Biogeochemical Elimination of Chromium (VI) from Contaminated Water

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Abstract: Ferrous iron [Fe(II)] reductively transforms heavy metals in contaminated groundwater, and the bacterial reduction of indigenous ferric iron [Fe(III)] to Fe(II) has been proposed as a means of establishing redox reactive barriers in the subsurface. The reduction of Fe(III) to Fe(II) can be accomplished by stimulation of indigenous dissimilatory metal-reducing bacteria (DMRB) or injection of DMRB into the subsurface. The microbially produced Fe(II) can chemically react with contaminants such as Cr(VI) to form insoluble Cr(III) precipitates. The DMRB *Shewanella algae* BrY reduced surface-associated Fe(III) to Fe(II), which in batch and column experiments chemically reduced highly soluble Cr(VI) to insoluble Cr(III). Once the chemical Cr(VI) reduction capacity of the Fe(II)/Fe(III) couple in the experimental systems was exhausted, the addition of *S. algae* BrY allowed for the repeated reduction of Fe(III) to Fe(II), which again reduced Cr(VI) to Cr(III). The research presented herein indicates that a biological process using DMRB allows the establishment of a biogeochemical cycle that facilitates chromium precipitation. Such a system could provide a means for establishing and maintaining remedial redox reactive zones in Fe(III)-bearing subsurface environments.

Key Words: metal reduction, chromium, bacteria, bioremediation, biobarrier, iron, permeable reactive barrier.

Introduction

Chromium(VI) compounds have been used extensively in the manufacture of alloys, the electroplating industry, the manufacture of dyes and pigments, the preservation of wood, in the leather tanning industry, and as corrosion inhibitors in conventional and nuclear power plants (Hayes, 1997; Langard, 1980). Due to leakage, poor storage, and improper disposal practices, Cr(VI) has become one of the most frequently detected contaminants at hazardous waste sites (National Research Council, 1994; Watts, 1997). The spills are often extensive, and the frequent presence of co-contaminants such as chlorinated aliphatics, radionuclides, metals, and organic solvents makes Cr(VI) remediation an environmental challenge (James, 1994; Pagilla and Canter, 1999; Watts, 1997).

Cr(VI) is highly soluble and therefore easily transported with ground water (Bartlett and James, 1988). It is toxic and mutagenic to humans and other organisms (Committee on Biologic Effects of Atmospheric Pollutants, 1974; Gibb, 1999; Keith and Telliard, 1979; Watts, 1997). In its reduced trivalent state [Cr(III)], chromium forms insoluble hydroxides (Hug and Laubscher, 1997; Richard and Bourg, 1991) and remains immobile under most environmentally relevant pH and redox conditions (Blowes *et al.*, 1997; James, 1996; Lantz, 1992; Pohland *et al.*, 1993; Richard and Bourg, 1991). Cr(III) is less toxic than Cr(VI) and is neither readily taken up by organisms or plants nor

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transferred through the food chain (Hayes, 1997; National Research Council, 1994).

Traditional remediation technologies such as excavation with subsequent treatment or pump and treat have often proven to be expensive and can create a long-term liability for the responsible party (Mackay and Cherry, 1989; Seaman *et al.*, 1999). Technologies to treat large volumes of soils and groundwater inexpensively are lacking. Passive treatment systems, such as subsurface barriers, offer an alternative to traditional cleanup technologies because they minimize the need for expensive operating equipment and human exposure to contaminants. Treatment systems relying on geochemical processes have attracted increased attention in recent years.

Permeable reactive subsurface barriers made from iron metal have been used successfully for the elimination of a variety of contaminants, including chlorinated solvents (O'Hannesin and Gillham, 1998), heavy metals (Cantrell *et al.*, 1995; Gould, 1982), and radionuclides (Fiedor *et al.*, 1998). The iron metal is usually introduced into the aquifer using trench and fill methods. However, the installation of trenches can become uneconomical at depths of more than approximately 12 m (Fortner, 1995; Scott *et al.*, 1998). Thus, alternative technologies allowing for the establishment of redox reactive zones in the subsurface have been proposed and implemented in the field. These technologies involve the injection of small Fe(0) particles either under high pressure or after hydraulic or pneumatic fracturing of the aquifer (Cantrell *et al.*, 1997; Landis and Vidumsky, 1999).

In situ redox manipulation has also been suggested for sites where trench and fill methods are impractical (Seaman *et al.*, 1999). Cr(VI) can be reduced by ferrous iron, sulfides, and natural organic compounds (Wittbrodt and Palmer, 1996). In several field studies, the reduction of Cr(VI) has been attributed specifically to the presence of ferrous iron (Anderson *et al.*, 1994; Henderson, 1994; Pagilla and Canter, 1999; Patterson *et al.*, 1997). Industrially, the addition of ferrous iron as a reductant represents a common treatment scheme for Cr(VI) contaminated water (Eary and Rai, 1988). Additionally, the use of ferrous iron in subsurface barriers for the remediation of chromium has been proposed (Blowes et al., 1997; Powell *et al.*, 1995). During a field demonstration at the Department of Energy (DOE) site in Hanford (Washington), an inorganic reductant (sodium dithionite, $Na_2S_2O_4$) was injected into the aquifer and allowed to reduce indigenous Fe(III) minerals to Fe(II) (Scott *et al.*, 1998). The dithionite-containing carbonate-bicarbonate buffer (pH 11) was injected into the subsurface and reduced between 60 and 100% of the Fe(III) present in the Hanford sediments. The resulting reductive capacity of the reduced aquifer material was calculated to be sufficient for the treatment of 51 to 85 pore volumes (or 7 to 12 years) of groundwater at 1 ppm Cr(VI) and 9 ppm dissolved oxygen (Scott *et al.*, 1998).

Dissimilatory Metal-Reducing Bacteria (DMRB) have been found to use a wide range of iron minerals as electron acceptors at ambient groundwater pH (Caccavo, Jr. *et al.*, 1992; Fredrickson and Gorby, 1996; Roden and Zachara, 1996). DMRB are widely distributed in both pristine and contaminated terrestrial, aquatic, and subsurface environments (Lovley, 1995). They gain energy for growth by coupling the oxidation of organics or H_2 to the dissimilatory reduction of Fe(III) minerals and other metals.

Amonette *et al.* (2000) and Gorby *et al.* (1994) demonstrated that DMRB can reduce Fe(III) oxides, which can in turn reduce carbon tetrachloride (CT) to chloroform (CF) and Cr(VI) to Cr(III) according to the following reactions:

$$2\ Fe^{2+} + CCl_4 + H^+ \leftrightarrow 2\ Fe^{3+} + CHCl_3 + Cl^- \quad (1)$$

$$3\ Fe^{2+} + CrO_4^{2-} + 5\ H^+ \leftrightarrow 3\ Fe^{3+} + Cr(OH)_3\downarrow^* + H_2O \quad (2)$$

* Depending on the Fe(II)/Cr(VI) ratio and reaction time, reaction 2 will result in the precipitation of pure chromium-hydroxides or mixed iron-chromium-hydroxides with iron and chromium in different ratios (i.e., $Fe_xCr_{3-x}(OH)_3$, x=0,1,2,3) (Cary *et al.*, 1977; Powell *et al.*, 1995).

If DMRB can repeatedly generate surface reactive ferrous iron from indigenous iron minerals in the subsurface, they could potentially be used to establish *in situ* bioreactors or permeable reactive barriers. A viable remediation strategy based on this approach must provide continuous reduction and precipitation of chromium from contaminated groundwater. The continuous reduction of Cr(VI) relies on the renewal of Fe(II) through microbial reduction of the Fe(III) present. To our knowledge, researchers have yet to demonstrate that DMRB are able to repeatedly generate surface reactive Fe(II) for the chemical reduction of Cr(VI) to Cr(III) in the presence of iron-chromium precipitates. Therefore, we used laboratory-scale model systems to evaluate the potential for such a process.

Materials and Methods

Experimental

Test Organism and Culture Methods. *Shewanella algae* BrY (Caccavo, Jr. *et al.*, 1992; Rossello-Mora *et al.*, 1994) was used as the model DMRB in these

studies. *S. algae* BrY was maintained on tryptic soy agar (40 g/L, Difco Laboratories) at ambient temperature and grown to the late log, early stationary phase in tryptic soy broth (30 g/L, Difco Laboratories) at 150 rpm for 15 h at ambient temperature. Cultures were centrifuged at 5860 × g for 20 min at 4°C, washed twice in phosphate-buffered saline solution (PBS) to remove nutrients, and resuspended in the same buffer (Caccavo, Jr. *et al.*, 1996). *S. algae* BrY cells immediately after this treatment were defined as vegetative cells and were used after resuspension and dilution in oxygen-free minimal medium. Starved cells were obtained by aseptically stirring cell suspensions on magnetic stir plates at room temperature. Cells were harvested after 7 weeks of starvation at which point Fe(III) reductase activity was not detectable anymore under nongrowth conditions (Caccavo, Jr. *et al.*, 1996). Centrifugation and suspension in oxygen-free minimal medium served to remove cell debris from the starvation cultures. Starved cells were used in parts of this study because we have previously demonstrated that they are transported more effectively through porous media than vegetative cells and would therefore have an advantage in subsurface bioaugmentation with DMRB (Bouwer *et al.*, 2000).

Amorphous Iron Coating. Artificially iron-coated sand was obtained by soaking 200 g of quartz sand (40 mesh, Unimin Corp., Emmet, ID) for several days in twice its volume of 1 N HNO_3. Subsequently, it was washed with distilled water until the pH of the rinsewater exceeded 5.0 and mixed with 400 mL of a suspension of amorphous iron(III)oxyhydroxide (Lovley and Phillips, 1986). This suspension was prepared by dissolving 13.8 g $FeCl_3$ in 400 mL of deionized water and slowly adjusting the pH to 7.0 using NaOH. A layer of clear liquid formed on top of the suspension after the mixture of sand and iron(III)oxyhydroxide was briefly shaken and allowed to settle. The clear liquid was decanted after approximately 12 h of settling time and replaced with an equal volume of 0.1 m*M* NaCl at pH 7.0. The entire overlying suspension was decanted after an additional 12 h, leaving only the sand. The above procedure was repeated four additional times over the course of 96 h. The coated sand was washed to remove loosely attached iron, allowed to air dry, and passed through a 2-mm sieve to break up larger aggregates. The coating procedure yielded a coating of amorphous iron(III)oxyhydroxide of about 1.3 ± 0.12 mg Fe(III) per gram of sand. No Fe(II) was detected on the sand using the analytical method described below.

Batch Experiments, Iron Reduction. Oxygen-free minimal medium containing $NaHCO_3$, 2.5; NH_4Cl, 1.5; KH_2PO_4, 0.6; KCl, 0.1 (in grams per L of deionized water); vitamins 10 mL per L, and trace minerals 10 mL per L (Balch *et al.*, 1979; Lovley and Phillips, 1988) amended with 10 m*M* lactate was prepared under an N_2:CO_2 (80:20) gas atmosphere (Balch and Wolfe, 1976). The pH of the medium was approximately 6.9 and remained stable throughout the experiments. Oxygen-free minimal medium (9 mL) and 1 mL of bacterial culture, either starved or vegetative, were added to test tubes containing 1 g of iron-coated sand, and the vials were crimp sealed using thick butyl rubber stoppers (Bellco Glass, Vineland, NJ). All manipulations were performed under an oxygen-free N_2/CO_2 purge. The resulting bacterial concentrations were approximately 1.5×10^7 colony forming units (CFU) per mL. Control vials were established lacking either bacteria or lactate and all vials were incubated statically in the dark. Triplicate sets of vials were sacrificed periodically to determine the amount of ferrous iron in the supernatant and on the sand separately.

Batch Experiments, Chromium Reduction. A subset of vials containing biologically generated Fe(II) was autoclaved to eliminate microbial activity and thus to distinguish between chemical and biological Cr(VI) reduction. Cr(VI) (0.15 mg) was injected from a K_2CrO_4 stock solution resulting in a final concentration of 15 mg/L. Cr(VI) concentrations in the aqueous phase were monitored over time as described below. Vials were sacrificed and analyzed for Cr(VI) and total Cr in the aqueous and solid phase separately at the end of each experiment. Controls containing oxidized iron oxyhydroxide coated sand were established to account for abiotic losses of Cr(VI).

Experiments evaluating the ability of *S. algae* BrY to reduce Cr(VI) directly were performed by injecting Cr(VI) into vials containing active *S. algae BrY* cells and oxygen-free minimal medium supplemented with 10 m*M* lactate. Cr(VI) concentrations were monitored over time as described below.

Regeneration of Ferrous Iron in the Presence of Chromium Precipitates and Repeated Chromium Reduction. A set of vials containing microbially reduced iron was sterilized by autoclaving and then repeatedly injected with a Cr(VI) solution until their reduction capacity for Cr(VI) was exhausted. The capacity of these vials to reduce Cr(VI) was regarded as exhausted if 48 h after the last injection of 0.05 mg Cr(VI), more than 0.05 mg/vial (=5 mg/L) Cr(VI) were still detected. Vials were sacrificed for analysis of Fe(II), total Fe, Cr(VI), and total Cr after exhaustion of the Cr(VI) reduction capacity. A subset of the remaining vials was reinoculated with *S. algae* BrY to evaluate the potential for regeneration of reduced iron through the addition of DMRB. Reinoculated vials were sacrificed over time for iron analysis. Vials that showed evidence of Fe(II) production were autoclaved and their ability to repeatedly reduce Cr(VI) was tested as described above.

Column Experiments. Porous media columns were established to evaluate the microbially mediated geochemical elimination of Cr(VI) from contaminated water in flow through systems (Figure 1). Polytetrafluorethene (PTFE) tubes (5 cm in length and 1.1 cm in diameter) fitted with stainless steel nuts and ferrules, and PTFE and Tygon® Tubing (Cole Palmer) were established. Each column was filled with 7 g of iron-coated sand, resulting in a packed bed length of 6.3 cm, with a porosity of approximately 45%. The columns were covered with aluminum foil to prevent photo-reactions. A PTFE grid was placed at the inlet and outlet of each column to prevent washout of sand into the outlet or settling of sand into the inlet tubing. The columns were operated upflow with a peristaltic pump at flowrates of 4 mL/min and up to 15 columns were operated independently from each other via a manifold.

Each column was inoculated with approximately 6×10^8 CFU of starved *S. algae* BrY, and the columns were operated in semibatch mode. Approximately 20 mL of oxygen-free minimal medium supplemented with 10 m*M* lactate was pumped through each column every 2 days. Columns were sacrificed over time, and the sand was analyzed for Fe(II) and total Fe. After evidence of Fe(II) production, a Cr(VI) solution was injected concurrently with a tracer (fluorescein) at a flow rate of 0.45 mL/min, resulting in a residence time of approximately 6 min in the columns. Inlet and outlet concentrations of Cr(VI) and the tracer concentrations were monitored over time. Columns were sacrificed and analyzed for both total Cr and Cr(VI) after Cr(VI) injection.

A subset of columns was reinoculated with starved *S. algae* BrY cells after depletion of the Cr(VI) reduction capacity. The ability of the columns to reduce Cr(VI) again was assessed as described above after allowing adequate time for iron reduction.

Analytical

Iron Quantification. Amorphous iron-coated sand was analyzed for both total iron and ferrous iron [Fe(II)]. The method described by Lovley and Phillips (Lovley and Phillips, 1987 and 1988) was modified as follows. Instead of 0.5 *M* HCl, 2.5 *M* HCl was used, which proved to be sufficient for the solubilization of all Fe from the sand after incubation on a horizontal shaker for 30 min at 150 rpm. Fe(II) was determined by adding 5 mL of 2.5 N hydrochloric acid to 1 g of iron-coated sand. Total iron was quantified by adding 5 mL of 0.25 N hydroxylamine solution in 2.25 N hydrochloric acid to 1 g of iron-coated sand. Aliquots of the acid extracts were added to 5 mL of a solution of 1 g ferrozine (Sigma, St. Louis, MO) in 1 L 750 m*M* HEPES buffer at pH 7 and the absorbance was determined spectrophotometrically at 562 nm. Assays were performed on dry sand samples where possible. In most cases, however, these had to be performed on wet samples in order to minimize oxidation of Fe(II) to Fe(III) after destructively sampling the batch vials or columns. Because no Fe(II) was detected in the supernatant of the batch vials or effluent of the columns, inaccuracies based on this procedure were considered minimal.

Chromium Quantification. Aqueous Cr(VI) was quantified using diphenylcarbazide (Urone, 1955). A 500 µL sample was added to 10 mL 0.2 N sulfuric acid and vortexed. A diphenylcarbazide (DPC) solution (0.5 mL, 0.25% of DPC in 100% acetone) was added and the absorbance was read at 540 nm. The detection limit of this method is approximately 0.1 mg/L.

Cr(VI) was extracted from the sand using a modified procedure described by Vitale *et al.* (1994). A 0.28 M Na_2CO_3/0.5 M NaOH solution (5 mL) was added to 1 g of sand, mixed, and heated to 90 to 95°C for 60 min. The vials were swirled repeatedly during the heating process and deionized water was added, if needed, to replace losses due to evaporation. The vials were allowed to cool to room temperature and the total volume was filtered through a 0.2 µm nylon membrane (Fisher Scientific, Pittsburgh, PA). The pH was adjusted to 7.5 using concentrated HNO_3 and 0.5 mL of the DPC solution was added. The pH was adjusted to 1 with concentrated H_2SO_4 and the total volume of the sample was adjusted to 11 mL with 0.2 N H_2SO_4 before the absorbance was read at 540 nm.

Aqueous phase total Cr was determined after filtration using a modified standard method (Anon., 1992). One drop of methyl orange indicator solution was added to 1 mL of sample. Concentrated NH_4OH was added until the solution turned yellow, diluted H_2SO_4 (1:2 from concentrated H_2SO_4) was added until the indicator turned red, and the total volume was adjusted to approximately 5 mL. Boiling chips were added, the vial was closed with a rubber stopper, and a 20-gauge needle was inserted as exhaust. The solution was heated to a boil. One drop of a $KMnO_4$ solution (40 g/L) was added and boiling was continued. If fading occurred, another drop of $KMnO_4$ was added and boiling was continued for at least 2 min after the addition of the last drop of $KMnO_4$. Sodium azide (NaN_3, 5 g/L) was added in 0.1-mL increments and boiling continued until the color disappeared. The vials were allowed to cool after the addition of 1 drop of concentrated H_3PO_4. Concentrated H_2SO_4 was used to adjust the pH to 1.0, after which the whole content of each vial was filtered through a 0.2 µm nylon filter. The volume was adjusted to 10.5 mL using 0.2 N H_2SO_4 and 0.5 mL of DPC solution were added before measuring the absorbance at 540 nm.

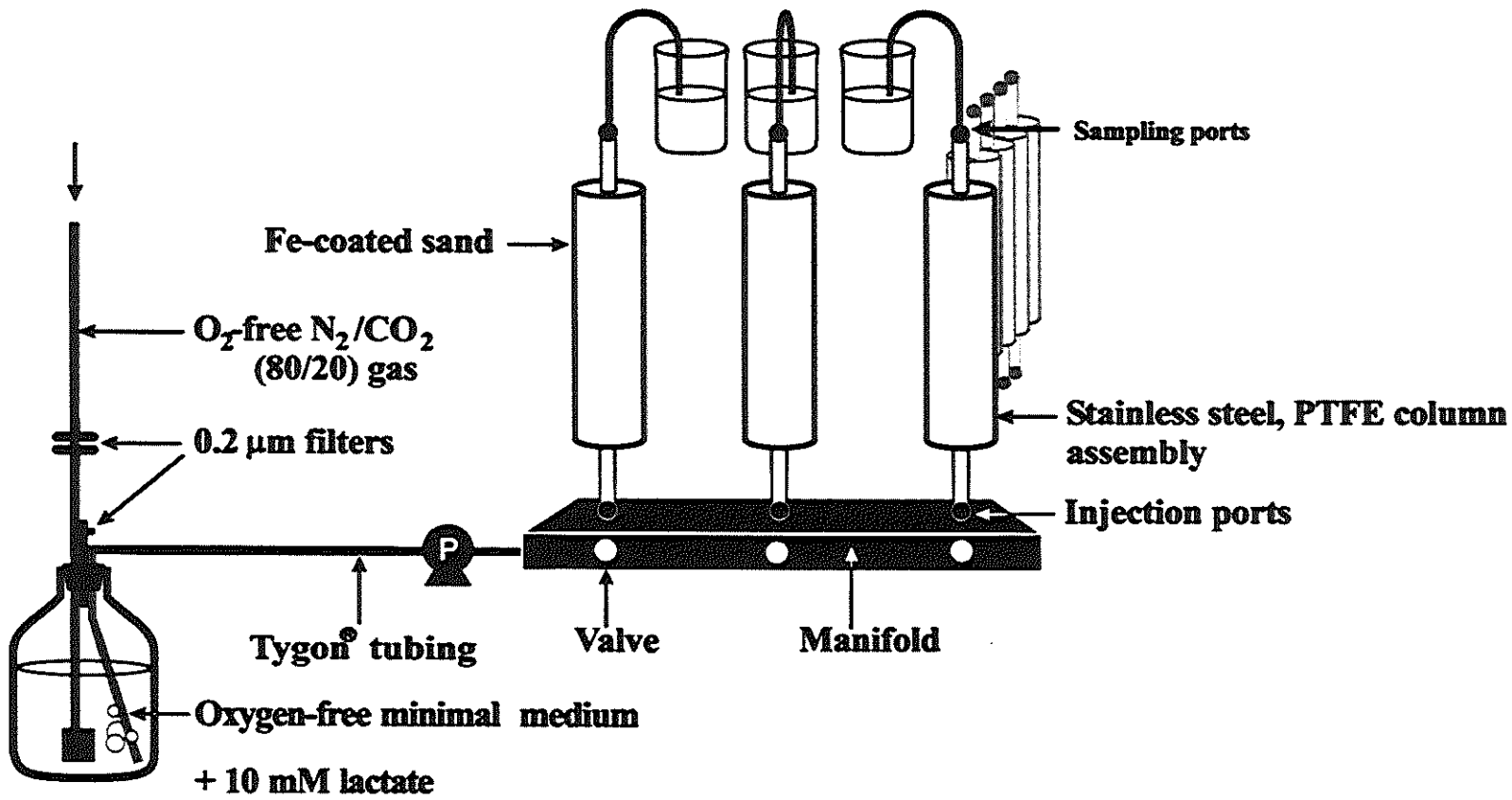

Figure 1. PTFE, stainless steel column assembly for studying microbial Fe(III) reduction and Cr(VI) reduction in flow through systems. Each column contained 7 g of iron coated sand. Up to 15 columns were operated independently using a manifold. A reduced copper catalyst at 400°C served to remove residual oxygen from the nitrogen/carbon dioxide gas mixture.

Total Cr from the sand was determined analogously, substituting the 1 mL liquid sample with 1 g of Fe-coated sand.

Results and Discussion

Microbial Fe(III) Reduction

S. algae BrY can use Fe(III) as electron acceptor to grow in the absence of oxygen with lactate as the electron donor. Starved and vegetative cells of *S. algae* BrY reduced surface-associated ferric iron [Fe(III)] to ferrous iron [Fe(II)] over time (Figure 2). The reduction was faster in vials inoculated with vegetative bacteria than in those inoculated with starved bacteria. Vegetative cells reduced approximately 90% (1.19 ± 0.09 mg) of the ferric iron available on the sand (1.3 ± 0.12 mg) within 24 h. Starved cells exhibited a slower rate of Fe(III) reduction and reduced approximately 80% (1.04 ± 0.02 mg) of the ferric iron within 10 days. Control vials lacking either lactate or *S. algae* BrY cells showed no evidence of Fe(II) production, indicating that the observed reduction was due to electron donor-dependent microbial activity.

It has been shown that starved cells of *S. algae* BrY reduce soluble ferric pyrophosphate slower than vegetative cells (Caccavo, Jr. *et al.*, 1996). The data presented here are consistent with these results. Although starved cells of *S. algae* BrY reduce surface-associated Fe(III) at a slower rate than vegetative cells, the amount of Fe(II) produced by starved cells after 10

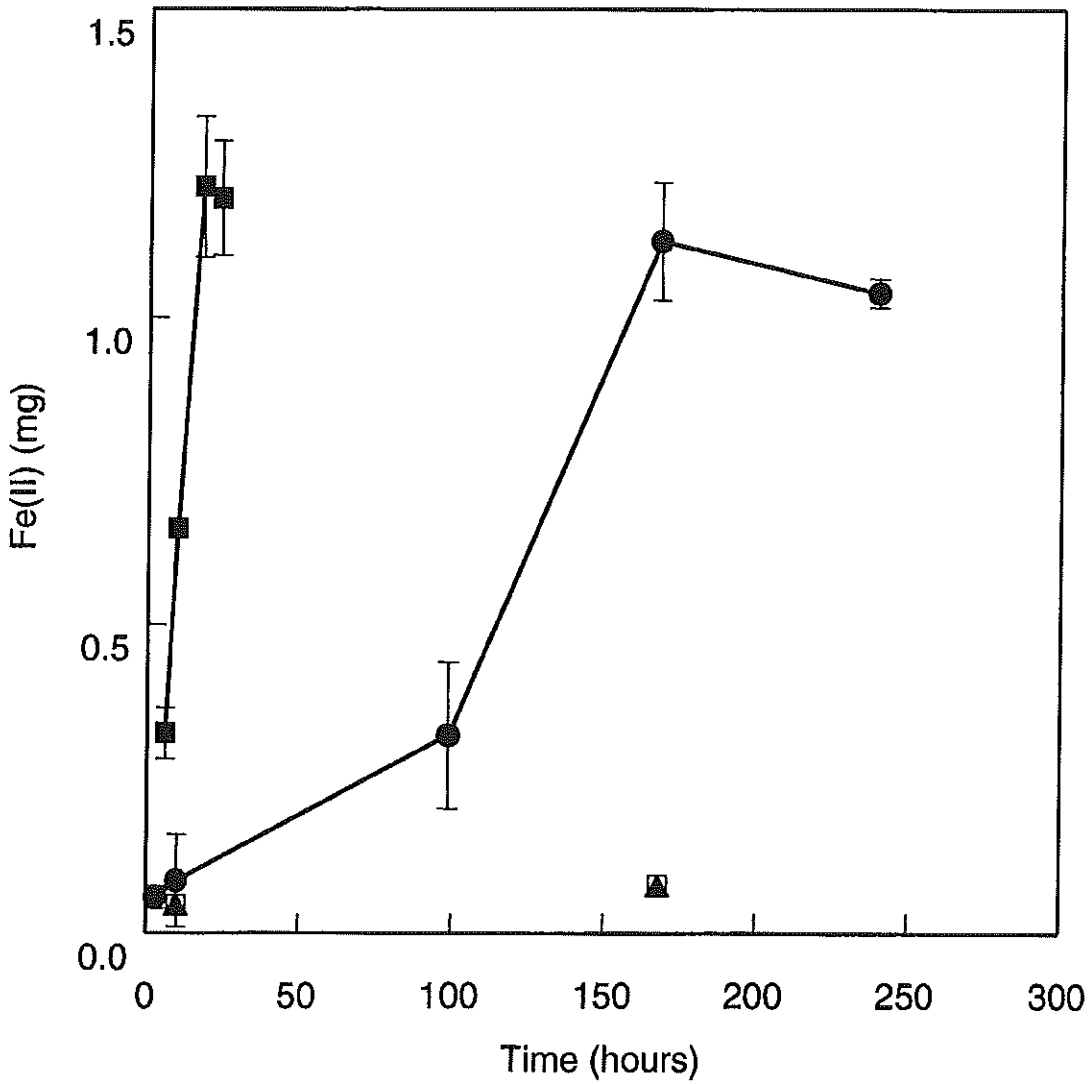

Figure 2. Production of Fe(II) from amorphous Fe(III) oxyhydroxide-coated sand by starved (●) and vegetative cells (■) of *S. algae* BrY. Every system contained 1 g of sand coated with 1.3 ± 0.12 mg of Fe(III) initially. Controls lacking either lactate (▲) or *S. algae* BrY (□) did not produce significant amounts of Fe(II). Error bars represent the standard error of the means (n = 3).

days is only slightly lower than the amount produced by vegetative cells.

Most of the Fe(II) produced (≥ 95%) remained associated with the sand surfaces as observed by separate analyses of the supernatant and the sand (data not shown). The fact that most of the reduced iron remains surface associated is important in view of a possible field application because surface-associated Fe(II) is more reactive with dissolved contaminants than dissolved Fe(II) (Kriegman-King and Reinhard, 1994). Furthermore, in aquifers dissolved Fe(II) would be transported out of the system over time with the flowing groundwater minimizing the efficacy of this technology.

The injection of DMRB into the subsurface and the stimulation of DMRB in the subsurface to enhance contaminant transformation has been proposed (Caccavo, Jr. *et al.*, 1996, Gorby *et al.*, 1994). Here we demonstrate that starved *S. algae* BrY cells, which can be transported through porous media better than vegetative cells (Bouwer *et al.*, 2000), can reduce surface-associated Fe(III) and produce surface-associated, reactive Fe(II). Thus, zones of surface reactive Fe(II) could be established in contaminated aquifers by stimulating naturally occurring DMRB or by bioaugmenting the contaminated subsurface with starved DMRB.

Cr(VI) Reduction by Microbially Produced Fe(II)

The reduction of Cr(VI) by Fe(II) has been documented using soluble Fe(II) (Seaman *et al.*, 1999) and surface-associated Fe(II) in natural aquifer material (Caccavo, Jr. *et al.*, 1996; Gorby *et al.*, 1994; Kriegman-King and Reinhard, 1994). We demonstrated that Cr(VI) concentrations decreased immediately after injection in the experimental vials containing biologically produced, surface-associated Fe(II) (Figure 3). The reaction of Fe(II) with Cr(VI) occurred so fast in these vials that at the first sampling event, after 30 s, the concentration of Cr(VI) was already significantly lower than the control concentration of 15 mg/L. Vials that were sterilized immediately before the injection to eliminate microbial activity showed the same initial decrease, providing evidence that chemical processes are governing the reduction of Cr(VI) by Fe(II) initially. Control vials containing oxidized iron oxyhydroxide-coated sand did not show a significant decrease in Cr(VI) over the duration of the experiment, indicating that sorption of Cr(VI) to the iron-coated sand was negligible. The initial increase in Cr(VI) concentrations in the control vials can be attributed to incomplete mixing before the first sampling event. Because the reaction of Cr(VI) with Fe(II) was expected to occur rapidly, the systems were sampled as soon as possible after addition of Cr(VI), that is, after 30 s. Thus, there was only a limited time available for mixing and the resulting incomplete mixing might account for initially lower Cr(VI) concentrations.

On the basis of the stoichiometry of reaction 2, the vials contained sufficient Fe(II) to completely reduce the Cr(VI) added (0.15 mg, resulting in an initial concentration of 15 mg/L). However, 11 min after the injection of Cr(VI), the reduction of 15 mg/L Cr(VI) remained incomplete. This incomplete conversion of Cr(VI) could be due to mass transport limitations within the experimental systems or the presence of Fe(II) sites, which were not reactive with Cr(VI). Powell *et al.* (1995) stated that the reactivity of surface-associated Fe(II) can vary depending on the mineralogy. Although no mineralogical analyses were performed during this research, the white color of the precipitate during biological iron reduction and the high carbonate content of the buffer suggest that siderite ($FeCO_3$) formed at least partly on the sand grains. Siderite has been described as only moderately reactive with dissolved phase contaminants relative to other iron minerals (Agrawal and Tratnyek, 1996; Blowes *et al.*, 1997). This may explain the fraction of moderately reactive and nonreactive Fe(II) in the batch vials. Diffusion limitations (Agrawal and Tratnyek, 1996), and the accumulation of chromium precipitates can also limit Cr(VI) reduction by surface reactive Fe(II).

The vials were analyzed for total chromium and Cr(VI) at the conclusion of each experiment. The majority (> 95%) of the chromium recovered from the sand was recovered as Cr(III) (data not shown) demonstrating that Cr(VI) was transformed to Cr(III) in the presence of biologically produced surface-associated Fe(II).

Complete conversion of Cr(VI) within 24 h was only observed in the vials containing active *S. algae* BrY cells. This decrease suggests either direct reduction of Cr(VI) by *S. algae* BrY or an indirect mechanism in which *S. algae* BrY rereduced ferric iron resulting from reaction 2 to ferrous iron, which then again reduced Cr(VI) to Cr(III). Direct microbial reduction of Cr(VI) by metal-reducing bacteria and soil indigenous microbial populations has been reported (Bader *et al.*, 1999; Bopp and Ehrlich, 1988; Turick *et al.*, 1998). We demonstrated that direct microbial reduction of Cr(VI) by active *S. algae* BrY is possible, although significantly slower than the chemical reduction with surface reactive Fe(II). Regardless of whether surfaces were available (quartz sand or iron-coated sand) or not, active *S. algae BrY* cells did not significantly reduce 5 mg/L Cr(VI) within 11 min (Figure 4). However, the concentration of Cr(VI) dropped to below 1 mg/L in all vials containing *S. algae* BrY after 24 h, indicating slow direct microbial

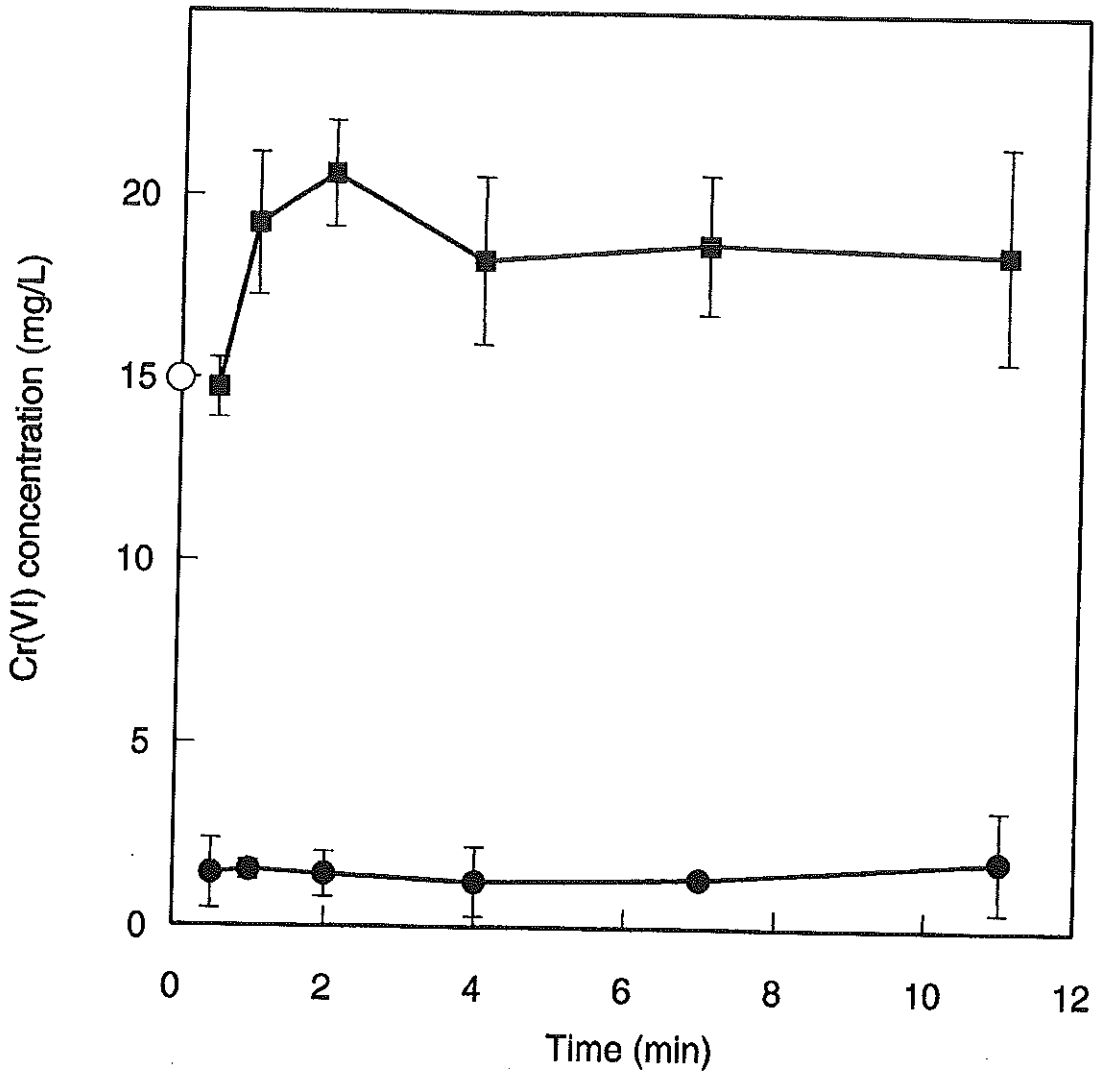

Figure 6. Cr(VI) transformation in vials with initially exhausted Cr(VI) reduction capacity. (●) after reinoculation and incubation with *S. algae* BrY, (■) without reinoculation. The Cr(VI) spike increased the initial Cr(VI) concentration in the vials by 15 mg/L (indicated by ○). Error bars represent the standard error of the means (n = 3).

Three different processes are likely to be responsible for the reductive precipitation of chromium. Cr(VI) may react chemically with surface reactive Fe(II) or active *S. algae* BrY cells may directly reduce Cr(VI) to precipitate Cr(III). These processes, in addition to DMRB-mediated biogeochemical cycling of Fe(III) back to Fe(II) (process 2 in Figure 8), may explain the attenuated increase of Cr(VI) toward the end of the experiment. All three processes together with the limited reduction capacity of the columns would cause the slow but continued reductive precipitation of chromium within the columns. These processes would result in Cr(VI) effluent concentrations that approach the influent concentration asymptotically. Likely, all three processes occur within the columns. Therefore, it is surprising that only approximately 1% of the total reduction capacity of surface-associated Fe(II) for Cr(VI) is utilized after approximately four pore volumes. Also unexpectedly, Cr(VI) concentrations in the effluent are almost equal to the influent concentration.

To evaluate the possibility of regenerating the reactivity of the columns by injection of *S. algae* BrY, the remaining columns were reinoculated after the depletion of the Cr(VI) reduction capacity. Approximately 2 months after reinoculation, Fe(II) production in these columns was evident and Cr(VI) was injected a second time concurrently with fluorescein. The normalized breakthrough curves were very similar to those plotted in Figure 7 (data not shown), indicating that the reduction capacity of the columns was regenerated by the reinoculation of *S. algae* BrY.

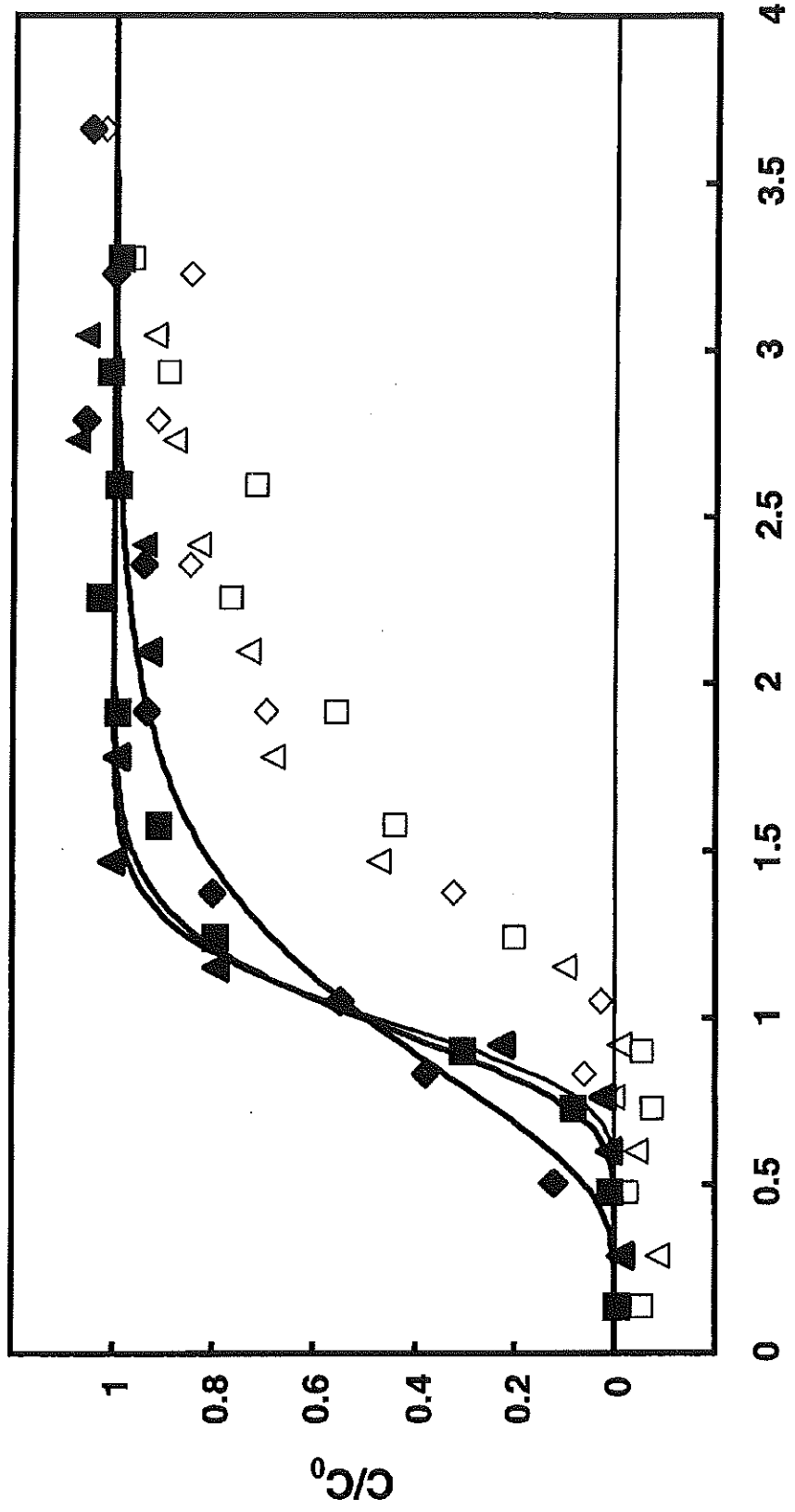

Figure 7. Normalized breakthrough curves of a fluorescein tracer (■, ▲, ◆) (corresponding model fits indicated by solid lines) and their corresponding Cr(VI) breakthrough data (□, △, ◇) through columns containing biologically produced Fe(II). The influent Cr(VI) concentration was 6.6 ± 0.1 mg/L for every column.

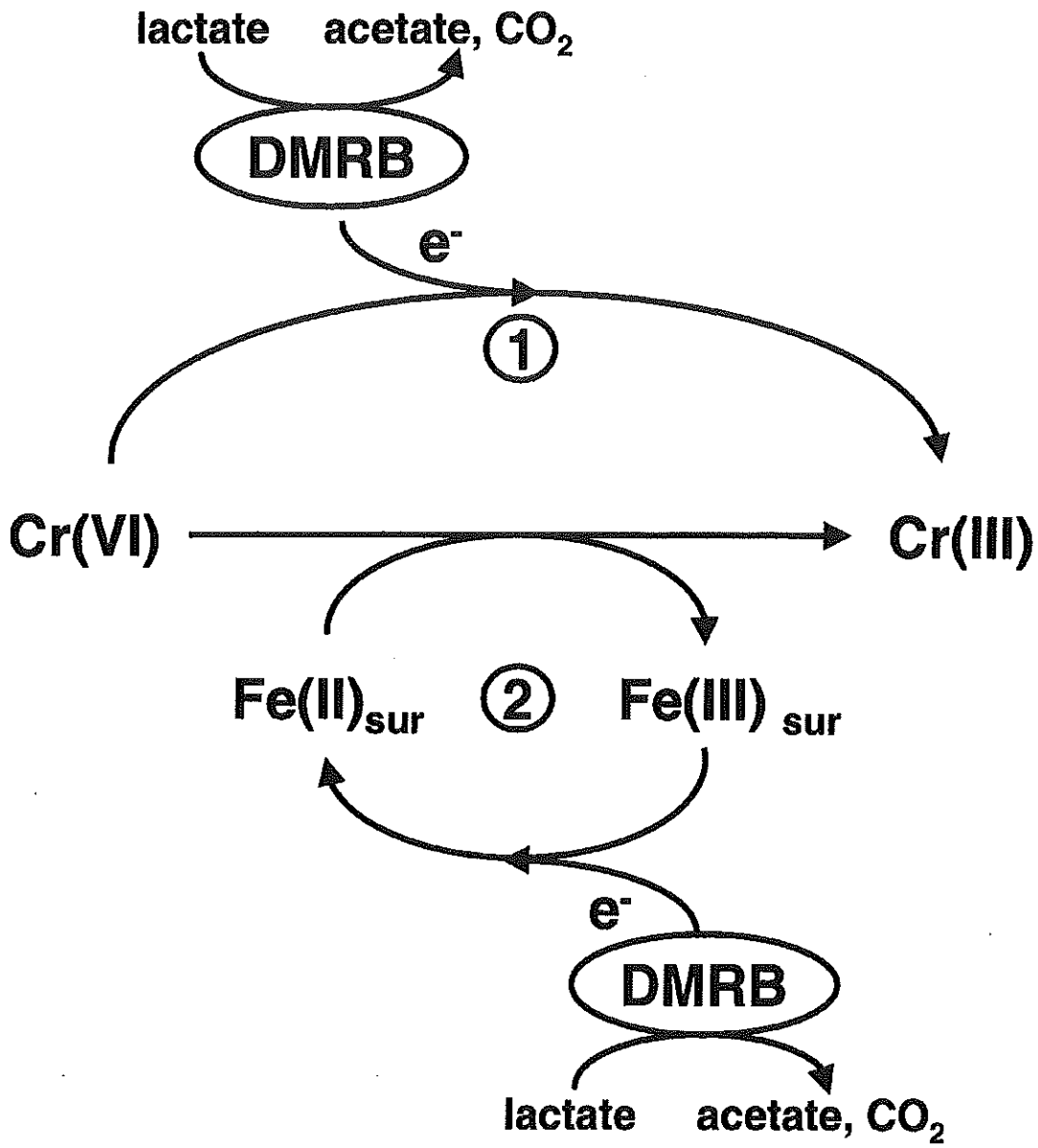

Figure 8. Processes governing the fate of Cr(VI) in environments containing surface associated iron and dissimilatory metal-reducing bacteria (DMRB). (1) Direct Cr(VI) reduction by DMRB; (2) chemical reduction of Cr(VI) by surface associated ferrous iron ($Fe(II)_{sur}$) and biological rereduction (regeneration) of surface associated ferric iron ($Fe(III)_{sur}$) to $Fe(II)_{sur}$ by DMRB.

Conclusions

The results presented here suggest the possibility of artificially establishing a biogeochemical cycle (Figure 8) in Cr(VI) contaminated subsurface environments. DMRB reduce ferric iron [Fe(III)] to ferrous iron [Fe(II)], which in turn reduces Cr(VI) to Cr(III), while Fe(II) is oxidized (back) to Fe(III). DMRB can then rereduce the Fe(III) to Fe(II) to close the biogeochemical cycle.

The use of DMRB potentially allows the establishment and maintenance of redox reactive zones (permeable subsurface barriers) in Fe(III)-bearing subsurface environments. The applicability of this technology to any given field site will depend on the iron minerals present in the subsurface and the ability to produce surface reactive Fe(II) from the iron minerals present in the aquifer. The amount and type of iron minerals present in a soil might vary from one to several hundred g/kg of soil and is strongly dependent on the geology at any given site (Cornell and Schwertmann, 1996). The ability of indigenous organisms to reduce the iron minerals present might be limited. Thus, bacterial iron reduction might be achieved by stimulating indigenous microorganisms or, if microbial iron reduction cannot be sufficiently supported,

by augmenting the site with metal-reducing bacteria. The use of starved metal-reducing bacteria has potential for such an approach. Starved bacteria are transported better through porous media (Bouwer *et al.*, 2000) and, as shown here, can produce surface reactive Fe(II) from amorphous iron (oxy) hydroxides.

However, like in most bioremediation strategies, some limitations and concerns have to be considered. High initial Cr(VI) concentrations or the presence of other toxic compounds might slow down or prevent biological iron and chromium reduction. High or low pH values as well as a lack of electron donor can also hinder biological iron reduction and, subsequently, chromium reduction.

The process described herein does not remove chromium from the aquifer but immobilizes it by precipitation. Although Fe(III)-Cr(III) hydroxides and pure Cr(III) hydroxide have very low solubilities and are unlikely to result in chromium concentrations above the drinking water limit, the reoxidation of Cr(III) compounds is a concern. Microbially mediated reoxidation of Cr(III) is not a proven concern in systems containing natural soil material (Bader *et al.*, 1999; James, 1994); however, manganese oxides have been found to quickly oxidize Cr(III) in soils (Bartlett and James, 1979; Rai and Zachara, 1988). Therefore, the application of oxidants such as potassium permanganate or hydrogen peroxide, commonly used in certain remediation schemes, can potentially lead to a remobilization of immobilized chromium.

Future research is needed and will address the identification of mineral phases developing during the microbial reduction of iron (hydr)oxides and iron chromium (hydr)oxides. The stability of these precipitates will be assessed and the recycling frequency of the ferric/ferrous iron redox-couple in the presence of Cr(VI) will be evaluated in batch and flow through systems.

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