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Abstract

Production of nuclear fuels for weapons and electric energy has resulted in groundwater uranium contamination at Department of Energy (DOE) sites. Reduction of uranium by dissimilatory metal-reducing bacteria (DMRB) is an effective approach for *in-situ* bioremediation of these sites. In this process, an organic electron donor is typically delivered through a well into groundwater in order to promote the biological reduction of soluble and toxic U(VI) to insoluble and less toxic U(IV). A key challenge is mixing the organic electron donor with U(VI) in groundwater where laminar flow conditions prevail. A potential solution is to enhance reaction beyond the scale of physical mixing by promoting extracellular electron shuttling. Growing evidence suggests that extracellular electron shuttling can occur by either diffusion of aqueous phase electron shuttles (e.g., H_2, quinones) between syntrophs and/or DMRB, or through direct electron transfer between cells through metallic-like appendage (i.e., nanow-

ires). In this project, we used pore scale, microfluidic experiments in order to elucidate cell-to-cell electron transport that can potentially enhance U(VI) reduction beyond the scale of physical mixing with an organic electron donor. Batch studies were performed to develop DMRB cultures, and to evaluate their growth with different electron acceptor and donor conditions. DMRB cultures from batch studies were used to inoculate pore scale, microfluidic reactors. The microfluidic experiments allowed direct imaging of microbial growth over various mixing length scales. *Anaeromyxobacter dehalogenans Strain K* and a mixed ground water culture were used, and we hypothesize that these organisms will enhance reaction beyond physical mixing scales by facilitated electron transfer.

Introduction

The end of the Cold War era shifted focus from the processing of nuclear weapons to the cleanup of uranium contamination at Department of Energy (DEO) groundwater sites (Hyun, Davis, Sun, & Hayes, 2012). To reduce this contamination, effective strategies for in-situ remediation of groundwater are being investigated. In particular, reduction by dissimilatory metal-reducing bacteria (DMRB) is an approach that could facilitate these needs (Wall & Krumholz, 2006). Studies done at the Oak Ridge Integrated Field Research Challenge (IFC) have shown that various strains of bacteria can grow in an anaerobic environment to reduce and immobilize uranium (Thomas et al., 2010). Reduction of uranium involves a two electron transfer, resulting in the transformation of uranium from its +6 oxidation state, U(VI), to its +4 oxidation state, U(IV). Electrons are provided from an electron donor, which is subsequently oxidized in this reaction. In this biological process, DMRB bio-films form, and they immobilize and reduce uranium (Valocchi, 2011; Lovley, Phillips, Gorby, & Landa, 1991).

Currently, models show that bacterial growth and the reduction of metals are limited by physical mixing zones. This limitation results in slow and sometimes ineffective reduction of metals in areas where electron donors and metals do not easily mix, e.g., Oak Ridge IFC. At such sites, for example, clay and mineral oxide nanopores sequester metals and make them inaccessible for bacteria resulting in a slow reduction process through diffusion by the surrounding water (Valocchi, 2011). It has been suggested that the reduction process could be enhanced by either diffusion of aqueous phase electron shut-

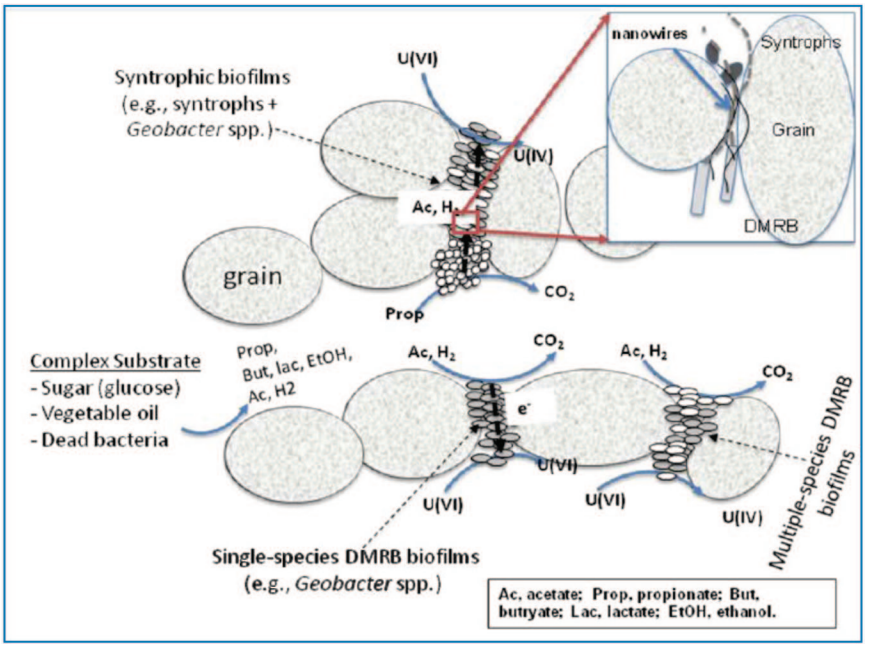

Figure 1. Illustration of enhanced electron transfer by syntrophic and similar species. (Valocchi, 2011)

tles or through a metallic-like appendage of DMRB that directly react with sequestered uranium (Valocchi, 2011; Lovley, Phillips, Gorby, & Landa, 1991; Childers, Cluto, & Lovley, 2002). These metallic-like appendages are often referred to as nanowires.

A variety of aqueous phase electron shuttles has been identified. Among the most widely studied are quinones. Multiple studies have demonstrated their ability to enhance iron reduction (Childers, Cluto, & Lovley, 2002) Natural organic matter can also act as an electron shuttle, and this may be due to the presence of quinones in these complex organic molecules (Valocchi, 2011). Intermediate or final end products of microbial processes can also act as aqueous phase electron shuttles. One very relevant example involves syntrophs. They degrade complex organics to acetate and/or hydrogen, and these end products serve as electron donors for DMRB. Syntrophs require close association with an acetate or hydrogen utilizing organisms, because elevated concentrations of these compounds inhibit syntroph activity. One example of a syntrophic association is the conversion of organic wastes to methane in anaerobic digesters (Lovley, 2011). Figure 1 depicts a synthophic relationship with DMRB.

There is also evidence that extracellular electron transfer occurs via bacterial nanowires (Reguera et al., 2005), suggesting that direct cell-to-cell contact may be responsible for transporting electrons over distances that exceed physical mixing scales. Research on iron and manganese reduction using *Geobacter sulfurreducens* has shown that under electron acceptor limited conditions, the bacteria can synthesize appendages, or nanowires, that extend beyond their physical mixing zone to reach sequestered metals (Childers, Cluto, & Lovley, 2002). If the gene(s) for nanowire synthesis and function is (are) expressed for U(VI) reduction, reduction of sequestered U(VI) could be enhanced. (Gorby, 2006). Figure 1 depicts direct electron transfer among DMRB. A better understanding of cell-to-cell interaction is required in order to determine if reduction of U(VI) can be enhanced beyond physical mixing scales in groundwater (Valocchi, 2011).

The main objective of this research project is to understand cell-to-cell interactions and enhance techniques for bioreduction of uranium along mixing zones at DOE sites.

For this project, three hypotheses will be tested:

1. Reduction of U(VI) occurs via a syntrophic mechanism, where electrons from complex substrates are delivered to DMRB via aqueous phase diffusion of H_2 between syntrophs and DMRB.
2. Electrons are transported between syntrophs, from syntrophs to DMRB, or between DMRB by direct extracellular electron transfer through nanowires.
3. Electron transfer by H_2 or direct electron transfer through nanowires enhances U(VI) reduction beyond the physical mixing zone between electron donor and U(VI).

Due to the large scope of the project, my research efforts as a summer research opportunity student at the University of Illinois focused on only one aspect of the project. Specifically, I evaluated electron transfer using alternative metals, i.e., chromate and selenite, because of their ease of handling and facile reduction compared to uranium. Chromate reduces from its highly soluble and toxic form as Cr(VI), to the less soluble and less toxic form Cr(III) (Nancharaiah, Dodge, Venugopalan, Narasimhan, & Francis, 2010). Selenite reduces from its toxic and soluble form as Se (IV) to the insoluble and less toxic form as Se (0) (He & Yao, 2009).

The objective was to determine if metal reducing bacteria grow in the presence of chromate or selenite. The specific tasks were to:

1. Measure chromate and selenite reduction in batch experiments, and determine if selected microbes can reduce the metals.
2. Evaluate chromate and selenite reduction in a microfluidic pore structure (micromodel) using the same bacteria as in task 1, and determine whether bio-films form in a flow-through system.
3. Determine in both batch and microfluidic experiments if chromate and selenite precipitate, and if they form on microbial appendages or in solution away from bacteria.

To conduct these tasks, two bacterial cultures, *Anaeromyxobacter dehalogenans Strain K* and a mixed ground water culture, were grown under anaerobic conditions in both batch and a microfulidic flow-cell experiment. Micromodels contain a two-dimensional pore network fabricated in silicon wafers using standard photolithography methods. The pore networks consist of a homogenous set of regularly spaced cylinders representing interspersed soil particles. Micromodels were used to evaluate selenite reduction at the pore scale under representative groundwater flow conditions.

Microbial Nanowires

Previously, biological electron transfer was thought to occur by electron tunneling or hoping. However, recent evidence suggests that certain bacteria can produce metallic-like conductive wires by means of pilus-like appendages (Gorby, 2006; Malvankar & Lovley, 2012). Electron transfer occurs in such wires via delocalization of electrons as shown in Figure 2. Direct electron transfer through such wires does not require thermal activation. In addition, when cooled, pili conductivity increases exponentially (a characteristic of

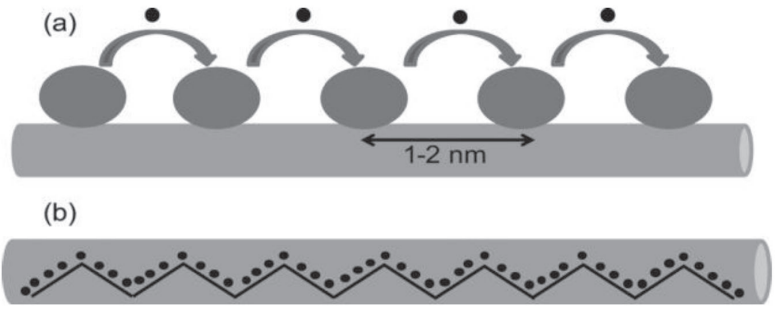

Figure 2. (a) Electron transfer by hopping or tunneling (b) Metalic-like conductivity-like conductivity. (Malvankar & Lovley, 2012)

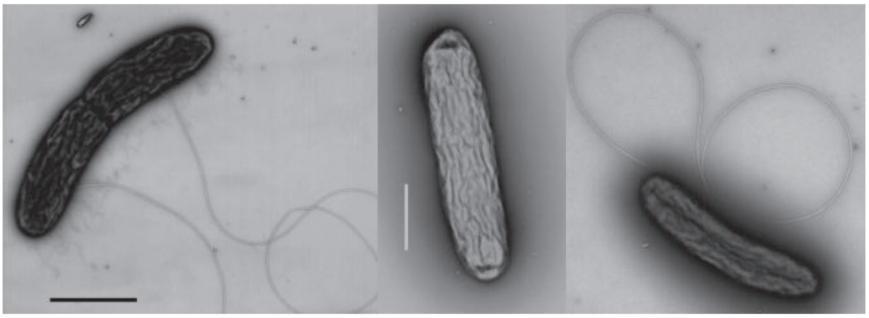

Figure 3. Images of *G. sulfurreducens* grown in the presence of Mn(IV) Oxide (left), Fe(III) Citrate (center), Fe(III) Oxide (right). (Childers, Cluto, & Lovley, 2002)

quasi-one-dimensional organic metals). In contrast, in a typical system where diffusion (hopping or tunneling) is the electron transfer mechanism, conductivity would exponentially decrease upon cooling (Malvankar & Lovley, 2012).

Studies with Geobacter sulfurreducens show that the bacteria grow pili or nanowires only when grown using insoluble Fe(III) oxide or Mn(IV) oxide, and not when grown using the soluble form of these metals. This shows that the bacteria use an alternative strategy to promote electron transfer when there is a depletion of electron acceptors (Fe(III) or Mn(IV)) in the soluble state. They express the ability to synthesize suitable appendages or nanowires to extend beyond their physical mixing zone (Childers, Cluto, & Lovley, 2002; Malvankar & Lovley, 2012). Figure 3 depicts *G. sulfurreducens* grown with insoluble Fe(III) oxide or Mn(IV) oxide and soluble Fe(III)-citrate. These images show the development of nanowires in the *G. sulfurreducens* grown with Fe(III) oxide and Mn(IV) oxide.

Experimental Methods

Bacteria and Growth Medium

A. dehalogenans Strain K and a mixed groundwater culture were obtained from Dr. Robert Sanford in the department of Geology at the University of Illinois at Urbana-Champaign. *A. dehalogenans Strain K* is a metal reducing, Gram-negative, slender rod-shaped bacterium that exhibits both aerobic and anaerobic growth (Sanford, Cole, & Teidje, 2002). The mixed groundwater culture is native to a DOE con-

taminated site. The specific bacterium in the mixed culture has not yet been identified.

The cultures were grown in 100 mL of strictly anaerobic basal medium in 125 mL serum bottles. Bacterial growth was stimulated by the coupling of sodium acetate (CH_3COONa) as electron donor and sodium nitrate ($NaNO_3$) as electron acceptor at a concentration of 3.75 and 1.25 mM, respectively. Nitrate (1.25 mM) and acetate (3.75 mM) were re-amended to serum bottles once the concentration of nitrate was reduced. Note, on most occasions, that after the first re-amendment, it took two days to reduce the second re-amendment and one day to reduce the third re-amendment. This was not consistent for all experiments. *A. dehalogenans Strain K* and the groundwater culture were incubated at 30° C and room temperature, respectively.

Batch Experiments

Batch experiments using chromate and selenite were conducted under anaerobic conditions in 125 mL serum bottles using *A. dehalogenans Strain K* and the groundwater culture grown initially on only acetate and nitrate. The cultures were inoculated with sodium chromate ($Na_2CrO_4 \cdot 4H_2O$) or sodium selenite (Na_2SeO_3) at a concentration of 100 μM after the nitrate concentration was less than 15μM. Nitrate should be a low concentration to ensure rapid metal reduction. Abiotic controls were also performed to differentiate reduction between biological and abiotic processes. Metal reduction experiments with *A. dehalogenans Strain K* and the groundwater culture were incubated at 30° C and at room temperature, respectively. Abiotic controls were performed at these same temperatures. Visual analysis of reduction for each culture was recorded. An illustration of batch experiment procedures is provided in Figure 4.

Micromodel Fabrication

Micromodels were fabricated in silicon. Silicon wafers have <100> crystal orientation and P type boron doping, are prime grade, and have a thickness of 500 μm and a diameter of 100 mm. For fabrication, a digitized image of the micromodel pore structure including inlet and outlet holes was created in AutoCAD, and transferred to a chrome-plated glass mask. The image was then transferred via ultra-violet light to a silicon wafer coated with SPR-220 photoresist. The wafer was then developed in AZ 400K developer. Pore structures were then etched into the wafer using inductively coupled plasma – deep reactive ion etching (ICP-DRIE). Analysis with a profilometer

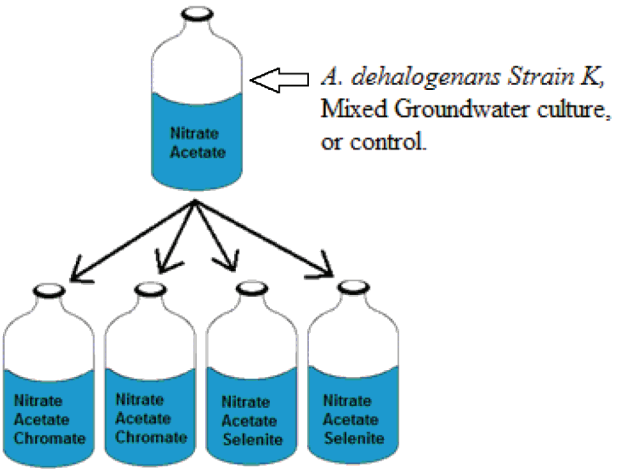

Figure 4. Illustration of batch experiments. Four samples were inoculated for each mixture.

determined an etching depth of 13 µm. After etching was complete, the microfluidic cell was heated to 1100°C in an oxidation tube furnace to create a silicon dioxide layer (130 nm) on the surface with surface properties similar to sand or quartz in groundwater. Holes were etched through the backside of the wafer to create inflow and outflow ports. The device was sealed by anodically bonding a Pyrex glass wafer of 500 µm thickness to the silicon. A Disco dicing saw was used to cut wafers into separate micromodels. Nanoports were bonded to each micromodel in an oven (Sentry 2.0 microprocessor) with a two-stage ramp up to 174°C. Impermeable polymer and halar tubing were connected to inlets and outlet. Ports and tubing were obtained from IDEX Health and Science. An illustration and image of the micromodel are shown in Figures 5 and 6.

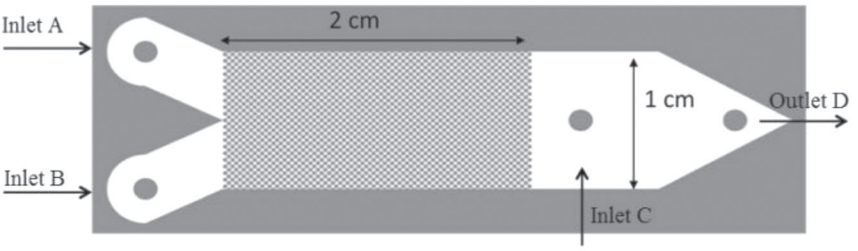

Figure 5. Illustration of micromodel. (Werth, 2010)

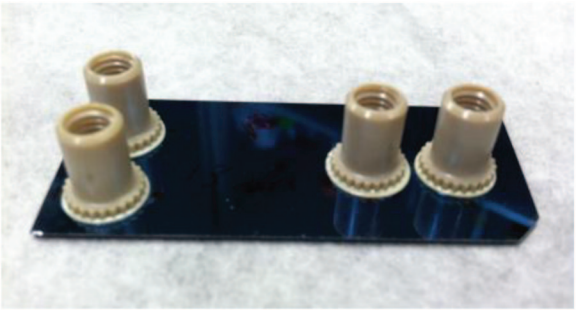

Figure 6. Image of micromodel with nanoports.

Micromodel Experiment

The micromodel experiment was conducted under strict anaerobic conditions using a syringe pump as shown in Figure 7. First, isopropanol (IPA) was degassed by sonicating under a vacuum for 5 min-

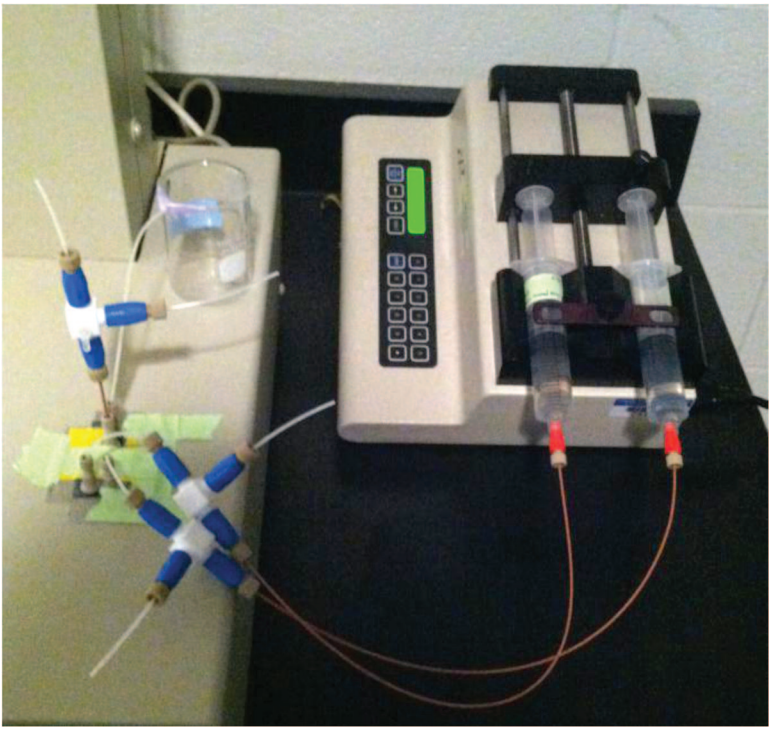

Figure 7. Image of syringe pump and micromodel.

utes. Two syringes were filled with the degassed IPA and purged through inlets of the micromodel to remove gas bubbles that may alter flow paths or disrupt the growth of bacteria. Next, water was degassed by sonicating under a vacuum for 45 minutes. Then, two syringes were filled with deionized and degassed water and purged through the inlets to flush out the IPA. Degassing prevents bubble formation in syringes and the micromodel.

After the micromodel was saturated with water, a two-stage process was conducted to establish biomass and introduce metals. Stage 1: Two syringes (denoted 1.A and 1.B) were prepared, and their solutions were purged through the system at a rate of 75 μL/hr until biofilms were established. Syringe 1.A consisted of *A. dehalogenans Strain K* in growth medium that contained remaining acetate (electron donor) and depleted nitrate; syringe 1.B consisted of pure growth medium and 1.25 mM nitrate (electron acceptor). Both mixtures were degassed before using by sonicating for 5 minutes under a vacuum. Stage 2: Two syringes (denoted 2.A and 2.B) were prepared to introduce and reduced metals. Their solutions were purged through the system at a rate of 15 μL/hr. Syringe 2.A consisted of pure media and 3.75 mM acetate (electron acceptor); syringe 2.B consisted of pure media, 24 μM nitrate (electron donor), and 60 μM selenite (electron donor). Again, both mixtures were degassed by sonication under vacuum for 5 minutes. Micromodel images were captured with an inverted microscope (Epiphot 200 series, Nikon) using a 20× objective.

Microfluidic experiments needed degassed liquids because bubbles in syringes and reactor cause issues. The media recipe used in this experiment needed CO_2 with bicarbonate to stabilize the pH. When samples were degassed, pH levels increase due to CO_2 exhaust. The pH in these experiments was around 8 to 8.5. Since growth is optimal at a pH of 7, the growth medium buffering system has been modified to contain HEPES, a nonvolatile buffer.

Results and Discussion

Batch Experiments

Images of batch experiments are shown in Figure 8. Images were taken after 10 days of inoculation with chromate and selenite. The bottle with *A. dehalogenans Strain K* (Figure 8a) showed the most re-

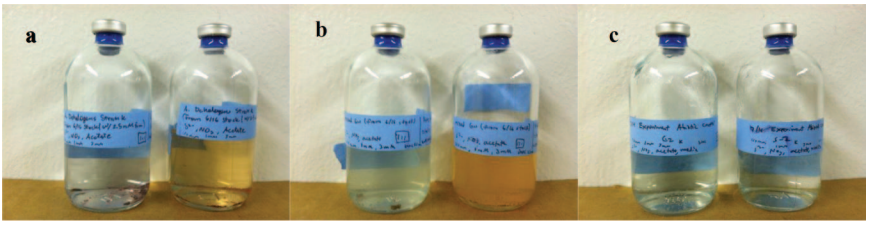

Figure 8. Each image contains chromate to the left and selenite to the right. (a) A. dehalogenans Strain K, (b) groundwater, and (c) abiotic controls respectively.

duction, based on the large amounts of precipitates in the bottles. Precipitates in this culture developed quickly, which is logical because *A. dehalogenans Strain K* is a known metal reducer. Groundwater cultures (Figure 8b) showed little precipitation or change in color. The batch experiment continues and future imaging might give evidence of precipitation by the groundwater culture. Abiotic controls (Figure 8c) show small signs of precipitates due to chemical reaction. This complicates interpretation of biogenic precipitation, and therefore needs further evaluation.

Micromodel Experiment

During inoculation (Stage 1), *A. dehalogenans Strain K* biomass was purged through system for 7 days. At the end of this time, biomass clumps were observed in the pore structure. During Stage 2, electron donor and electron acceptors were purged through the system. Precipitates formed in areas of biomass growth along the center mixing zone. Figure 9.a,b shows a montage image of the mixing zone using the 5× and 20× optical objectives, respectively. Images of single pores with biomass growth and precipitates are shown in Figure 10. Figure 10.c,d show signs of possible contamination of other bacterium, due to the variable size of the filaments. Some filaments are much larger than the typical *A. dehalogenans Strain K* bacterium. Figure 10.d depicts both of these bacteria in the same area for comparison. The images were taken 7 days after Stage 2 began. These images indicate that biofilms of *A. dehalogenans Strain K* can be established in these micromodels. Selenite reduction to Se(0) was proven and confirmed with Raman spectroscopy.

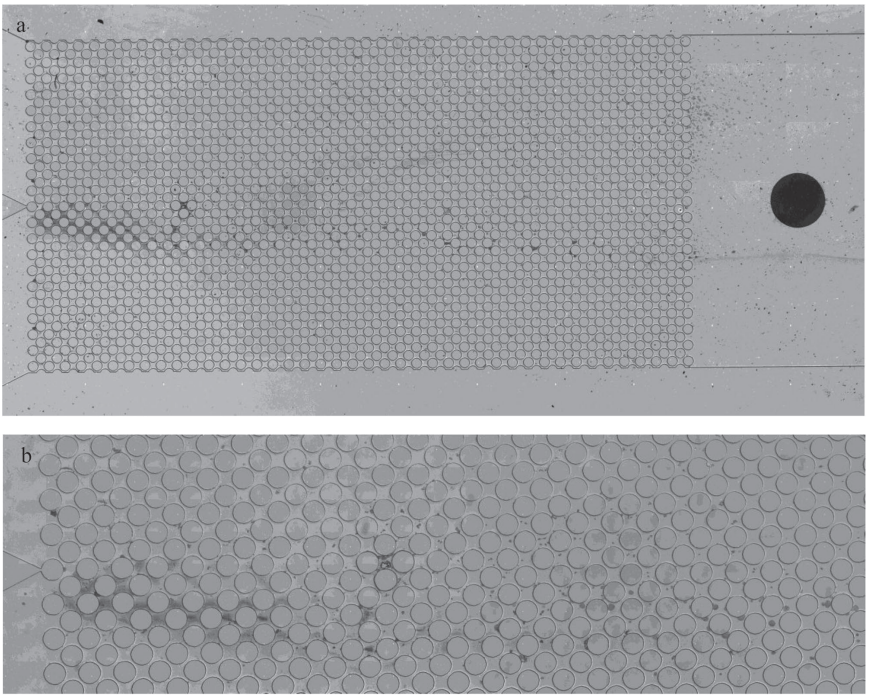

Figure 9. Montage image of micromodel depicting biomass growth and precipitates. (a) 5× optical (b) 20× optical.

Implications for Future Research

Experimental Models

Batch experiments will be conducted once more. The new batch experiments will be evaluated using ion chromatography in order to quantify the amount of selenite and chromate reduced. New micromodel experiments will be conducted with the same bacterial cultures using chromate and selenite as electron acceptors. Micromodel effluent will be periodically collected and tested to quantify metal reduction. Biofilm growth will be imaged when significant changes are observed. Montaged images of biofilm will be created and analyzed to determine growth rates over time. A third stage will be added to the micromodel experiments, where only 100μM of chromate or selenite will be purged from one syringe, and the same acetate solution from the second syringe.

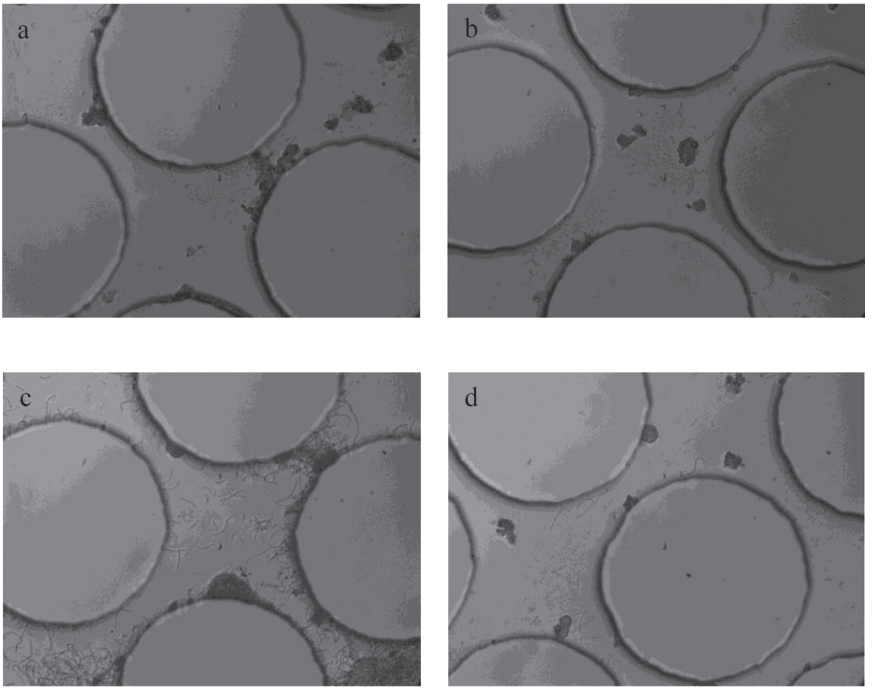

Figure 10. Images of biomass growth and precipitates. Four different locations taken during the same time.

Nanoporous Wall Barrier Micromodel

A new nanoporous wall barrier microfluidic device will be fabricated. An illustration of the micromodel is shown in Figure 11. The device is intended to stimulate the growth of nanowires in bacterial cultures by separating the bacteria from electron acceptors via a nanoporous wall barrier. This will give the bacteria an acceptor limited environment, thus inducing the synthesizes of nanowires. Theoretically, bacteria will use nanowires to directly transfer the electrons from e-acceptor to e-donor. Effluent samples will be analyzed to determine metal reduction. To determine if reduction is through nanowire direct electron transfer and not mixing by diffusion, micromodels will be fabricated with various nanoporous wall thickness.

Equation 1 (Crofts, 1996) shows that as the distance x is increased the time of diffusion increases by a squared factor. If there is a decrease in the reduction as the wall thickness increases, reduction is

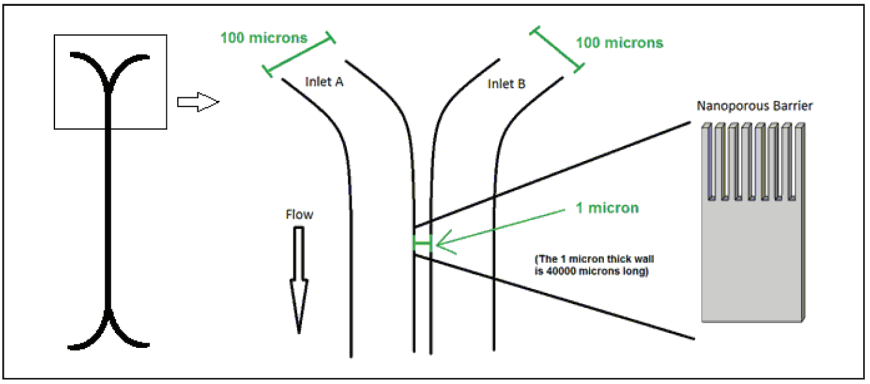

Figure 11. Illustration of nanoporous wall barrier micromodel.

through diffusion. If the reduction stays constant over increasing x distances, nanowires may be directly transferring electrons.

Diffusion equation over a distance is given (Crofts, 1996) as:

$$\langle x^2 \rangle = q_i D t \tag{1}$$

where:

$\langle x^2 \rangle$ — mean-square displacement (x is the mean distance from the starting point that a molecule will have diffused in time, t)

q_i — numerical constant which depends on dimensionality

D — diffusion coefficient

t — time.

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