FORMATION OF REDOX-REACTIVE SUBSURFACE BARRIERS USING DISSIMILATORY METAL-REDUCING BACTERIA

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ABSTRACT

This paper describes research focusing on the development of a biologically driven permeable redox-reactive subsurface barrier for the elimination of groundwater contaminants. Dissimilatory Metal-Reducing Bacteria (DMRB) have the ability to reduce a wide range of naturally occurring Fe(III)-minerals. In Fe-rich aquifers, DMRBs could be used to establish a zone of reduced Fe, which would serve as a source of electrons for the redox-based elimination of groundwater contaminants such as heavy metals and chlorinated solvents. Column studies showed that starvation of the DMRB *Shewanella alga* BrY facilitates the transport of this organism through a porous medium. Starvation could, therefore, provide an effective means for delivering these organisms into desired subsurface areas where their metabolism could provide a mechanism for the bioremediation of groundwater contaminants.

Key words: *bacterial transport, biobarrier, starvation, metal reduction*

INTRODUCTION

Recent advances in redox-reactive subsurface barrier technology have demonstrated significant potential for in-place treatment of groundwaters contaminated with chlorinated organics and heavy metals. This remediation concept is based on channeling contaminated groundwater (by means of subsurface containment walls, grout curtains, or biofilm barriers) through a zone of low redox potential, which serves as a source of electrons for remediation-related chemical and biological reactions, such as heavy metal precipitation and reductive dechlorination. Permeable reactive subsurface barriers have been accepted into the U.S. EPA's SITE (Superfund Innovative Technology Evaluation) Program, and several field-scale and full-scale systems have been installed in locations including California, Colorado, Kansas, North Carolina, and Belfast (Northern Ireland) (U.S. EPA, 1996). Redox-reactive subsurface barriers are commonly built by excavating deep trenches and refilling them with zero-valent iron. The barriers are designed to remain reactive over several years until the reactive components are exhausted. The costs of establishing these redox-reactive subsurface barriers by excavation and placement of zero-valent metal into the aquifer, however, can be very high and, therefore, provide incentive to develop lower-cost alternatives. This paper describes research elucidating the feasibility of an alternative technology for the establishment of redox-reactive subsurface barriers based on bioaugmentation with Dissimilatory Metal-Reducing Bacteria (DMRB).

DMRB have been shown to facilitate degradation of chlorinated organics and precipitation of heavy metals (Caccavo et al., 1996b; Caccavo et al., 1996c; Caccavo and Olson, 1995; Gorby et al., 1995; Caccavo et al., 1994; Gorby et al., 1994; Caccavo et al., 1992; and Lovley et al.,

1991). DMRB can directly reduce these contaminants enzymatically. Alternatively, DMRB can enzymatically generate ferrous iron from naturally occurring ferric iron minerals. Microbially produced ferrous iron can chemically dechlorinate chlorinated organics, reductively immobilize metals and radionuclides (e.g. Cr^{6+}, U^{6+}, Co^{3+}), or reductively degrade nitroaromatic compounds (Heijman et al., 1995; Klausen et al., 1995; and Heijman et al., 1993).

A permeable-reactive biobarrier could be established in the subsurface by stimulating naturally occurring DMRB populations or inoculating contaminated environments with pre-adapted or genetically engineered DMRB. The economic feasibility of bioaugmenting large volumes of soil *in situ* depends on the ability to transport DMRB inocula over distances of tens to hundreds of meters. Additionally, the DMRB inoculum must survive, effectively compete with indigenous populations, and maintain the capacity for enzymatic metal reduction. Therefore, research is needed to improve our ability to effectively deliver bacteria to targeted areas in the subsurface, as well as to ensure their survival and successful competition.

Bacterial transport through porous media is influenced by many parameters, including the unique properties of the bacterial cells, solution chemistry, porous media characteristics, and physical properties of the environment such as interstitial fluid velocity (Tan et al. 1994, Gannon et al. 1991b).

Cell properties that influence bacterial transport include surface proteins, (extracellular) polysaccharides, pili, filaments, motility, chemotaxis, cell size, cell shape, surface charge, hydrophobicity, buoyant density, growth state, and nutritional status (Harvey et al., 1997; Weiss et al., 1995; Camper et al., 1993; Gannon et al., 1991a; Peterson and Ward, 1989; Fletcher and Loeb, 1979; Costerton et al., 1978; Brown et al., 1977; Fletcher, 1977; Fletcher, 1976; Marshall, 1976; and Fletcher and Floodgate, 1973). Solute characteristics such as ionic strength, pH, temperature, presence of specific ions, concentration of dissolved organic matter, and presence of surfactants have also been shown to influence bacterial attachment to surfaces (Johnson and Logan, 1996; Gross and Logan, 1995; McCaulou et al., 1995; Jackson et al., 1994; Mills et al., 1994; Kinoshita et al., 1993; Martin et al., 1992; Fontes et al., 1991; Gannon et al., 1991b; van Loosdrecht et al., 1987; Sharma et al., 1985; and Absolom et al., 1983). Porous media properties such as mineralogy (e.g., Fe coating), organic matter and clay content, grain and pore size distribution, surface charge, and surface roughness can also influence bacterial attachment to surfaces (Johnson and Logan, 1996; Sharma and McInerney, 1994; Mueller et al., 1992; Scholl et al., 1990; Peterson and Ward, 1989; and Geesey and Costerton, 1979).

In full-scale engineered systems, however, manipulating the solute chemistry (e.g., lowering the ionic strength, changing the pH, changing the temperature of the groundwater) or manipulating aquifer characteristics to facilitate bacterial transport may not be economically feasible. Focusing on these parameters is, therefore, not anticipated to result in the development of a competitive

bioremediation technology based on augmentation with bacteria.

Manipulating the bacterial inoculum to facilitate bacterial transport through porous media appears to be more promising. However, attempts to facilitate transport by the addition of surfactants or the (genetic) manipulation of the cell surface have mostly resulted in a rapid decrease in activity and viability of the bacteria, or did not result in the desired increase in transport distance (Gross and Logan 1995). Starvation of bacteria, however, has been shown to be an effective means of facilitating bacterial transport through porous media.

Starved bacteria have been described in deep-sea environments and in the terrestrial subsurface to depths greater than 9000 ft. Some of these bacteria were estimated to have survived there for hundreds of years (Amy and Haldeman 1997). Starvation of bacteria results in size reduction and a decrease in metabolic activity until the bacteria approach complete dormancy (Kjelleberg 1993). Starved bacteria have been injected into porous media and resuscitated *in situ* to form bacterial plugs in high-permeability regions. Their vegetative counterparts, however, adhered so avidly to the surfaces close to the injection point that they plugged up the porous medium almost immediately and did not permit microbial transport over greater distances (Cusack et al., 1992; Lappin-Scott et al., 1988a; Lappin-Scott et al., 1988b; and MacLeod et al. 1988). Cusack et al. (1992) predicted that starved bacteria could penetrate at least 200 m through consolidated sands.

Previous studies in our laboratory have used starved bacteria to generate low-permeability biofilm barriers in porous media designed to contain groundwater flow and inhibit contaminant migration (Cunningham et al., 1997). Based on this previous research, it is anticipated that we can establish a biologically driven permeable redox-reactive barrier by injecting starved DMRB into iron-rich aquifers. Such redox-reactive sites could then be used to remediate groundwater contaminants such as chlorinated organics, nitroaromatic compounds, heavy metals, and radionuclides.

This paper evaluates the feasibility of a DMRB-driven redox-reactive subsurface barrier for the cleanup of contaminated groundwater. The transport of starved and vegetative DMRB through porous media, the reduction of solid-phase Fe(III)-species by starved dissimilatory metal-reducing bacteria, and preliminary studies monitoring the fate of chromate in the presence of microbially reduced subsurface material were addressed.

MATERIALS AND METHODS

Preparation of Starved Cells

Cells of *Shewanella alga* BrY were aerobically grown to the late-exponential, early-stationary phase in tryptic soy broth (30 g/L; Difco, Detroit, MI) at room temperature on a rotary shaker at 150 rpm for 15 hours. The cells were harvested by centrifugation (6000 rpm, 4°C, 20 min) using a Sorvall Instruments (Newton, CT) centrifuge, model RC5C, washed 3 times in phosphate-buffered saline solution (PBS; 8.5 g/L NaCl, 0.96 g/L K_2HPO_4, 0.61 g/L KH_2PO_4, pH 7.0), and then re-

suspended in PBS. Washed *S. alga* BrY cells were either used directly (vegetative state) or starved by aseptically stirring them on a magnetic stir plate at room temperature. Starved cells were incorporated into transport experiments after 7 weeks of starvation.

Fe-Coating of Quartz Sand

Quartz sand (40 mesh, Unimin Corp., Emmet, ID) was soaked in approximately twice its volume of 1 N HNO_3 for at least three days, rinsed with deionized water (10-15 times) and allowed to soak in deionized water overnight. The deionized water was discarded and 200 mL of a solution of amorphous Fe(III)-hydroxide was added. The amorphous iron solution was freshly prepared by dissolving 34.5 g of $FeCl_3$ in 1 L deionized water and slowly adjusting the pH to 7 using 5 N NaOH. The Fe(III)-hydroxide-sand mixture was vigorously shaken and allowed to settle for several hours. The overlying water was then decanted and replaced with a 0.1 mM NaCl-solution and allowed to settle again overnight. The next morning, the Fe(III)-hydroxide-NaCl solution was discarded completely and replaced with freshly made Fe(III)-hydroxide solution. This process was repeated three more times before the coated sand was allowed to air dry. The dried sand was sieved through a 1.19 mm sieve to remove large aggregates. The resulting mineral coating contained 1.34 ± 0.12 mg Fe per gram of sand, 95% of which was Fe(III).

Fe(III)-Reduction Experiments

Vials containing 9 mL of anaerobic minimal medium (Caccavo et al., 1996b) were prepared containing either soluble Fe(III) (as ferric pyrophosphate, Sigma, St. Louis, MO), artificially Fe(III)-coated sand, or subsurface material from Hanford, WA. All anaerobic media were boiled and cooled down under an oxygen-free N_2 (80 vol %) - CO_2 (20 vol %) atmosphere, sealed gas tight, and sterilized by autoclaving. Stock solutions of lactate and ferric pyrophosphate were treated the same way. Bacterial cultures were prepared by centrifuging aerobically grown or starved BrY cells and re-suspending them in anaerobic minimal medium. The bacteria were injected into each vial (except for the controls without bacteria, which received the same volume of sterile anaerobic minimal medium). The initial cell concentration was approximately 1.1×10^6 colony forming units per mL (CFU/mL) in the experiments using Ferric-Pyrophosphate and Hanford subsurface material as Fe(III) sources, and approximately 5.5×10^6 CFU/mL in the experiments using artificially Fe-coated sand. All additions were made maintaining anoxic conditions using sterile syringes and needles.

Analytical Techniques

The concentration of Fe(II) was determined using the ferrozine assay, as described previously (Lovley and Phillips, 1987). Fe(II) and Fe(III) species were solubilized in 2.5 N HCl. In order to determine the concentration of Fe(III), hydroxylamine-HCl (Alfa Aesar, Ward Hill, MA) was used to reduce Fe(III) to Fe(II). The concentration of total iron was then determined with the ferrozine

assay. The amount of Fe(III) was determined as the difference between the concentration of total iron and the concentration of Fe(II). Chromate was analyzed by the sym-diphenylcarbazide method (Urone, 1955).

Bacterial Transport Studies

Cells of *S. alga* BrY, starved or vegetative, were injected into porous media columns filled with 40 mesh quartz sand. Columns of two different dimensions were used: 10 ft (~3 m) long and 1 in (2.54 cm) in diameter, and 1 ft (~30.5 cm) long and 0.5 in (1.27 cm) in diameter. The column inlet and outlet were layered with nylon mesh and pea gravel to prevent settling of sand into the inlet tubing and washout of sand through the outlet tubing. The columns were equipped with sample ports at the influent and effluent points. The pore volume of each system was determined utilizing Na-Fluorescein (Aldrich, Milwaukee, WI) as a tracer.

The columns were prepared for inoculation by flushing with 2 pore volumes 0.5 % NaOCl, 1 pore volume sterile PBS, 2 pore volumes 0.01 M sodium-thiosulfate to eliminate residual chlorine and 2 pore volumes sterile PBS. A commercial kit was used to assay the column effluent for residual chlorine (HACH, Loveland, CO). The columns were inoculated with 2 pore volumes of starved or vegetative cells of *S. alga* BrY suspended in PBS. Transport of bacteria was monitored by sampling the effluent of the columns for culturable cells, determined as colony-forming units on tryptic-soy agar (40 g/L; Difco, Detroit, MI; incubation for 24 hours at 30°C). The distribution of sorbed cells throughout the columns was determined at the end of the experiments by sectioning the columns and analyzing the sand cores for culturable cells after desorption from the sand. Desorption of cells was accomplished by diluting a desorption solution, as described in Camper et al. (1985), 1:4 in PBS. An aliquot (5 ml) of this solution was added to 1 g of sand in a test tube. The test tube was placed on a horizontal shaker for 30 min and then homogenized with a vortexer. The supernatant was sampled immediately after coarse particles had settled and plate counts were performed as described above.

RESULTS AND DISCUSSION

Starvation and Resuscitation of Shewanella alga BrY

Shewanella alga BrY is a facultatively anaerobic bacterium that has been extensively studied and characterized (Caccavo et al., 1997; Caccavo et al., 1996a; Caccavo et al., 1996b; and Caccavo et al., 1992). The starvation of *S. alga* BrY results in a gradual decrease in the mean cell volume from 0.48 mm^3 to 0.2 mm^3 and a dramatic decrease in endogenous metabolic activity (Caccavo et al., 1996b). Starved *S. alga* BrY cells can be resuscitated with a variety of electron acceptors including soluble Fe(III) sources, Fe(III)-bearing subsurface minerals, ferric-oxyhydroxide, Co(III)-EDTA, and oxygen (Caccavo et al., 1996b). In addition to an electron acceptor, an electron donor, such as lactate or hydrogen, is also required for the resuscitation of

starved cells. The length of the lag phase prior to resuscitation increases proportionally with the duration of starvation.

The resuscitation of starved *S. alga* BrY cells (starvation time 8.5 weeks) with artificially Fe(III)-coated sand is shown in Figure 1. After an initial lag phase, the injected cells of *S. alga* BrY reduced the surface associated Fe(III) minerals, resulting in increasing Fe(II) concentrations over time. The final mass of Fe(II) produced in the systems (~1.1 mg Fe^{2+}) is only slightly below the total amount of Fe(III) added to the system (~1.3 mg). Bacteria-free controls did not show an increase in Fe(II) concentrations, suggesting that the Fe reduction was due to microbial activity.

Bacterial Transport through Porous Media

The transport experiments were carried out in up-flow porous medium reactors at flow rates of approximately 10 mL/min in the 10 ft columns and 2 mL/min in the 1 ft column systems, resulting in interstitial fluid velocities of approximately 11.5 cm/min and 3.5 cm/min, respectively. The studies compared the transport of starved *S. alga* BrY cells relative to vegetative *S. alga* BrY cells. The results showed that starvation is an effective means of increasing *S. alga* BrY transport through a homogenous porous medium such as quartz sand.

The breakthrough curves of starved and vegetative *S. alga* BrY cells through 1-ft-long porous media columns filled with quartz sand are shown in Figure 2. The cell numbers in the effluent were normalized with respect to the influent cell concentration, and the flow-through volume was normalized as pore volume. The influent cell concentrations, the interstitial flow velocities, the flow rates, and the duration of these studies are listed in Table 1. Figure 2 clearly shows that starved *S. alga* BrY cells were more effectively transported than vegetative cells. The effluent concentration of starved cells approached approximately 40% of the influent concentration after less than one pore volume of flow through the column. However, the effluent concentration of vegetative cells did not reach more than approximately 6% of the influent concentration, even after 2 pore volumes of bacterial culture had been injected. The distribution of sorbed cells throughout the columns at the end of the experiments supports these observations (Figure 3). While the counts of vegetative cells sorbed to the porous medium decreased rapidly with distance from the point of injection (from 2.7×10^7 CFU/g sand in the first two inches of the column to 1.8×10^6 CFU/g sand in the last two inches of the column), the starved cells were more evenly distributed throughout the column.

Facilitation of bacterial transport by starvation becomes even more evident in the 10-ft columns. The passage through 10 ft (~3 m) of quartz sand lead to a 5-log removal of vegetative cells (Figure 4). However, there was only a 1.5 log removal of starved cells. The influent concentrations of starved and vegetative cells were approximately equal and are listed in Table 2, along with the interstitial flow velocities, the flow rates, and the duration of these experiments.

The transport data were supported by quantification of the cells sorbed to the quartz sand along the flow path. More vegetative cells adhered to the porous medium than starved cells (Figure

5). High concentrations of vegetative cells within the first few feet of the injection were observed, while the starved cells were more evenly distributed throughout the columns. No vegetative cells could be detected farther than 4 ft (~1.2 m) away from the injection point. The detection limit for culturable cells was 10^3 CFU/g sand for sorbed cells and 10^2 CFU/mL for planktonic cells.

Results from the 1-ft and 10-ft column studies clearly indicate that starvation of *Shewanella alga* BrY provides a means of enhancing bacterial transport in quartz sand porous media. As observed by Cusack et al. (1992), Lappin-Scott et al. (1988a & 1988b), and MacLeod et al. (1988), vegetative celLs tend to strongly sorb to the porous medium close to the injection point. Starvation, in contrast, appears to decrease the tendency of bacteria to attach to quartz sand, thus facilitating a more homogeneous distribution of cells along the flow path.

CONCLUSIONS AND FUTURE RESEARCH

Starved cells of *Shewanella alga* BrY can be resuscitated with artificially Fe(III)-coated sand and Fe(III)-bearing subsurface material, resulting in the production of surface associated Fe(II). Starvation of *S. alga* BrY cells significantly enhances their transport through saturated quartz sand relative to vegetative *S. alga* BrY cells. These results suggest that a permeable redox-reactive biobarrier technology based on bioaugmentation with DMRB is possible. The feasibility of this technology will be further assessed in experiments designed to examine the influence of heterogeneities (e.g. presence of Fe(III)-bearing surfaces) on the transport of starved DMRB. Contaminant reduction by microbially reduced Fe minerals will also be evaluated in flow-through column systems.

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Table 1. Influent concentrations, interstitial fluid velocities, flow rates, and duration of bacterial transport studies through 1-ft columns filled with clean quartz sand.

Inoculum	Interstitial fluid velocity [cm/min]	Flow rate [mL/min]	Influent cell concentration Co [CFU/mL]	Duration of experiment [min]
Vegetative bacteria	3.4 + 0.7	2.0 + 0.4	$4.5 \times 10^7 + 7.7 \times 10^6$	45
Starved bacteria	3.7 + 0.2	2.1 + 0.1	$6.0 \times 10^6 + 2.9 \times 10^6$	45

Table 2. Influent concentrations, interstitial fluid velocities, flow rates, and duration of bacterial transport studies through 10-ft columns filled with clean quartz sand. No standard deviations are available for these data.

Inoculum	Interstitial fluid velocity [cm/min]	Flow rate [mL/min]	Influent cell concentration Co [CFU/mL]	Duration of experiment [min]
Vegetative bacteria	11.5	10	3.9×10^7	150
Starved bacteria	11.5	10	2.6×10^7	150

Figure 1. Resuscitation of starved *Shewanella alga* BrY cells on amorphous Fe(III)-hydroxide coated sand. ◆ setups containing starved bacteria, ■ bacteria-free controls.

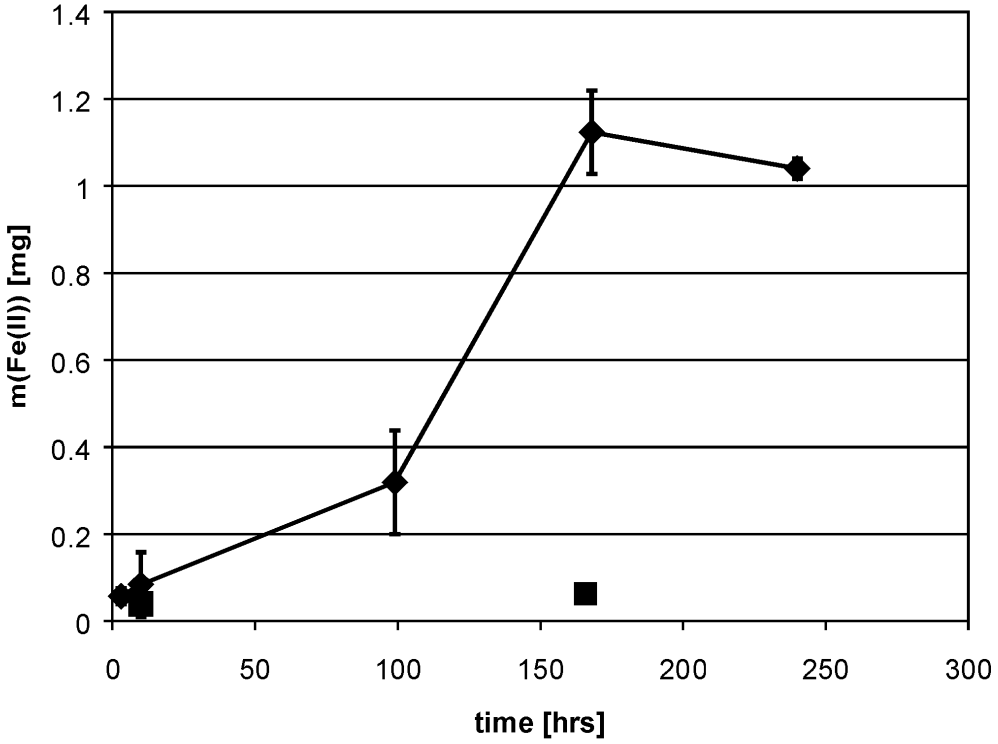

Figure 2. Breakthrough of starved and vegetative *Shewanella alga* BrY cells through 1 ft-long porous media columns filled with clean quartz sand. See Table 1 for further details. Starved cells: ▲, vegetative cells: ◆

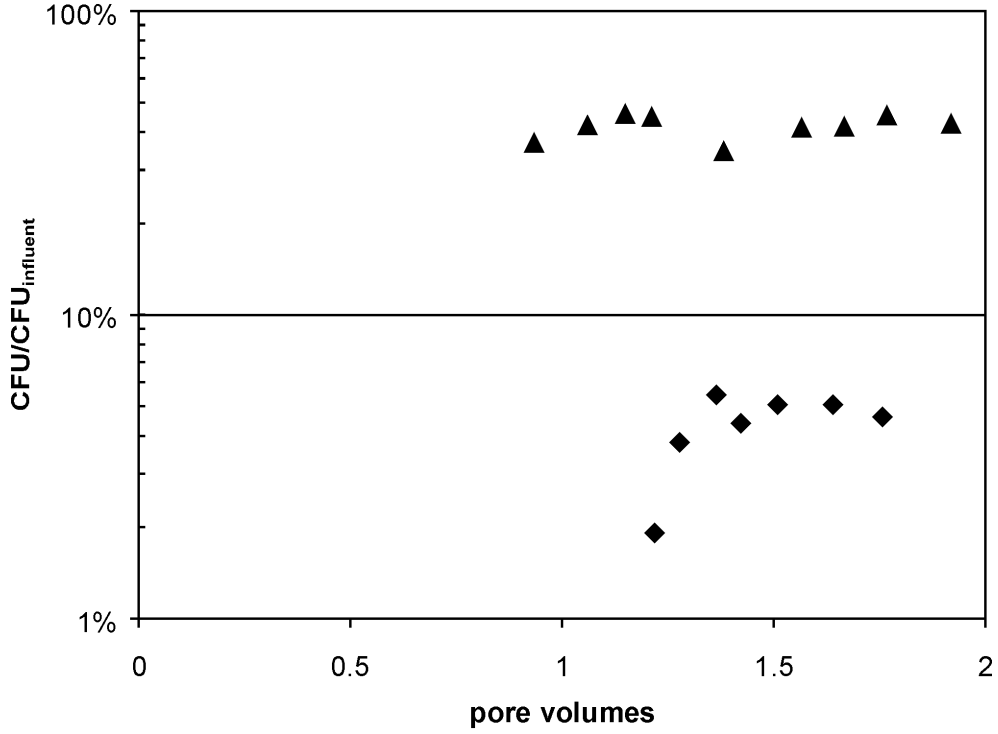

Figure 3. Distribution of starved (▲) and vegetative (◆) cells sorbed to the quartz sand throughout the 1-ft columns after injection of 2 pore volumes of cell suspension. See Table 1 for further details. The error bars represent one standard deviation.

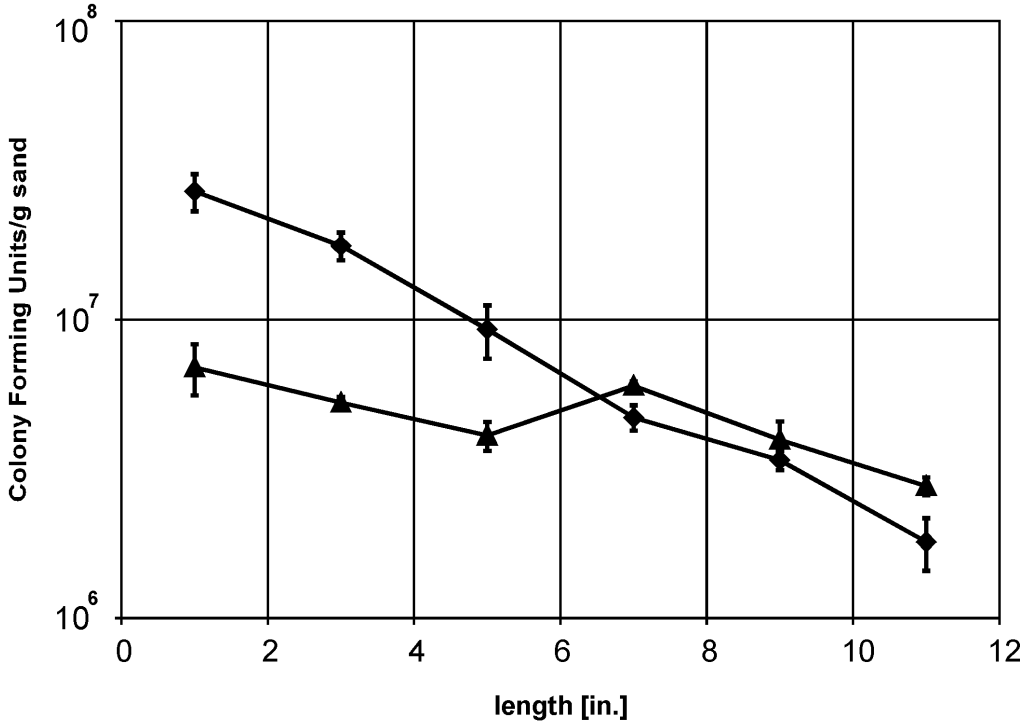

Figure 4. Breakthrough of starved and vegetative *Shewanella alga* BrY cells, measured as colony-forming units (CFU), through 10-ft-long porous media columns filled with clean quartz sand. Cell concentrations are normalized to influent cell concentrations, elapsed time (flow through volume) is normalized to pore volumes. See Table 2 for further details. Starved cells: ▲, vegetative cells: ◆.

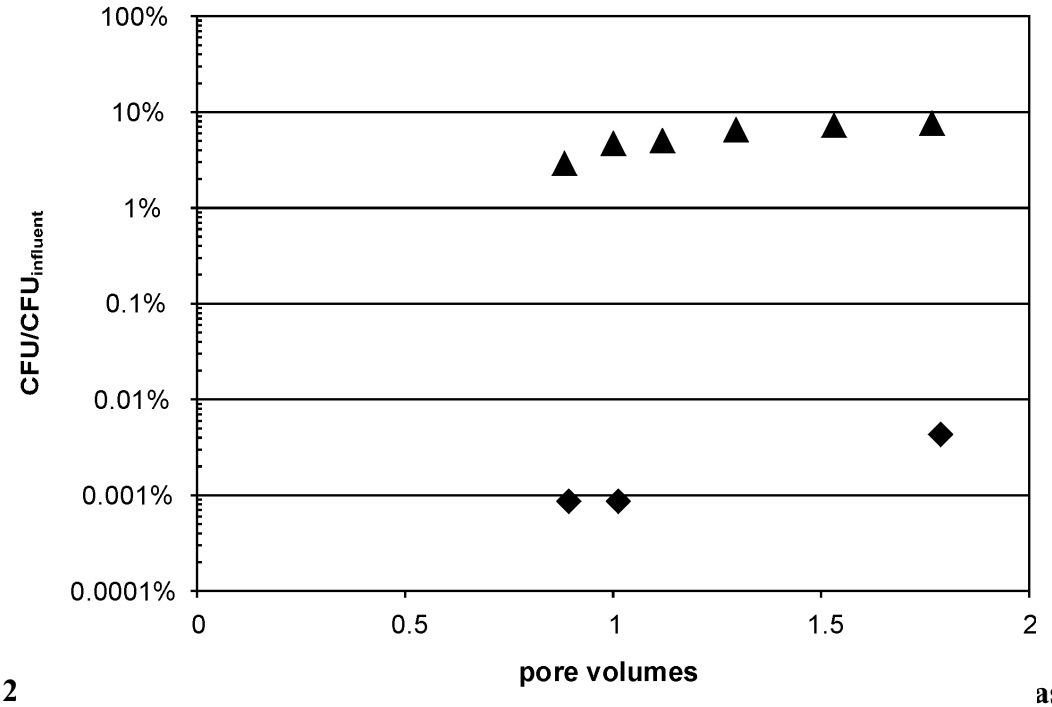

Figure 5. Distribution of starved (▲) and vegetative (◆) cells sorbed to the quartz sand throughout the 10-ft columns after injection of 2 pore volumes of cell suspension. See Table 2 for further details. The error bars represent one standard deviation.