Environ. Sci. Technol. **1999**, *33,* 1847−1853

Ferrous Iron Removal Promotes Microbial Reduction of Crystalline Iron(III) Oxides

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Semicontinuous cultures were used to assess the effect of aqueous Fe(II) removal on the dissimilatory reduction of crystalline Fe(III) oxides by *Shewanella alga* strain BrY. Aqueous phase replacement in semicontinuous cultures (average residence time of 9 or 18 days) resulted in a 2−3-fold increase in the cumulative amount of Fe(II) produced from synthetic goethite reduction over a 2-month incubation period, compared to parallel batch cultures. A more modest (maximum 30%) but significant stimulation of natural subsoil Fe(III) oxide reduction was observed. The extended Fe(III) reduction resulted from enhanced generation of aqueous Fe(II) which was periodically removed from the cultures. A concomitant stimulation of bacterial protein production was detected, which suggested that Fe(II) removal also promoted bacterial growth. A simulation model in which Fe(II) sorption to the solid-phase resulted in blockage of surface reduction sites captured the contrasting behavior of the batch vs semicontinuous Gt reduction systems. Our findings indicate that elimination of Fe(II) via advective transport could play a significant role in governing the rate and extent of microbial Fe(III) oxide reduction in sedimentary environments.

Introduction

Surface chemical reactions involving Fe(III) oxides play a central role in the geochemistry of soil and sedimentary environments (*1*). One such reaction with broad environmental significance is the reduction of Fe(III) oxides coupled to the oxidation of organic matter under anaerobic conditions (*2, 3*). In addition to contributing to the oxidation of sedimentary organic matter and generating soluble ferrous iron (a widespread groundwater contaminant), microbial Fe(III) oxide reduction can have an important influence on the persistence and mobility of various metal, radionuclide, and organic contaminants (*2, 4, 5*).

Microbial Fe(III) oxide reduction may also play an important role in facilitating the reductive immobilization of certain metal and radionuclide species (e.g. Cr, U), either by supporting the growth and maintenance of metal-reducing bacterial populations which can enzymatically reduce soluble, oxidized forms of these metals to insoluble reduced phases (*6*), or in the case of Cr by producing aqueous and/or solid-phase Fe(II) which can abiotically reduce Cr(VI) to insoluble Cr(III) oxides (*7*). Because perpetuation of such contaminant metal reduction processes will depend on the presence of active metal-reducing bacterial populations, the ongoing capacity for endogenous Fe(III) oxides to serve as the primary source of electron acceptor required for cell growth and maintenance becomes an important issue with regard to contaminant fate and transport. Oxidized contaminant metals may often not be present at concentrations sufficient to support continued cell growth, and in some cases bacterial growth with contaminant metals may not be possible for physiological reasons (*6*).

The above considerations suggest the need for information on how geochemical parameters influence the long-term potential for Fe(III) oxide reduction and maintenance of metal-reducing bacterial populations in anaerobic soils and sediments. Recent studies with the dissimilatory FeRB *Shewanella alga* suggest that adsorption and/or precipitation of Fe(II) compounds on Fe(III) oxide and FeRB cell surfaces (hereafter referred to collectively as Fe(II) sorption) is responsible for the cessation of Fe(III) oxide reduction activity in batch culture experiments (*8, 9*). Subsequent work has shown that aqueous Fe(II) ligands such as dissolved inorganic carbon or nitrilotriacetic acid as well as solid-phase components such as Al_2O_3 oxides or clay minerals can enhance the long-term extent of synthetic goethite reduction by *S. alga* by delaying or retarding the accumulation of Fe(II) on oxide and cell surfaces (*10*). These findings suggest the possibility that removal of Fe(II) by advective transport (e.g. via groundwater flow or recharge of shallow aquifers) could play an important role in governing the rate and extent of microbial Fe(III) oxide reduction in subsurface sedimentary environments. In the present study, we used semicontinuous cultures to test the hypothesis that advective Fe(II) removal can promote microbial reduction of natural and synthetic crystalline Fe(III) oxides by retarding the accumulation of sorbed Fe(II). We also tested whether aqueous Fe(II) complexants (synthetic chelators, humic acid) could further enhance the long-term extent of oxide reduction by accelerating removal of Fe(II).

Materials and Methods

Organism and Culturing Procedures. The dissimilatory Fe(III)-reducing bacterium *Shewanella alga* strain BrY (*11, 12*) was used as a test organism. Batch and semicontinuous cultures (SCs) were initiated in 60 mL serum bottles with 20−50 mmol Fe(III) L^{-1} in the form of synthetic goethite (Gt) or Fe(III) oxide-rich subsoil. The basal culture medium (30 mL/bottle) contained 10 mM Pipes buffer (pH 6.8), 18 mM NH_4Cl, and a low level of PO_4^{3-} (0.044 mM) compared to previous media used to cultivate Fe(III)-reducing bacteria (*8, 13*). Carbon and energy were provided by 8 mM malate and 30 mL of H_2 (10 mL overpressure) in the bottle headspace, respectively. Although malate has the capacity to complex Fe(II) and thereby influence the outcome of microbial Fe(III) oxide reduction experiments, previous studies have demonstrated that its presence at a concentration of 8 mM does not have a significant impact on the long-term extent of synthetic Gt reduction (*9*) or on Fe(II) sorption to Gt (Urrutia, M. unpublished). In one experiment, 30 mM lactate served as a combined carbon and energy source. All cultures were inoculated with washed (10 mM Pipes, pH 6.8) *S. alga* cells that had been grown to late exponential phase (16 h) in aerobic tryptic soy broth medium. The cells were added to a final cell density of ca. 10^8 mL^{-1}, equivalent to ca. 40 mg protein L^{-1}.

Fe(III) reduction was allowed to proceed for 6−7 days before medium replacement commenced in the SCs. After that, mean residence times of 18 or 9 days were established

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10.1021/es9809859 CCC: $18.00
Published on Web 04/21/1999

TABLE 1. Characteristics of Fe(III) Oxide Materials

material	Fe(III) oxide content[a] (μmol/g dry wt)	surface area[b] (m^2/g)	Fe(II) sorption[c] sorption capacity[d] (μmol Fe^{II}/g dry wt)	Fe(II) sorption[c] sorption capacity[d] (μmol Fe^{II}/μmol Fe^{III})	Freundlich K[e]
synthetic Gt	1.79×10^4	55	0.25	0.020	0.62
CP subsoil	3.49×10^2	29	0.03	0.066	0.85
HC subsoil	6.73×10^2	41	>0.05	0.085	2.64

[a] Determined by citrate/dithionite extraction. [b] Determined by BET N_2 adsorption (Micromeritics Model Gemini). [c] Determined using $FeCl_2 \cdot 2H_2O$ in 10 mM Pipes buffer (pH 6.8) as described in ref *9*; the mass of solids per unit volume liquid in the sorption experiments was identical to that in the Fe(III) reduction cultures. [d] Assessed visually from isotherm plots. [e] Estimated by nonlinear least-squares fitting of sorption data in units of mmol Fe(II) L^{-1} (sorbed vs aqueous) to the Freundlich equation (*27*).

by replacing either 5 or 10 mL of the aqueous phase of the cultures with fresh anaerobic culture medium every 3−4 days. Medium was removed with a sterile 4″ 18G stainless steel needle without disturbing the solid-phase materials settled at the bottom of the bottle. An aliquot of the removed medium was acidified with 0.5 M HCl for subsequent analysis of aqueous Fe(II) concentration. A volume of fresh sterile medium identical to that removed was then added to the bottles, after which the bottles were thoroughly mixed and samples taken for determination of total (0.5 M HCl-extractable) Fe(II) and in some cases protein content as described below. An amount of H_2 gas equal to the amount of medium removed was then added to bottle headspace. Batch cultures which received no medium replacement were sampled for HCl-extractable Fe(II) and soluble Fe(II) in parallel with the SCs. Between samplings, the cultures were incubated statically at 30 °C in the dark. At 2-week intervals, the rubber stoppers in all bottles were replaced (working inside an anaerobic chamber) with new sterile stoppers, and 30 mL of H_2 was added to the bottle headspace.

Fe(III) Oxide Materials. A synthetic goethite (Gt), prepared and characterized as previously described (*9*), was employed for most of the experiments. Two Fe(III) oxide-rich subsoils (<106 μm fraction) were also used (see Table 1): a Holston/Cloudland, Typic Fragiudult from Tennessee (80−120 cm depth; designated HC) and a Cecil/Pacolet, Typic Hapludult from North Carolina (40−90 cm depth; designated CP). The soils differ in their clay mineralogy, with the HC being dominated by 2:1 layer silicates and CP by kaolinite (*14*). Previous studies (*8*) have shown that these two soils contain substantial quantities of microbially reducible Fe(III), the vast majority of which (>95%) is in the form of crystalline Fe(III) oxides (primarily goethite and hematite, respectively).

Fe(II) Complexants. The influence of three different potential aqueous Fe(II) complexants on Gt reduction in batch and SCs was evaluated: nitrilotriacetic acid (NTA), ethylenediamine tetraacetic acid (EDTA), 0.5 mM final concentration, and International Humic Substance Society Soil Humic Acid Standard (SHA), 100 mg L^{-1} final concentration. The NTA and EDTA were added to sterile culture medium from anaerobic filter-sterilized stock solutions, whereas the SHA was dissolved in culture medium prior to autoclaving.

The choice of 0.5 mM for the chelator additions represents a compromise between the relatively low levels present in subsurface contaminant plumes (<1 μM; *15*) and the mM levels used in previous experimental studies of the influence of chelators on bacterial Fe(III) oxide reduction (*10*, *16*, *17*). The choice of 100 mg L^{-1} for the humic acid addition, while high relative to most sedimentary environments, is substantially lower than levels used in recent studies of the influence of humics on Fe(III) oxide reduction (*18*, *19*).

Calculation of Cumulative Fe(II) and Protein Production. Cumulative Fe(II) production in the SCs was calculated at each sampling point from the sum of HCl-extractable Fe(II) plus the amount of Fe(II) eliminated with the aqueous phase during the current and previous medium replacements. Cumulative protein production was computed in a similar manner. However, protein measurements were only conducted every fourth sampling (every 2 weeks). Therefore, for those sampling times for which no protein data were available, protein content was estimated from the average of measurements made at the beginning and end of the corresponding 2-week interval.

Analytical Techniques. HCl-extractable Fe(II) (aqueous + solid-phase) was determined by extracting 0.5 mL culture samples in 5 mL of 0.5 M HCl for 2 h and then determining the Fe(II) content of the extracts with Ferrozine (*20*). Aqueous Fe(II) concentrations were determined by analyzing aliquots of 0.2 μm-filtered sample with Ferrozine. The total Fe(III) oxide content of the subsoils was determined by citrate/dithionite extraction followed by Ferrozine analysis as described previously (*8*).

The protein content of whole and aqueous phase culture samples was determined by the bicinchoninic acid (BCA) method after NaOH digestion. Because of the potential for Fe(III) oxides to adsorb proteins, standards (bovine serum albumin) were prepared in the same medium as the culture samples. Samples and standards were digested in 2.5 M NaOH for 15 min at 100 °C. Following cooling and neutralization with 2.5 M HCl, duplicate 1-mL aliquots of extract were mixed with an equal volume of freshly prepared BCA reagent (Pierce Chemical Co.) and incubated for 1 h in a 60 °C water bath. The colored extract was then passed through a 0.2-μm nylon filter, and the A_{562} was determined within 15 min. Protein contents are expressed in mg protein L^{-1} culture.

Results and Discussion

Synthetic Goethite Reduction. Medium replacement in the SCs resulted in a 2−3-fold increase in the extent of Gt reduction compared to parallel batch cultures (Figure 1). A clear trend toward continuously increasing cumulative Fe(II) production was evident in the SCs, whereas Fe(II) production approached an asymptote in the batch cultures. The higher volume of medium replacement led to a greater stimulation of Fe(III) oxide reduction.

Concentrations of aqueous Fe(II) ($Fe(II)_{aq}$) were slightly higher (0.1−0.2 mM) in the SC vs batch cultures. Although this may seem counterintuitive since $Fe(II)_{aq}$ was removed from the SCs during medium replacement, $Fe(II)_{aq}$ removal allowed for perpetuation of Fe(III) oxide reduction activity so that $Fe(II)_{aq}$ reaccumulated between dates of replacement. In contrast, concentrations of total (solid + aqueous) HCl-extractable Fe(II) were lower in the SC vs batch Gt cultures, by 50−100% toward the end of the experiments (see Table 2). This difference reflected primarily a decrease in solid-phase (sorbed) Fe(II). The extended Fe(III) reduction in the SCs was thus accounted for entirely by the $Fe(II)_{aq}$ removed during medium replacement. $Fe(II)_{aq}$ production accounted for 80−93% of cumulative Fe(II) production in the SCs, compared to ca. 25% in batch cultures (Table 2). The higher

TABLE 2. Fe(II) and Protein Production in Batch and Semicontinuous Cultures

medium	culture[a]	final HCl-Fe(II) (mmol L^{-1})[b,c]	cumulative Fe(II) (mmol L^{-1})[b]	% cumulative Fe(II) as Fe(II)aq[b]	cumulative protein (mg L^{-1})[b]
Gt, H_2/Mal	batch	1.27 ± 0.10	1.27 ± 0.10	23.48 ± 9.47	50.57 ± 2.56
	5 mL	0.84 ± 0.05	2.13 ± 0.04	81.57 ± 2.60	117.45 ± 4.38
	10 mL	0.57 ± 0.15	2.98 ± 0.09	89.76 ± 4.62	200.77 ± 5.27
Gt, lactate	batch	1.78 ± 0.23	1.78 ± 0.23	48.78 ± 3.48	ND[d]
	5 mL	1.11 ± 0.05	3.14 ± 0.12	85.01 ± 0.34	ND
	10 mL	1.02 ± 0.28	4.29 ± 0.18	93.25 ± 8.73	ND
Gt, H_2/Mal + NTA	batch	1.51 ± 0.03	1.52 ± 0.01	30.13 ± 1.33	62.80 ± 4.77
	5 mL	0.80 ± 0.05	2.24 ± 0.02	77.18 ± 1.56	98.69 ± 5.43
	10 mL	0.51 ± 0.02	2.86 ± 0.17	75.60 ±1.79	146.29 ± 5.62
Gt, H_2/Mal + EDTA	batch	1.65 ± 0.10	1.65 ± 0.10	36.70 ± 4.02	ND
	5 mL	0.82 ± 0.06	2.50 ± 0.08	64.28 ± 0.64	ND
	10 mL	0.91 ± 0.07	4.41 ± 0.21	73.94 ± 3.69	ND
Gt, H_2/Mal + SHA	batch	1.76 ± 0.17	1.76 ± 0.17	73.12 ± 2.00	225.79 ± 19.76
	5 mL	1.12 ± 0.04	2.20 ± 0.05	75.03 ± 2.47	425.70 ± 59.02
	10 mL	0.98 ± 0.16	2.93 ± 0.15	76.72 ± 4.23	708.03 ± 70.39
CP soil, H_2/Mal	batch	3.67 ± 0.20	3.67 ± 0.20	ND	ND
	5 mL	2.74 ± 0.19	4.39 ± 0.29	36.95 ± 3.23	ND
	10 mL	2.57 ± 0.03	5.11 ± 0.12	57.81 ± 6.92	ND
HC soil, H_2/Mal	batch	5.32 ± 0.38	5.32 ± 0.38	ND	ND
	5 mL	3.06 ± 0.35	4.74 ± 0.52	40.89 ± 1.94	ND
	10 mL	2.59 ± 0.18	5.27 ± 0.88	54.40 ± 4.14	ND

[a] Five milliliters and 10 mL refer to SCs receiving 5 and 10 mL medium replacements on each sampling date. [b] Mean ± SD of triplicate cultures. [c] HCl-extractable Fe(II) on the final sampling date; in batch systems, final HCl−Fe(II) equals cumulative Fe(II). [d] ND = not determined.

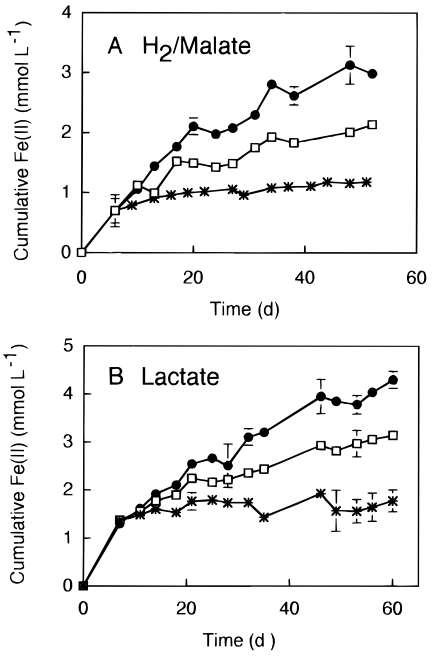

FIGURE 1. Cumulative Fe(II) (aqueous + solid-phase) production in batch (*) and semicontinuous synthetic Gt (50 mmol L^{-1}) cultures receiving 5 (□) or 10 (●) mL medium replacement every 3−4 days, with H_2/malate (A) or lactate (B) as the energy/carbon source. Medium replacement was carried out just prior to sampling the cultures for 0.5 M HCl-extractable Fe(II) content. Error bars show ± 1 SD of triplicate cultures; bars not visible are smaller than symbol.

proportion of aqueous to total Fe(II) production and lower concentration of sorbed Fe(II) in the SCs suggest that medium replacement enhanced Fe(III) reduction by altering the equilibrium between dissolved and "sorbed" Fe(II) such that the inhibitory effect of Fe(II) sorption on oxide reduction was reduced. This intepretation is consistent with previous studies which have demonstrated that Fe(II) sorption onto oxide and Fe(III)-reducing bacterial cell surfaces is the primary cause for cessation of Fe(III) oxide reduction in batch cultures (*9*). Recent experiments on soluble Fe(III)-citrate reduction by *S. alga* have verified that it is the association of Fe(II) with oxide and FeRB cell surfaces—rather than feedback inhibition of the reductase enzyme system by Fe(II)—that is primarily responsible for the cessation of Fe(III) reduction (*21*).

The possibility existed that periodic renewal of energy, carbon, or nutrients— as opposed to Fe(II) elimination— could have contributed to the enhanced Fe(III) reduction activity in the SCs. Of all the substrates that may have been limiting, phosphate (present at 0.044 mM in the culture medium) was the most logical possibility, given that both nitrogen, carbon, and energy were present in vast excess. However, a separate batch culture experiment in which phosphate was added (in an amount equivalent to that added during the 10-mL replacement in the SCs) every 3−4 days indicated that additional phosphate did not lead to an increase in synthetic Gt reduction (data not shown). This result supports the interpretation that removal of Fe(II), rather than nutrient renewal, was responsible for stimulation of Gt reduction in the SCs. To our knowledge these findings represent the first experimental demonstration that advective Fe(II) removal can promote microbial Fe(III) oxide reduction.

Subsoil Fe(III) Oxide Reduction. Medium replacement increased the extent of Fe(III) oxide reduction in the CP subsoil cultures (Figure 2), although not to the same degree observed in the synthetic Gt cultures. In contrast, no significant stimulation of reduction was detected in the HC subsoil cultures. The lesser stimulatory effect of medium replacement on subsoil Fe(III) oxide reduction vs synthetic Gt reduction may be related to the ability of clay minerals in the soils to bind Fe(II) and thereby function as Fe(II) sinks which "compete" with aqueous phase replacement as a mechanism for attenuating Fe(II) accumulation on Fe(III) oxide and FeRB cell surfaces. In other words, the effect of medium replacement was less because the clay minerals themselves acted as effective sinks for evolved Fe(II). This interpretation is consistent with (i) the Fe(II) sorption capacity of the subsoils, which when normalized to Fe(III) oxide content is 3−4 times higher than the synthetic Gt (Table 1), and (ii) our recent finding that layer silicates can act as Fe(II) sinks which enhance the long-term extent of synthetic Gt reduction (*10*). By the same line of reasoning, the fact that medium replacement stimulated reduction of CP more than

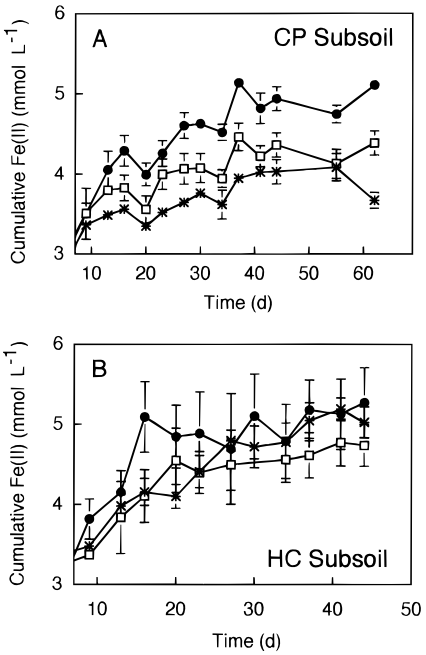

FIGURE 2. Cumulative Fe(II) (aqueous + solid-phase) production in batch and semicontinuous cultures containing CP (25 mmol Fe(III) L^{-1}) or HC (40 mmol Fe(III) L^{-1}) subsoils. Symbols as in Figure 1. Error bars show SD of triplicate cultures; bars not visible are smaller than symbol. *x* and *y* axes start at nonzero values in order to emphasize differences between treatments.

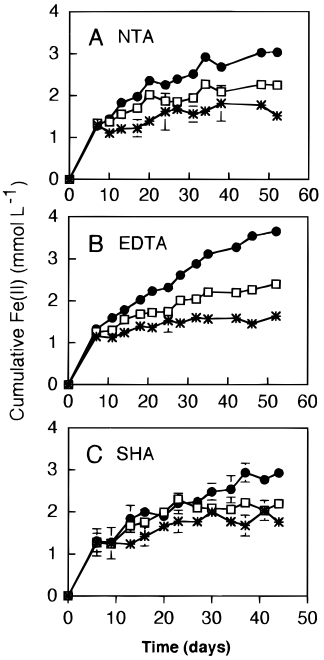

FIGURE 3. Cumulative Fe(II) (aqueous + solid-phase) production in batch and semicontinuous synthetic Gt (50 mmol L^{-1}) cultures amended with 0.5 mM NTA (A), 0.5 mM EDTA (B), or 100 mg L^{-1} IHSS Soil Humic Acid (C). Symbols as in Figure 1. Error bars show ± 1 SD of triplicate cultures; bars not visible are smaller than symbol.

HC subsoil can likely be attributed to HC's greater Fe(II) sorption capacity and higher affinity for Fe(II) (as indicated by Freundlich isotherm *K* values; see Table 1), since more effective Fe(II) binding would be expected to attenuate the influence of advective Fe(II) removal on oxide reduction. Our results do not necessarily mean that advective Fe(II) removal is not expected to promote the long-term extent of Fe(III) oxide in clay-rich sediments, but rather that the effect might take longer to manifest (e.g. after the onset of anaerobic conditions) than in clay-poor sediments due to the ability of the clay minerals to serve as alternative Fe(II) sinks.

Influence of Aqueous Fe(II) Complexants. Aqueous Fe ligands (e.g. NTA, EDTA) have been shown to stimulate microbial Fe(III) oxide reduction by (i) complexing and dissolving Fe(III) from the oxide surface (*10, 16, 17*), and (ii) complexing biogenic Fe(II), thereby retarding Fe(II) sorption to oxide and FeRB cell surfaces (*10*). An additional hypothesis of this study was that periodic renewal of fresh (i.e. non-Fe-complexed) chelator would enhance the stimulatory effect of medium replacement on Fe(III) oxide reduction in SCs by one or both of these mechanisms. In the case of humics, we anticipated that both Fe(II) complexation (*22*) as well as electron shuttling from quinone functional groups (*18, 19*) could enhance oxide reduction and that periodic influx of fresh humics would amplify these effects. In general, our results did not support these expectations. The presence of 0.5 mM NTA, 0.5 mM EDTA, or 100 mg L^{-1} SHA did not substantially increase the stimulatory effect of 5 or 10 mL medium replacements (Figures 1 and 3; Table 2), nor did they significantly increase the fraction of cumulative Fe(II) production accounted for by aqueous Fe(II) in the SCs (Table 2). These results suggest that the periodic removal of the Fe(II)-rich aqueous phase altered the equilibrium between sorbed (which inhibits reduction) and aqueous Fe(II) enough so that no major effect of the chelators was observed.

The presence of 0.5 mM NTA or EDTA did lead to a minor increase in the long-term extent of Gt reduction in batch cultures with H_2/malate as an energy/carbon source (Table 2). This result agrees with previous findings which demonstrated that NTA and (to a lesser extent) EDTA enhance the extent of Gt reduction by complexing evolved Fe(II) (*10*). Batch cultures containing soil humic acid produced ca. 50% more Fe(II) than their humic-free counterparts. Although this finding may indicate that the quinone electron shuttle mechanism stimulated Fe(III) reduction, it is not possible to assess whether the observed effect was due to electron shuttling or Fe(II) complexation by the humics. The latter explanation is favored by higher ratio of aqueous to total Fe(II) in the SHA cultures (Table 2). In any case, the stimulation of Gt reduction was not nearly so profound as that observed in experiments with much higher (20-fold) humic concentrations (*19*).

Bacterial Growth. Measurements of bacterial protein content suggested that FeRB cell growth was stimulated in the SCs (Figure 4). A substantial portion (40−50%) of bacterial biomass was present in the aqueous phase of the SCs, i.e., not attached to the Fe(III) oxide. Hence, removal of culture medium resulted in removal of bacterial biomass. This biomass was consistently replaced by bacterial growth in the SCs. In contrast, only minor increases in protein were observed in the batch cultures during the course of the experiments. While the lack of major protein production in the batch cultures does not necessarily mean that no net growth occurred (it is possible that some cells died while other cells underwent reproduction, leading to a constant level of protein, or that the protein content of dead cells was not completely degraded over the time scale of the experiments), the key result is that much greater cumulative protein production occurred in the SC vs batch cultures (Figure 4). Given that energy/nutrient renewal was not likely responsible for stimulation of Fe(III) oxide reduction in the SCs (see above), we conclude that Fe(II) removal led to a systematic stimulation of FeRB growth coupled to Gt reduction in the SCs. Because the standing stock of protein at any given time in the SCs was consistently lower than that in batch cultures, it is clear that the FeRB cells in SCs were considerably more active, in terms of Fe(III) reduction and growth, than those in the batch cultures.

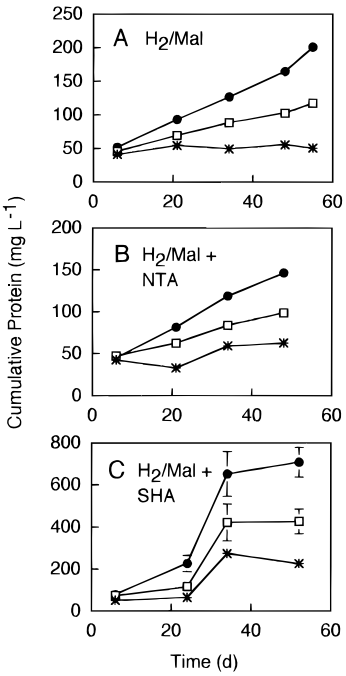

FIGURE 4. Cumulative protein production in batch and semicontinuous synthetic Gt cultures. Symbols as in Figure 1. Panels A−C show results of cultures with H_2/malate alone, H_2/malate + 0.5 mM NTA, and H_2/malate + 100 mg L^{-1} SHA, respectively. Error bars show ± 1 SD of triplicate cultures; bars not visible are smaller than symbol. Note difference in scale for panel C.

The presence of 0.5 mM NTA in the culture medium did not stimulate the production of bacterial cell protein in either batch cultures or SCs (Figure 4 and Table 2). However, the soil humic acids stimulated a 4-fold increase in protein in the batch cultures, and cumulative protein production in the soil humic SCs was 2−4-fold higher than in no-humic cultures (Figure 4). It is interesting that the humics stimulated protein production so much, given that the difference in total Fe(II) production was not nearly so dramatic (Table 2). As a result of this phenomenon, the ratio of protein to Fe(II) production was 3−4-fold higher in the humics-containing than in the no-humics cultures. The stimulation of cell growth by the humics was likely due to their use as an electron acceptor. This explanation is consistent with the previous demonstration that *S. alga* can grow with humic substances as the sole electron acceptor (*18*). While some of the electrons transferred to the humics were undoubtedly shuttled to the Gt (thereby regenerating oxidized humics available once again for enzymatic reduction), it appears that this humic recycling mechanism (*19*) was not a major factor in our experiments—given that cumulative Fe(II) production was not stimulated in proportion to the vastly increased cell growth in the humics-containing cultures.

Simulation Model. A simulation model was developed to illustrate how sorption of Fe(II) can limit the long-term extent of oxide reduction and how advective removal of aqueous Fe(II) could stimulate Fe(III) oxide reduction by retarding the accumulation of sorbed Fe(II). Our intention was to develop a simple model that could serve as a basis for future development of more sophisticated mechanistic models of bacterial Fe(III) oxide reduction.

(a) Model Design. The model depicts production and speciation of Fe(II) during microbial reduction of synthetic Gt in a batch vs "open" reactor system. A dilution rate constant of 0.1 d^{-1} was used to simulate advective Fe(II) removal in the open reactor system. Bulk Fe(II) production was governed by a first-order rate equation in which Fe(III) reduction is dependent on the concentration of free Fe(III)

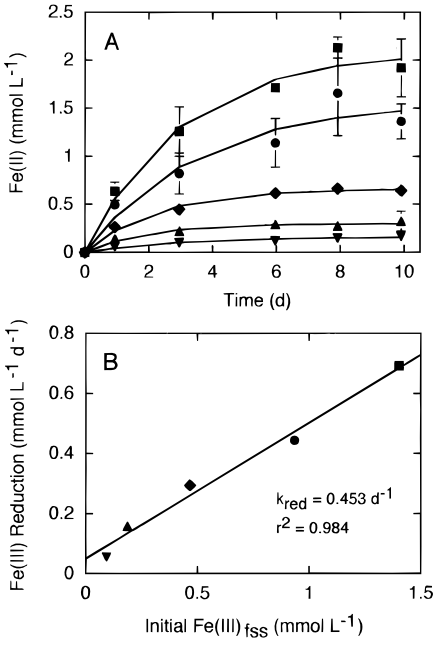

FIGURE 5. Kinetics of synthetic Gt reduction by *S. alga*. Panel A: time course of Gt reduction with a range of starting Fe(III) concentrations: ▼, 5 mmol L^{-1}; ▲, 10 mmol L^{-1}; ◆, 25 mmol L^{-1}; ●, 50 mmol L^{-1}; and ■, 75 mmol L^{-1}. Error bars show ± 1 SD of triplicate cultures; bars not visible are smaller than symbol. Solid lines are nonlinear least-squares regression fits of the data to the following equation which depicts Fe(II) accumulation from first-order consumption of a finite number of "free" surface Fe(III) reduction sites: $Fe(II) = Fe(III)_{fss}^0[1 - \exp(-k_{red}t)]$, where Fe(II) is the concentration of Fe(II) at time t, $Fe(III)_{fss}^0$ is the initial concentration of free surface sites, and k_{red} is a first-order rate constant. Both $Fe(III)_{fss}^0$ and k_{red} were allowed to vary in the curve-fitting procedure. Panel B: Initial Fe(III) reduction rate vs initial free Fe(III) surface site concentration (see text). Initial Fe(III) reduction rates were computed from the first-derivative of the nonlinear regression fits in panel A evaluated at $t = 0$.

oxide surface reduction sites ($Fe(III)_{fss}$):

$$\frac{dFe(II)}{dt} = k_{red}Fe(III)_{fss}$$

This formulation assumes that Fe(III) reduction was independent of electron donor and nutrient concentrations, in accordance with the excess of these substrates in our culture systems. The first-order reduction rate constant k_{red} was estimated from initial rates of synthetic Gt reduction as a function of oxide concentration in batch culture experiments identical to those run in parallel with the SCs (Figure 5). The estimated value of k_{red} (0.453 d^{-1}) was similar to the average (0.387 ± 0.099 d^{-1}; $n = 5$) of the first-order rate constants determined from curve fits of Fe(II) concentration vs time to an equation depicting the accumulation of end-product from a first-order reaction acting on a finite reservoir of surface reduction sites (Figure 5A). The fact that both these approaches as well as previous studies of Fe(III) oxide reduction kinetics (*8*) support a first-order rate model for Gt reduction suggests that use of this rate model is a reasonable way to depict the dependence of oxide reduction rate on oxide surface site concentration in our experimental systems. Although it may have been possible to base the model on growth of the FeRB population using recently determined growth rate and biomass yield parameters for *S. alga* in batch culture systems identical to those employed in this study (*23*), we do not yet know how FeRB growth rates vary as a function of Fe(III) oxide surface site concentration or the abundance of Fe(II) sorbed to oxide and/or cell surfaces.

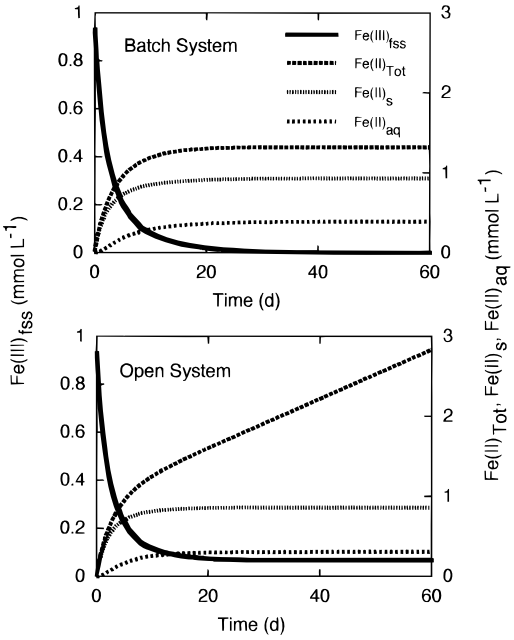

FIGURE 6. Simulation model results for batch and open (dilution rate of 0.1 d^{-1}) reactor systems. $Fe(III)_{fss}$ refers to "free" Fe(III) oxide surface sites (see text); $Fe(II)_{aq}$ and $Fe(II)_s$ refer to aqueous and sorbed Fe(II), respectively; $Fe(II)_{tot}$ refers to the cumulative amount of Fe(II) produced.

Thus inclusion of cell growth in the model is unwarranted at this time.

The initial abundance of Fe(III) oxide surface sites (0.93 mmol L^{-1}) was chosen in accordance with an assumed molar Fe(III) concentration of 50 mmol L^{-1}, the measured surface area of the oxide (55 m^2 g^{-1}), and the standard surface site density of 2.3 sites nm^{-2} recommended in ref *24*. Bulk Fe(II) was allowed to undergo equilibrium sorption to Fe(III) surface sites according to a Freundlich equation fit to Fe(II) sorption data for a mixture of 50 mmol L^{-1} Gt and a standard inoculum (same as that used in this study) of *S. alga* cells in Pipes-buffered growth medium (*9*).

To link the sorption of Fe(II) to the suppression of Fe(III) oxide reduction, Fe(III) surface sites occupied by sorbed Fe(II) were assumed to be unavailable for microbial reduction; in other words, a surface site with sorbed Fe(II) is no longer "free". This approach assumed that a sorbed Fe(II) occupies a single sorption site. It is important to note that because FeRB cells contribute significantly to the Fe(II) sorption capacity of our synthetic Gt culture systems (*9*), it may be appropriate to interpret the deactivation of Fe(III) reduction via Fe(II) sorption as the combined effect of sorption to binding sites on both FeRB cell and Fe(III) oxide surfaces—even though for the sake of simplicity the initial population of free reduction sites was defined only by those on the Gt surface.

The model was solved numerically over a 60-day simulation period using a stiff ODE solver algorithm described in ref *25*. Equilibrium sorption of Fe(II) onto Fe(III) surface sites was computed with a Newton–Raphson routine which operated within the ODE solver.

(b) Model Results. The model reproduced the basic trends observed in batch vs semicontinuous synthetic Gt reduction systems (Figure 6). Accumulation of sorbed Fe(II) ($Fe(II)_s$) in the batch system led to rapid blockage of free reduction sites, which in turn resulted in the cessation of Fe(III) reduction a few weeks into the simulation; this result is consistent with the batch culture data shown in Figures 1, 3, and 5. Advective removal of Fe(II) in the open system (residence time = 10 days) led to maintenance of a low but significant concentration of free reduction sites over time, which in turn allowed for continued Fe(III) reduction, resulting in a ca. 2-fold increase (relative to the batch system) in the total amount of Fe(II) produced during the 2-month simulation; this result is consistent with the SC data in Figures 1 and 3.

The basic agreement between the model and experimental results indicates that depicting inhibition of Fe(III) reduction through Fe(II) sorption is an appropriate first step toward modeling controls on the long-term extent of microbial Fe(III) oxide reduction. The success of this simple model suggests that pursuit of geochemically more sophisticated models (e.g. ones which include aqueous phase complexation, bulk-phase precipitation, surface precipitation, and Fe(II) sorption to non-oxide surface sites—including FeRB cells) will be justified when appropriate experimental data and modeling approaches become available. Other important steps will include incorporation of information on FeRB growth rate and biomass production as a function of Fe(III) oxide surface chemistry and incorporation of a mechanism for depletion of bulk Fe(III) oxide content in concert with the reductive dissolution of Fe(III) oxide surface sites.

Environmental Implications. Our results indicate that elimination of Fe(II) via aqueous phase transport could play a significant role in governing the rate and extent of microbial Fe(III) oxide reduction in sedimentary environments. These findings support the previous speculation (*8*, *9*) that advective Fe(II) removal was involved in facilitating the near-complete consumption of crystalline Fe(III) oxides within a shallow subsurface landfill leachate plume in Denmark (*26*). However, because the mean residence times in our SCs were probably considerably shorter than those in most subsurface environments and the rates of metabolic activity relatively high, quantitative extrapolation of our results to in situ subsurface conditions is difficult. Nevertheless, it is interesting to note that longer (600-day) model simulations using a 100-day residence time, and 10-fold lower k_{red} value, yielded total Fe(II) production values similar to those obtained in the 60-day simulation with a 10-day residence time and the k_{red} value for conditions of excess electron donor and nutrients. This agreement suggests that our 2-month experiments may give a reasonable impression of how crystalline Fe(III) oxide reduction might respond to advective Fe(II) removal over much longer periods of time in subsurface systems with slower rates of advective transport and metabolic activity. Experiments in reactor systems which more closely approximate in situ subsurface conditions will be required to evaluate this hypothesis.

In summary, the interaction between transport and Fe(II) sorption is likely to have a significant impact on the persistence of Fe(III) oxide reduction activity in subsurface environments. Of course, other geochemical interactions (in particular competitive sorption of Fe(II) and other cations and organics) may modify the influence of advection on oxide reduction. Such interactions may be a matter of significant concern in situations where the activity of FeRB is exploited for remediation of organic or metal/radionuclide contaminants.

Acknowledgments

This research was funded by Grant DE-FG07-96ER62321 from the U.S. Department of Energy, Office of Energy Research, Environmental Management Science Program. We thank Ginger Scott for technical assistance.

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Received for review September 23, 1998. Revised manuscript received February 23, 1999. Accepted March 9, 1999.

ES9809859