microbial activity.

In this study, we compared the long-term microbial reductive dissolution of a crystalline Fe(III) oxide in flow-through experimental columns to that occurring in closed-batch reactors. Our findings indicate that removal of biogenic Fe(II) via aqueous-phase transport in the column reactors decreased the passivating influence of surface-bound Fe(II) on oxide reduction activity, thereby allowing for virtually complete reductive dissolution of the oxide over a 6-month period.

MATERIALS AND METHODS

Goethite-coated sand preparation. Synthetic goethite-coated sand was prepared by air oxidation of $\text{FeCl}_2 \cdot 2\text{H}_2\text{O}$ (22) in a suspension of medium quartz sand (Sigma Chemicals). After Fe(II) oxidation was complete, the sand was washed repeatedly with distilled water and freeze-dried. The dried material had a Fe(III) content of $104 \pm 8 \mu\text{mol g}^{-1}$ (0.58% dry weight) (*n* = 6). A sample of the oxide mineral associated with the quartz sand was obtained by vigorously dispersing a 50-g portion of sand in 100 ml of distilled water, followed by lyophilization of the resulting suspension of fine-grained material. The oxide was analyzed by X-ray diffraction. The diffraction peaks obtained matched with

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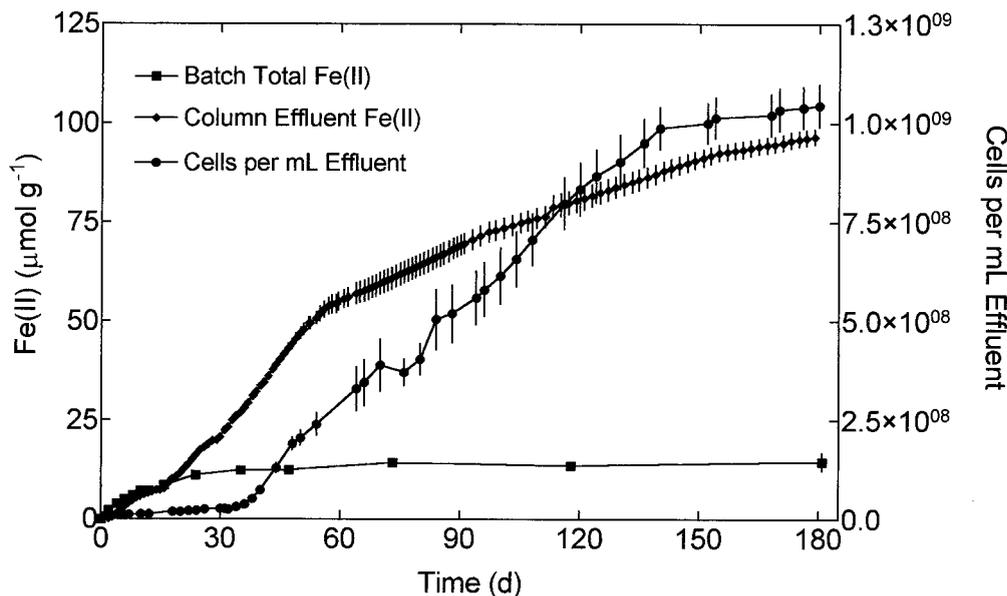


FIG. 1. Microbial reduction of synthetic goethite-coated sand in continuous-flow column reactors and batch cultures. Data for the column reactors (means of triplicate columns; error bars indicate standard deviations) are the cumulative amounts of aqueous Fe(II)- and Fe(III)-reducing bacteria collected in column effluent. Data for batch reactors are the means of duplicate cultures sacrificed for determination of aqueous and solid-phase Fe(II) at each sampling time; error bars indicate range of duplicates.

goethite and showed the broadening expected for the relatively small, high-surface-area particles formed during Fe(II) oxidation (22); no crystalline impurities were detected (data not shown).

Column and batch reactors. The flowthrough column reactors (Omnifit, Ltd.; 1.6 ml, total volume) were wet packed inside an anaerobic chamber with 2.2 g of synthetic goethite-coated sand and ca. 1-ml of culture medium containing ca. 5×10^7 cells ml^{-1} of the groundwater Fe(III)-reducing bacterium *Shewanella putrefaciens* strain CN32 (3). The final water content of the columns was ca. 40% (vol/vol). After an overnight equilibration period, the columns were flushed continuously (6-h residence time) in down-flow mode with a PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid)-buffered (10 mM, pH 6.8) artificial groundwater medium (1) containing 10 mM sodium lactate as a carbon and energy source together with inorganic nutrients (50 μM KH_2PO_4 , 500 μM NH_4Cl) and a mixture of vitamins and trace minerals (15). Effluent from the columns was collected in sterile, stoppered vials vented with a sterile 22-gauge needle to prevent pressure buildup. Direct microscopic counts of cells exported from the columns (see below) showed no evidence of bacterial strains other than *S. putrefaciens*. Parallel batch cultures (5-ml Wheaton serum vials) of similar total volume (2.2 g of goethite-coated sand plus 1.6 ml of culture medium) were established with artificial groundwater containing 30 mM lactate and inoculated with a quantity of FeRB cells comparable to that used in the column reactors.

Goethite-coated sand Fe(II) sorption experiment. Batch reactors containing 2.2 g of goethite-coated sand were amended with 2 ml of PIPES buffer containing ca. 10^9 cells ml^{-1} of *S. putrefaciens* strain CN32. Triplicate reactors were then amended with a 0.1-ml aliquot of $\text{FeCl}_2 \cdot 2\text{H}_2\text{O}$ stock solutions to achieve a range of final Fe(II) concentrations of 0.25 to 24 mmol liter $^{-1}$. The reactors were incubated overnight with gentle shaking, after which the concentration of Fe(II) remaining in solution was determined as described below.

Analytical procedures. Column effluent samples were analyzed for Fe(II) content using Ferrozine (24) and cell numbers by acridine orange direct count (7). Total Fe and Fe(II) concentrations in batch cultures were determined by citrate-dithionite and 0.5 M HCl extraction, respectively (21). The same methods were used to determine the total Fe and solid-phase Fe(II) content of the column reactors at the conclusion of the experiment. Concentrations of Fe(II) remaining in solution at the end of the Fe(II) sorption experiment were determined by Ferrozine analysis after filtering the suspension through a 0.2 μm syringe filter.

RESULTS AND DISCUSSION

A continuous efflux of aqueous Fe(II) from the column reactors occurred during the 6-month incubation period (Fig. 1). The cumulative amount of dissolved Fe(II) exported from the columns corresponded to $(95.0 \pm 3.7)\%$ ($n = 3$) of their original Fe(III) content, and wet chemical analysis revealed

that only $(6.5 \pm 3.2)\%$ of the initial Fe(III) content remained in the columns at the end of the experiment. The near-quantitative removal of Fe was visibly evidenced by extensive bleaching of color from the sand in the columns. In contrast to these results, Fe(II) production quickly reached an asymptote in batch cultures (Fig. 1), and only $(13.0 \pm 2.2)\%$ ($n = 3$) of the Fe(III) oxide content was reduced. No reduction of Fe(III) occurred in uninoculated batch cultures (data not shown). Previous work has shown that neither electron donor nor inorganic nutrient limitation are responsible for the minor degree of oxide reduction in closed (batch)-culture systems (20). Hence, the much greater degree of reduction observed in the flowthrough column reactors can be attributed to relief of Fe(II) inhibition of oxide reduction via advective Fe(II) removal.

In addition to its major impact on the degree of oxide reduction, aqueous-phase transport also promoted FeRB growth, as indicated by the sustained export of FeRB cells from the column reactors (Fig. 1). The onset of cell export at ca. 15 days was associated with a sharp increase in the rate of Fe(II) output. The number of FeRB cells exported from the column reactors, normalized to reactor volume, was more than 100-fold greater than the initial abundance of cells added to the reactors (ca. 2.5×10^7 ml^{-1}). Although cell counts were not conducted on that batch cultures, the maximum number of cells likely to have been produced in them can be estimated from published information on the number of FeRB cells generated during Fe(III) oxide reduction. An analysis of several studies of FeRB growth coupled to Fe(III) oxide reduction revealed a maximum value of 6.4×10^6 cells produced per μmol of Fe(II) (21). This value is close to the cumulative number of cells exported from the column reactors divided by the cumulative amount of Fe(II) exported from the reactors (4.5×10^6 cells/ μmol of Fe). Multiplying the volume-normalized amount of Fe(III) reduced in the batch reactors by the factor 6.4×10^6 yields a value of 7.3×10^7 cells ml^{-1} . This calculation suggests that cell growth in the batch reactors was

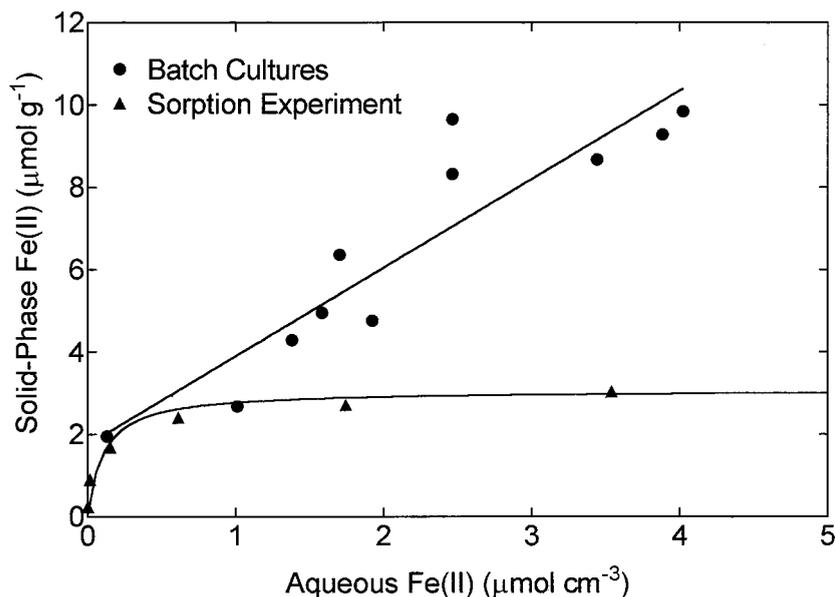


FIG. 2. Accumulation of solid-phase versus aqueous Fe(II) during synthetic goethite-coated sand reduction in the batch cultures, compared with solid- versus aqueous-phase Fe(II) partitioning during Fe(II) sorption experiments with the same goethite-coated sand. Data represent the means of duplicate (batch reactors) or triplicate (sorption experiment) samples. Solid lines represent curve fits of the batch culture and sorption experiment data to linear ($r^2 = 0.84$) and Langmuir ($r^2 = 0.84$) isotherms (25), respectively.

likely to have produced only an approximate doubling of the initial cell abundance, far less than the 100-fold increase which took place in the column reactors. The observed promotion of FeRB cell growth in the column reactors agrees with the recent finding that Fe(II) removal during medium replacement enhanced protein production by *Shewanella alga* (strain BrY) in semicontinuous culture systems (20).

The accumulation of solid-phase versus aqueous Fe(II) in the batch cultures was compared with independent data on Fe(II) sorption to a mixture of goethite-coated sand plus FeRB cells in order to assess the fate of Fe(II) in the batch system, an important consideration in relation to the mechanism of Fe(II) inhibition of oxide reduction (Fig. 2). Total solid-phase Fe(II) accumulation in the batch cultures far exceeded the measured Fe(II) sorption capacity of the mixed system, in agreement with previous experiments on synthetic goethite reduction (26). These findings suggest that bulk-phase mineral precipitation and/or surface Fe(II) precipitation were important sinks for Fe(II) in the batch cultures. Since the culture medium contained relatively low concentrations of phosphate ($50 \mu\text{M}$), vivianite [$\text{Fe}_2(\text{PO}_4)_3$] could not have been a major solid Fe(II) phase generated in these cultures. Hence, a combination of siderite (FeCO_3 , formed with inorganic carbon generated during lactate oxidation) together with $\text{Fe}(\text{OH})_2$ and/or mixed Fe(II)-Fe(III) phases (green rust, magnetite, or other spinel-like compounds [3]) were likely the main solid-phase end products of Fe(III) oxide reduction. Formation of such precipitates on or very near to oxide and FeRB cell surfaces can be viewed as a type of reductive corrosion which eventually impedes electron transfer from the cells to the oxide surface. When a small portion of the 6-month-old batch cultures was inoculated into fresh synthetic goethite-containing medium at the conclusion of the experiments, Fe(III) reduction activity resumed (data not shown), which suggests that the loss of reduction activity in the batch cultures could not be attributed to the death of the FeRB populations. Recent studies indicate that the simple presence of high concentrations of aqueous Fe(II)

does not inhibit Fe(III) reductase activity of FeRB (unpublished data). These data support the idea that it is the formation of solid Fe(II) phases in the zone of FeRB-oxide contact that stops the reduction process.

If the observed relationship between solid and aqueous Fe(II) accumulation in the batch cultures (Fig. 2) is interpreted as a simple linear sorption isotherm (where sorption indicates both adsorption and surface Fe(II) precipitation reactions), then it is possible to view advective aqueous-phase flux as a mechanism which moves the reaction system down the sorption isotherm, thereby holding the abundance of surface-bound Fe(II) at a level low enough for oxide reduction to remain favorable. This conceptual model provides a mechanistic explanation for how near-complete reductive dissolution of crystalline Fe(III) oxide phases could be achieved in the landfill leachate-contaminated aquifer investigated by Heron and Christensen (5). Sustained aqueous-phase transport, together with the potentially accelerating influence of Fe(II)-complexing agents (27) in the leachate, is likely to have maintained a pool of microbially reducible Fe(III) which was eventually exhausted during the oxidation of organic carbon compounds in the leachate.

Bacterial cell growth is recognized as an important parameter which regulates the advective transport of bacteria in saturated porous medium (4, 17). Particularly relevant in this regard is the process in which an attached cell gives rise to a mobile daughter cell that is free to migrate some distance before becoming attached (8). Our results indicate that advective Fe(II) removal during aqueous-phase flow promoted FeRB growth (see above), which in turn led to major cell export from the column. This effect suggests a previously unrecognized mechanism whereby water flow could enhance FeRB movement in the subsurface beyond its obvious role in advective transport.

In summary, our findings document an interaction between aqueous phase transport and surface chemical reactions at the bacterium-mineral interface which has fundamental implica-

tions for control of the rate and extent of Fe(III) oxide reduction, as well as FeRB growth and transport, in subsurface sediments. Simulation model results (20) suggest that short-term laboratory experiments such as those presented here may provide a reasonable indication of how Fe(III) oxide reduction could respond to advective Fe(II) removal over much greater periods of time in natural aquifer environments, which typically have much longer residence times and slower rates of metabolic activity than those in our experimental reactors. Consideration of the impact of aqueous phase flux on FeRB metabolism will be important for predicting the influence of metal-reducing bacteria on the fate and transport of metal and radionuclide contaminants in the subsurface. Immediate examples include the efficacy with which the FeRB may serve as agents for liberation of sorbed or coprecipitated contaminant metals through reductive dissolution processes designed to complement pump-and-treat restoration (2), for reductive immobilization and/or retardation of redox-sensitive metals and radionuclides such as Cr(IV), ⁶⁰Co(III), and ²³⁸U(IV) (11), or for hydrocarbon degradation in petroleum-contaminated aquifers (12, 13). In all cases, the coupling between stimulation of oxide reduction activity through advective Fe(II) removal and cell growth-promoted bacterial transport is likely to figure prominently in considerations of the spatial and temporal scales on which subsurface bioremediation strategies involving metal-reducing bacteria may be effectively implemented.

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