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An Experimental and Theoretical Approach to Visualize Dechlorinating Bacteria in Porous Media

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Introduction

Chlorinated solvents, such as trichloroethene (TCE), are common groundwater contaminants in the U.S. (Westrick et al., 1984). These compounds, which are sparingly soluble and denser than water, typically form globules and ganglia in the subsurface environment (Schwille, 1988) that bleed contamination into groundwater over long periods of time. As such, the efficacy of remediation by groundwater extraction (so-called "pump-and-treat") is limited by solvent dissolution in coarse grained sediments, and by diffusion in fine grained sediments, leading to estimated cleanup times typically approaching decades to centuries (National Research Council, 1994, Pankow and Cherry, 1996).

A number of alternative cleanup approaches have been developed that may accelerate remediation (EPA, 2001). One such approach is *in situ* bioremediation, which relies on microorganisms to degrade contamination in the subsurface. The microbiology is largely understood for the biodegradation of chloroethenes (e.g., Maymo-Gatell, 1997) and chloromethanes (e.g., Dybas et al., 2002), in which the relevant microorganisms use the contaminants as an energy and carbon source. Such microorganisms have been introduced into the subsurface at many contaminated sites to initiate or enhance the biodegradation of contaminants (Major et al., 2002, Dybas et al., 2002). At some sites, introduced microorganisms have been detected in downgradient wells (Major et al., 2002), indicating some degree of transport within permeable, coarse grained units of the subsurface. What has not been addressed adequately is how bioremediation can be used to remediate the fine grained sediments that bleed contamination in a diffusion-limited fashion. Specifically, it is unknown if introduced microorganisms will populate only coarse grained sediments in an aquifer, or if they can populate the interface between coarse and fine grained sediments, given their metabolic requirements. If microorganisms can populate such an interface, it would suggest that fine grained sediments could be enveloped with a "rind" of biological activity that consumes contamination as it diffuses out of the sediments. Furthermore, it is also unknown if microorganisms can survive for long periods of time on a diffusion-limited supply of contaminant emanating from the fine-grained sediments while still maintaining contaminant concentrations to below cleanup goals. If the survivability of microorganisms under these conditions could be demonstrated, then ideally bioremediation could be used as a long-term "polishing step" to keep contaminant concentrations in compliance, after pump and treat has removed the bulk of the contamination from permeable sediments.

The goal of this study is to understand how anaerobic dechlorinating bacteria are distributed in porous media following injection, in the context of the issues listed above. To address this goal, a series of experiments were conducted involving KB-1, a commercial microbial consortium containing *Dehalococcoides* bacteria, the only genus of organisms known to completely dechlorinate TCE into the benign end product ethene. KB-1 has been used to bioaugment

several TCE contaminated sites (Major et al., 2002). This study is comprised of three components:

1. *Development of a methodology for imaging the distribution of active KB-1 in transparent and translucent materials*. This effort has involved identifying a vital stain – a fluorescent dye which emits light at a specific wavelength when excited in metabolically active organisms – which could be used to macroscopically image the growth of KB-1 as a function of time.
2. *Analog porous media imaging*. Using the vital stain, this experiment aimed to image the activity of KB-1 in a translucent sandbox containing an initial concentration of TCE, subject to a steady-state flux of simulated groundwater containing nutrients. The sandbox was packed with different size classes of sand, emulating zones of differing hydraulic conductivity.
3. *Investigation of KB-1 migration at the aquifer scale*. This experiment entailed the development of a methodology to identify specific organisms in the KB-1 consortium in groundwater samples collected as part of an ongoing bioaugmentation treatability study conducted at LLNL's Site 300. The purpose of this experiment is to constrain the migration distance and metabolic activity time span of the injected KB-1 population across a scale of meters in a low-permeability aquifer setting.

Each of these components is discussed separately below.

1. Vital Stain Identification

As part of this study, a novel method for microscopic visualization of metabolically active, fluorescing anaerobic organisms was developed using vital stains which were mixed with the KB-1 culture using an airtight slide preparation protocol. Vital stains are fluorescent reagents (fluorogenic esterase substrates) which emit light upon excitation within a metabolically active cell. Calcein, for example, is a fluorescent dye which emits light at a wavelength of 515 nm (green) when excited by a 495-nm light wavelength source. After transport across the cell membrane, intracellular esterases alter the molecule, resulting in fluorescence. Because dead cells lack active esterases, only living cells fluoresce. A suitable concentration of such cells provides the opportunity to image the evolution of a KB-1 population under conditions favorable for growth.

1.1 Materials and Methods

KB-1 and reagents (i.e., candidate fluorescein dyes) were mixed in an anaerobic setting and the fluorescence was observed through airtight microscopic slide preparations.

1.1.1 KB-1 Culture Handling

Anaerobic shipments of KB-1 and its growth medium (≤100 ml) were received from SiREM Labs (the commercial vendor of the KB-1 culture) and refrigerated 24-72 hours until utilized. Typically, the culture was initiated the week prior to shipment. Upon receipt, the KB-1 culture was transferred into an anaerobic glove box (Coy Laboratory Products) and incubated for at least 24 hours prior to sampling or other manipulations. The internal atmosphere of the anaerobic box was maintained at approximately 10% CO_2/5% H_2/85% N_2. The glove box atmosphere was circulated through a Pt catalyst box, catalyzing the reaction of H_2 with residual O_2, an essential step to prevent exposure of the KB-1 organisms to O_2 which could destroy the culture. Ambient temperature was maintained between 21-25°C (i.e., room temperature). The KB-1 culture was kept from light except when manipulated.

1.1.2 Growth and Staining of KB-1

Sampling of the KB-1 culture entailed transferring 1.8 ml aliquots to small amber vials. The candidate fluorescent reagent(s) (Molecular Probes/ Invitrogen) were constituted at a concentration of 10 mM; 1.8 µL of the reagent solution was used to inoculate the 1.8-mL aliquots. The KB-1 culture and dye vials were kept in the anaerobic glove box. The procedure then called for a 170-µL aliquot to be removed and transferred to an Eppendorf tube for epifluorescent microscopy. The tube was spun for at least 5 minutes at 6,000 RPM (i.e., 2,000 g). The supernatant was pipetted off and the pellet was resuspended in 100-µL, 0.1-M PO_4 buffered pH 8.0 solution.

Microscope slide preparations were made inside the glove box at various following the addition of the fluorescent reagent to the KB-1 culture. The slides were prepared as soon as a few hours and up to a month post-addition of stain reagent(s). The slide preparations allowed for an airtight KB-1 + stain sample to be made for epifluorescent microscopy. A conventional glass microscope slide was used (75 x 25 x 1 mm) with a 13-mm diameter x 012-mm deep adhesive spacer (Secure-Seal, Molecular Probes/Invitrogen). This formed a well to which 10-20 µL of resuspended KB-1 + stain was added. Subsequently, a 1 oz.-, 18-mm glass cover slip was overlain upon this configuration. Finally, another adhesive spacer was placed over the glass cover slip, cementing it to the first spacer/glass slide.

1.1.3. Microscopic Visualization

Images were obtained using an inverted microscope, a Zeiss Axiovert 200M equipped with biological objectives. Epifluorescence illumination utilized an EXFO X-cite 120 illumination system and a Zeiss FITC Filter Set 44. The images were captured with a Zeiss Axiocam HRc REV 2 digital CCD camera. Images were primarily taken at 630X magnification, with or without use of oil immersion. The driver software used to collect the images was Axiovision Rel 4.3. Videos were produced using an upright microscope, Zeiss Axioskop. This microscope was equipped for epifluorescence using a 100W HBO burner, a FITC filter set, and a Nikon D90 12.3-megapixel SLR camera.

1.2 Results

A number of candidate flourescein dyse were tested, including Calcein-acetoxymethylester (calcein-AM), which is widely used for determining cell viability and was initially considered as a likely best-candidate vital stain. Although the Calcein AM dye yielded acceptable results, the most promising candidate dye identified was 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester, 5(6) CFDA, SE, which exhibited very strong fluorescence. Example imaging results are shown on Figures 1.1 through 1.5.

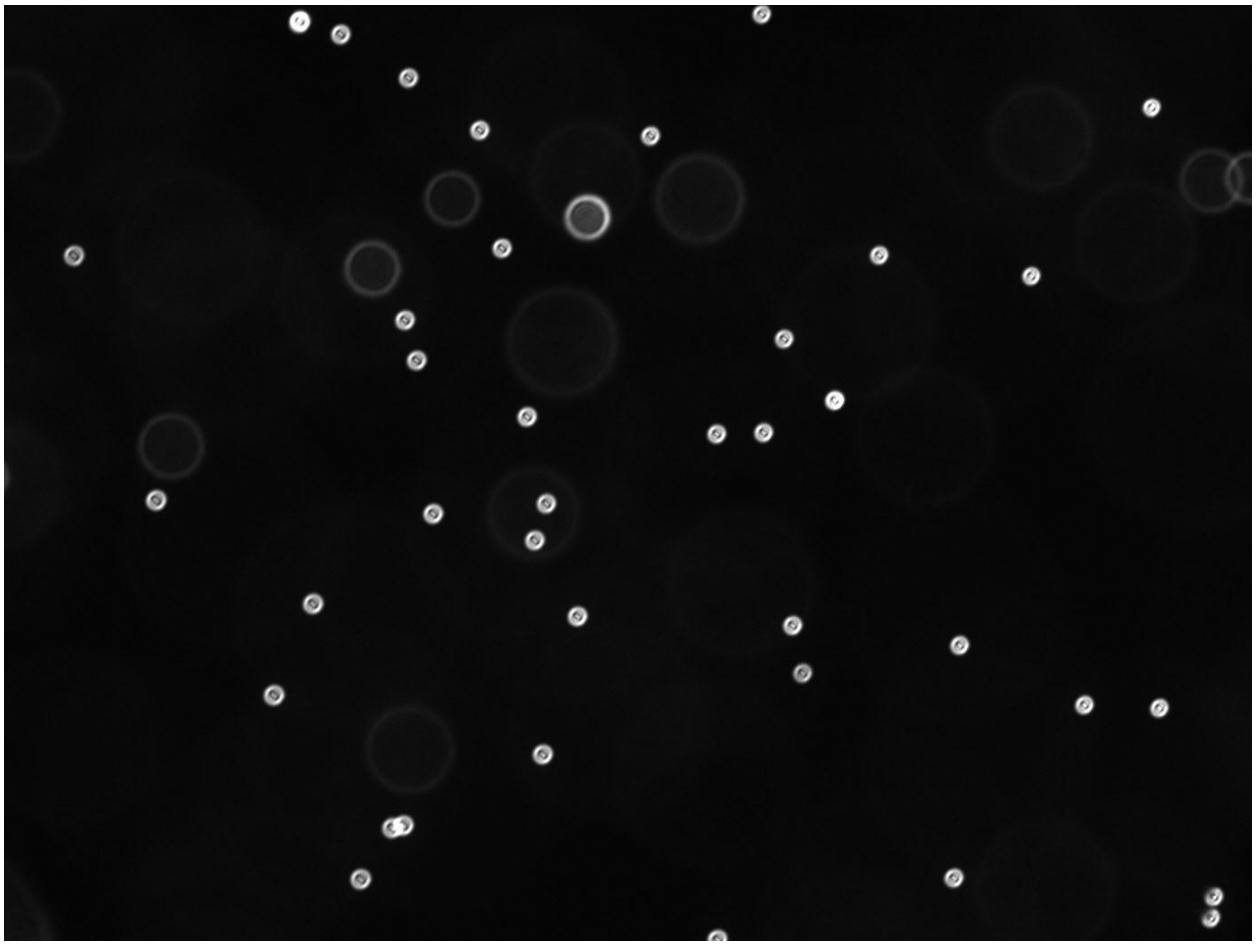

Figure 1.1. Photomicrograph of fluorescent microspheres used to test and calibrate imaging methodology (630X magnification, with FITC filter).

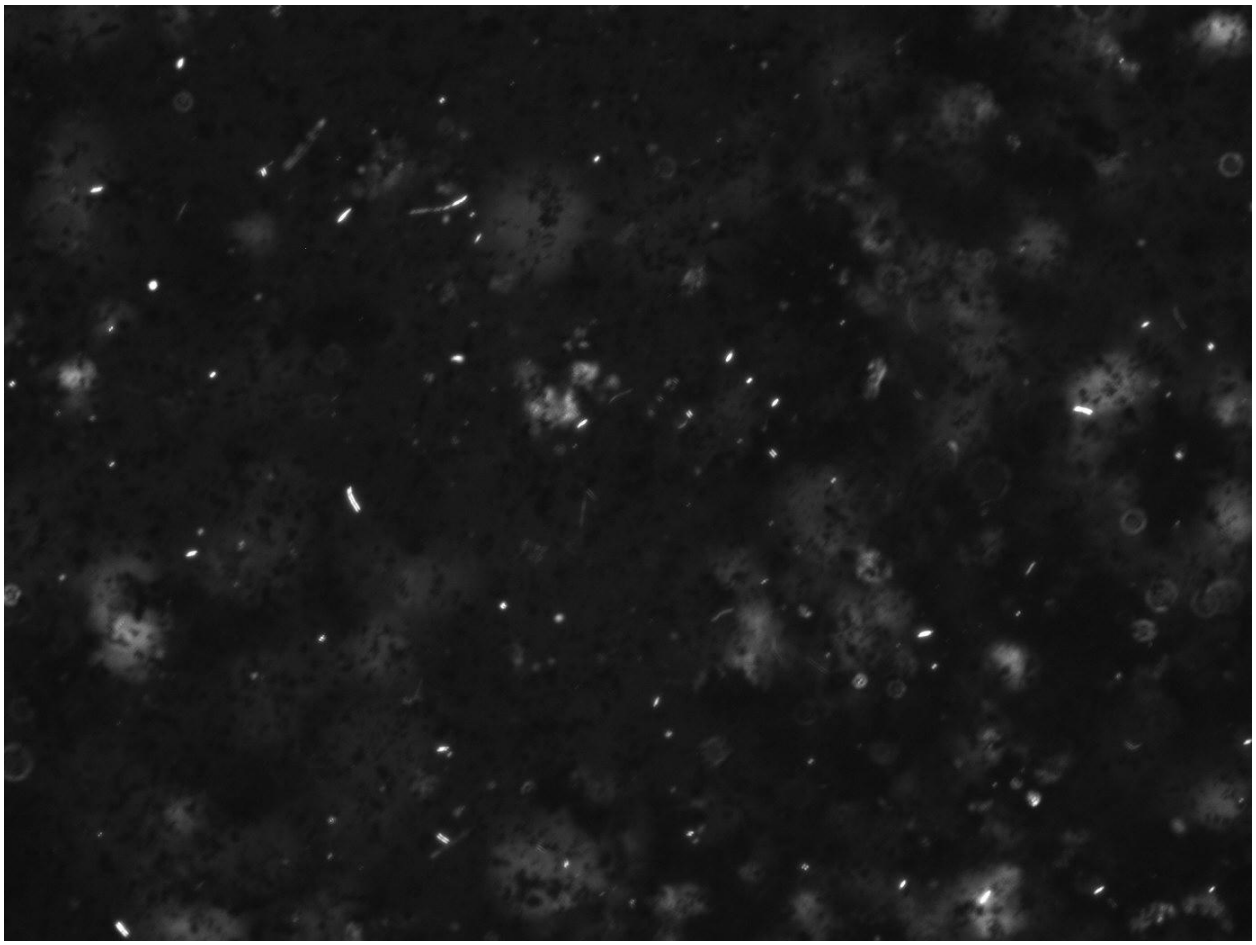

Figure 1.2. Photomicrograph showing KB-1 bacterial culture exposed to Calcein AM (200X magnification, with HD/FITC filter.

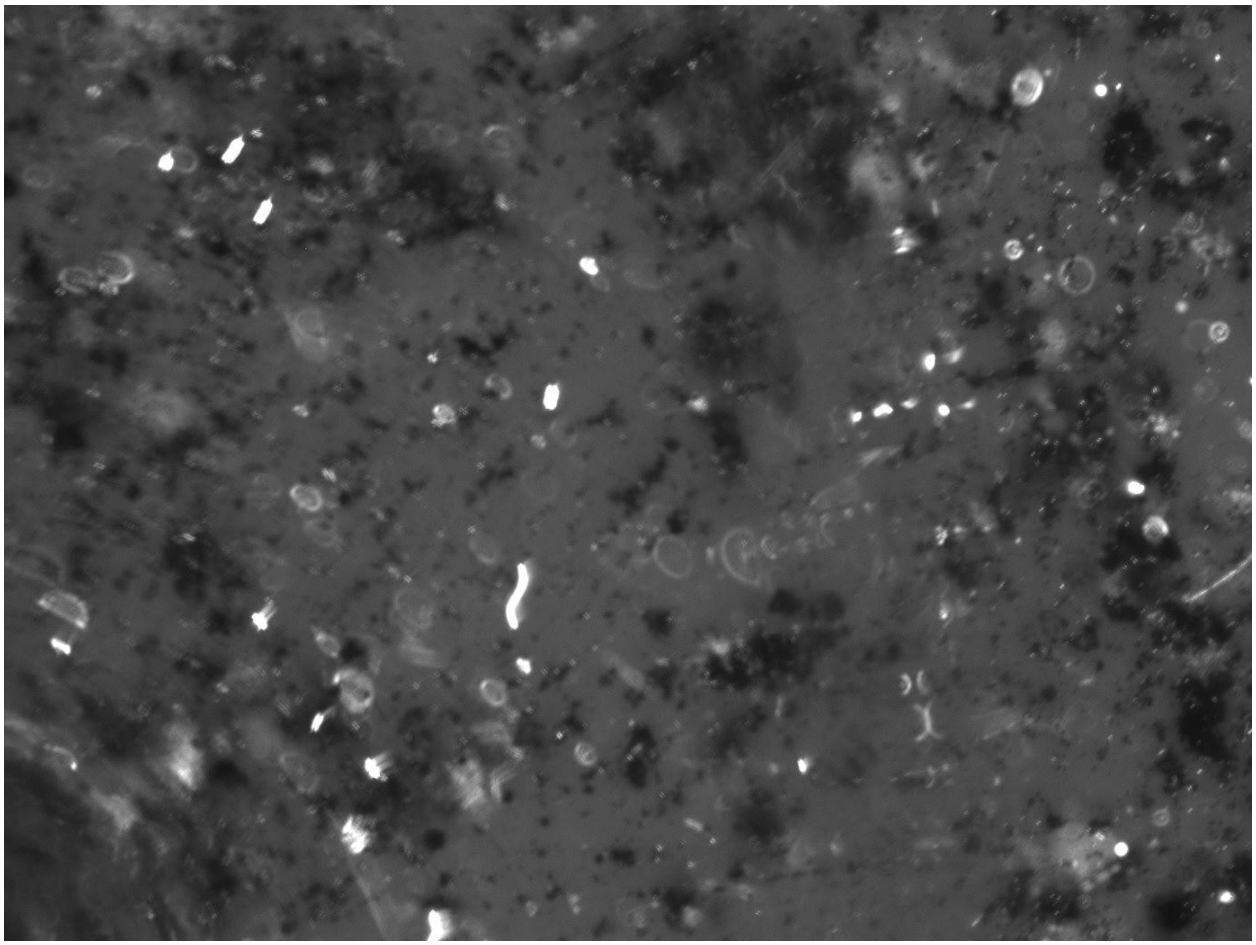

Figure 1.3. Photomicrograph showing KB-1 bacterial culture exposed to CFDA dye (200X magnification, with HD/FITC filter). Dark material is ferrous sulfide present in KB-1 growth medium as an oxygen scavenger.

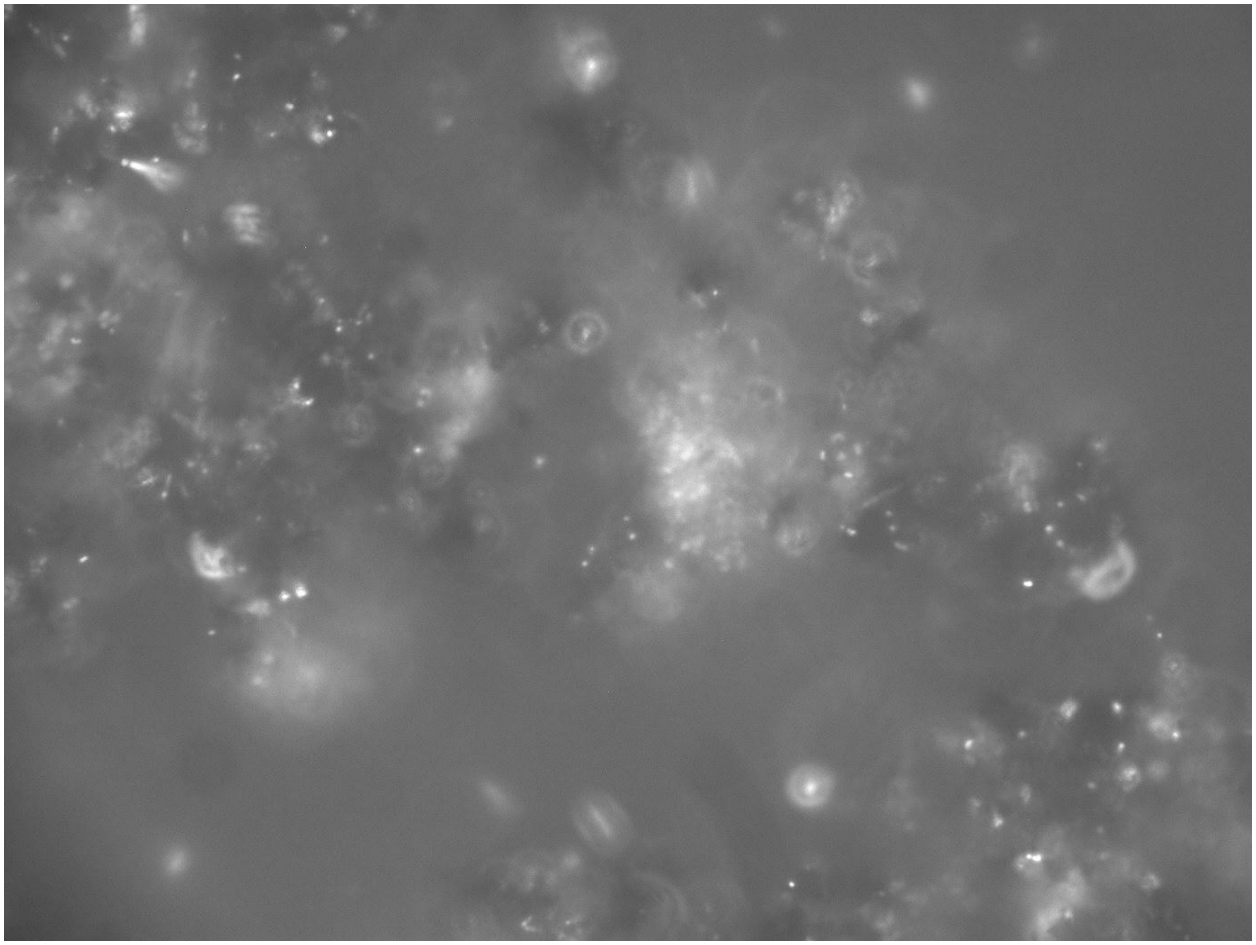

Figure 1.4. Photomicrograph showing KB-1 bacterial culture exposed to CFDA dye, with fluorescent microspheres for reference (630X magnification, FITC filter, oil immersion).

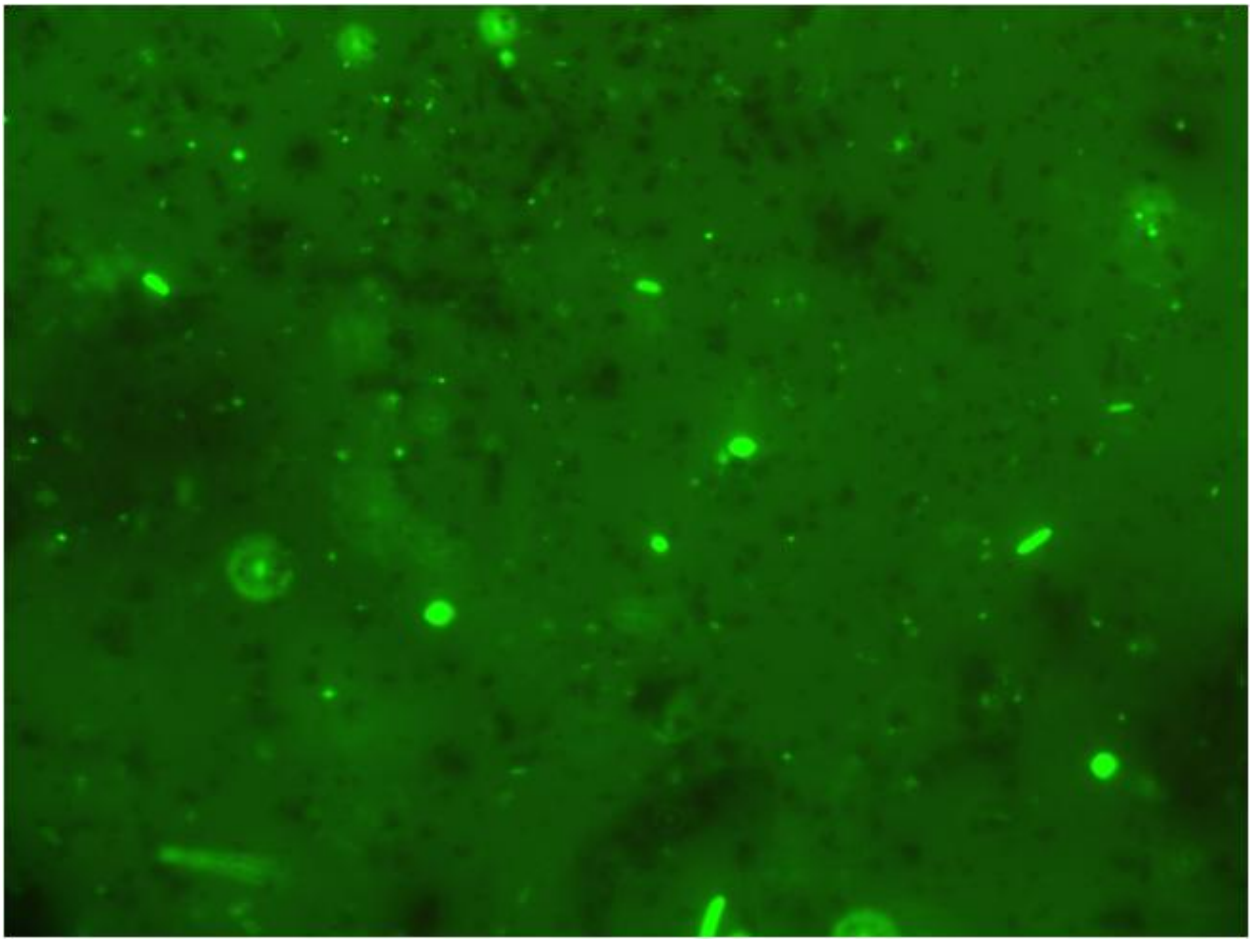

Figure 1.5. True-color photomicrograph of KB-1 bacterial culture exposed to CFDA dye (630X magnification, multidimensional imaging, oil immersion). Note differences in size and morphology of individual bacteria from the culture.

2. Analog Porous Media Imaging Experiments

2.1 Experimental Design

Experiments were designed for a translucent sand chamber with the objective of visualizing biological activity resulting from the injection of KB-1 into a layered sand system containing water partially saturated with TCE (Figure 2.1). To simulate the influence of low-permeability lenses, in which dissolved chlorinated solvents are expected to persist over long periods, the sand chamber was packed with 1/3 fine sand (Ottawa F-110) and 2/3 coarse sand (Ottawa F-30), which have hydraulic conductivities that differ by more than two orders of magnitude.

The sand chamber was constructed by compressing two sheets of 12-mm glass against a 1-cm-thick spacer resulting in a 30 x 60 x 1-cm chamber. A groove milled in the lateral spacers and separated from the sand by a fine stainless steel mesh allowed the establishment constant hydraulic head boundary conditions along each edge of the chamber. The top and bottom of the

chamber were sealed with no flow boundaries that included individual ports for filling and draining the chamber. The result was a completely water- and air-tight system. During all experiments, flow was pushed through the chamber at constant flow rate by a Masterflex pump motor with a ceramic flow-metering pump head (FMI, Inc.). The chamber was designed to allow flow across the width at constant flow rate with uniform hydraulic head along the lateral boundaries.

The two layers of sand were dry packed in the chamber by pouring the sand through a hopper system attached to the top of the chamber. The hopper contained a series of screens that randomized the falling sand grains, and a vibrator was attached the chamber frame to enhance settling of the sand grains. This approach minimized any settling of sand during subsequent experiments. Because settling during experiments made it difficult to quantitatively compare sequences of images acquired over time, improvement of this technique was a critical component in developing quantitative visualization techniques. Prior to beginning the experiments, the system was saturated by first purging with CO_2, followed by gradual filling from bottom to top with de-aerated, de-ionized water.

2.2 Preliminary Flow-Through Experiment

For biodegradation to provide an effective means for removing TCE from contaminated fine-grained, low-permeability sediments, the supply of TCE diffusing from low-permeability layers must be sufficient to meet the demands of the organisms in the KB-1 culture. As such, a preliminary experiment was designed to investigate the rate at which a contaminant diffuses from the low-permeability layer in the experimental system under typical subsurface conditions.

The chamber was first saturated by a solution of de-ionized water with 1/8 g/L FD&C Blue #1 dye. Subsequently, the dye solution was slowly displaced by steady flow of de-ionized water from left to right across the chamber. Images acquired during the experiment allowed direct quantification of the concentration boundary layer that formed along the layer between coarse and fine sand (Figure 2.2). During this experiment, the uniform gradient applied along each edge of the chamber resulted in linear flow across the chamber. This included flow through both the coarse and fine layers, though the flow was much slower in the fine-grained layer. In natural layered systems, flow will largely bypass the fine layers. Reduced advection in the fine-grained layers will cause the transient boundary layer observed at the interface in the experiments to become much more persistent because transport into the coarse-grained sediments will be largely controlled by diffusion.

2.3 KB-1 Experiment

A second experiment was carried out to evaluate imaging KB-1 culture growing within the chamber. This entailed replacing the broad-spectrum white light source with a filtered light source. To aid in visualizing the KB-1, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl

ester (CFDA) was introduced into the water in the chamber during the experiment. To optimize the conditions for culture growth, the de-ionized water in the chamber was completely displaced by the KB-1 growth media provided by SiREM Labs. In addition to a wide range of inorganic compounds included to favor growth, the growth media contained 25 mg/L of dissolved TCE, 10 μM CFDA, and a mixture of methanol/ethanol to provide a carbon source for the culture. KB-1 was then introduced to the chamber by connecting an airtight sample bag containing approximately 200 mL of growth media and active KB-1 culture to the chamber. After the culture was pumped into the chamber, the flow system was switched to allow recirculation of fluid through the chamber. The images shown in Figure 2.3 depict the evolution of emitted light from the chamber, normalized by an image of the chamber acquired immediately prior to injection of the KB-1 culture. Dark grays represent a reduction in light emission and light grays an increase. In general, the emitted intensity decreased over the two-week duration represented in these images. This suggests that there is a decay of the fluorescence over time (not observed during microscopic studies), or a loss of light transmission in the sand chamber, perhaps due to buildup of biofilms in the pore space. Interestingly, very little change in intensity was observed in the fine-grained layer. As with the conservative tracer experiment shown in Figure 2.2, it is likely that the injected KB-1 preferentially flowed through the coarse layer. The absence of measurable intensity shifts in the fine layer thus further supports the hypothesis that the dark region above the fine-coarse interface is related to biofilm accumulation.

2.3 Results

Taken together, the results of the flow-through experiments demonstrate that it is possible to directly image the distribution of active KB-1 organisms in translucent porous media. However, suspected biofilm accumulation, other than serving as a qualitative indicator of bacterial growth, appears to present a significant impediment to extracting long-term quantitative data on KB-1 growth and mobility. A key question is whether the suspected biofilm is associated with the KB-1 organisms or is the result of growth of unrelated organisms. This is important because the latter could be controlled through more through sterilization of the experimental apparatus, whereas the former would likely present an inherent challenge to applying the imaging technique to this particular consortium. Addressing this specific question would require detailed microbial characterization beyond the scope of the current study.

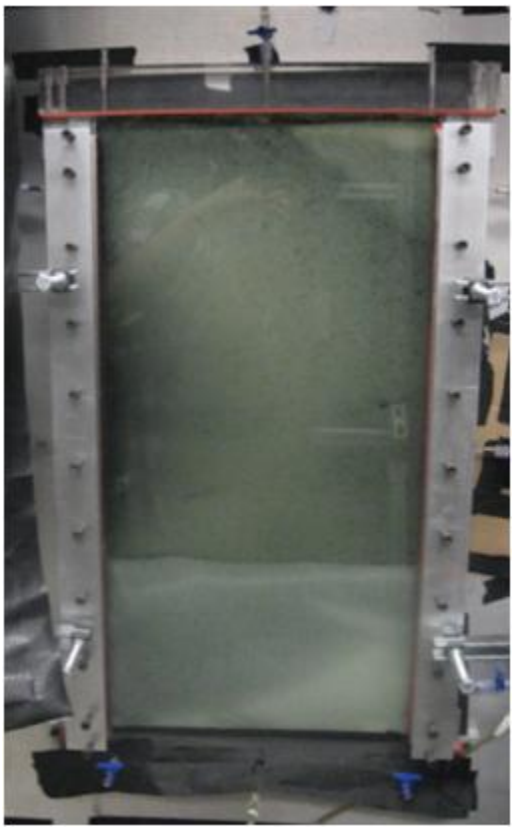

Figure 2.1. Test chamber filled with F110 sand (bottom) and F30 sand (top).

Figure 2.2. Left-to-right flow of de-ionized water displacing dyed water from the chamber.

Figure 2.3. Time sequence images spanning 16 days (left to right) of the KB-1 + CFDA flow-through experiment, with emitted light indicated by gray shading and suspected biofilm formation indicated by darken regions.

3. KB-1 Migration at the Aquifer Scale

The subsurface transport of organisms comprising the KB-1 culture, relative to the point of injection, was investigated at the field scale using a PCR amplification technique to identify KB-1 in groundwater samples collected at a bioaugmentation treatability test location. The treatability test, undertaken as part of the groundwater characterization and remediation effort at LLNL's Site 300, entailed the injection of both KB-1 and a carbon source, sodium lactate, which serves as an electron donor/oxygen scavenger, into an initially aerobic shallow aquifer characterized by a very low hydraulic conductivity. Geochemical evidence supporting the local development of anaerobic conditions as well as dechlorination reaction daughter products of TCE have been identified in the injection well as well as in a nearby monitoring well. A key question, therefore, is whether the reaction products were transported from the immediate injection well environment to the monitoring well location by advection or whether KB-1 organisms became active in the vicinity of the monitoring well. If the latter could be demonstrated, this would provide some insight into the mobility of KB-1 in low-permeability porous media.

3.1 Treatbility Study Background

Shallow groundwater in the vicinity of the B834/T2 area at Site 300 (Figure 3.1) is contaminated with high concentrations of TCE (exceeding 10 mg/L, or 2,000 times the maximum permissible regulatory concentration of 5 µg/L) and other dissolved chlorinated solvents as a result of historical usage in nearby buildings. As part of an attempt to address the contamination, a bioaugmentation treatability study was initiated in 2008 to evaluate the efficacy of KB-1 injected into the aquifer in dechlorinating the contaminants. Under the treatability study, sodium lactate was injected to facilitate the develpment anaerobic conditions conducive to the survival of the KB-1 organisms. An isotopic tracer (water supplied from the Hetch Hetchy reservoir in the Sierra, characterized by a different oxygen isotopic signature than local groundwater) was included in the injection sequence to provide a means for quantifying contaminant dilution resulting from the sodium lactate injection (as opposed to contaminant transformation via *Dehalococcoides*-facilitated dechlorination). Specifically, the sequence of the treatability study events included:

1. A total of approximately 10,600 L of Hetch-Hetchy water and1,060 L of Na-lactate were injected between June 2007 and December 2008 into well W-834-T2.
2. In August 2008, groundwater in the T2 area was bioaugmented with KB-1 after geochemical indicators (oxidation-reduction potential; concentrations of nitrate, manganese, and methane) suggested that anaerobic conditions had been established in the T2 area. Bioaugmentation entailed the injection of a 10-L slurry of KB-1 and its growth medium into well W-834-1825.

3. Groundwater water quality data, including concentrations of geochemical indicators, dissolved chlorinated hydrocarbon concentrations, and ethane concentrations were periodically measured in T2 area monitoring wells throughout 2008 and 2009. The appearance of ethene is specifically indicative of dechlorination reactions.

Groundwater quality data indicate anaerobic conditions were achieved in wells W-834-T2, W-834-1825, and W-834-1833 during the monitoring period. For example, methane in groundwater increased to maximum concentrations of 15,000 µg/L in well W-834-T2 and 19,000 µg/L in well W-834-1825 during the monitoring period. However, the methane production appears to be localized to the treatment zone in the immediate vicinity of these two wells, as methane levels in nearby well W-834-1833 were measured at a maximum of 50 µg/L. By late 2008, manganese concentrations increased from less than 0.10 mg/L to a maximum of 0.92 mg/L in groundwater from well W-834-T2 and to 10.0 mg/L in groundwater from well W-834-1825 during 2008, again suggesting the localized development of reducing conditions. Consistent with the development of a reducing environment, nitrate concentrations declined by up to an order-of-magnitude in the treatment zone as a result of denitrification by natural bacteria that convert NO_3 to N_2 gas.

In association with the changes in oxidation-reduction conditions, the total dissolved chlorinated hydrocarbon species concentrations in wells W-834-T2 and W-834-1825 declined significantly by mid-2009 (from 10-mg/L levels to 60.9 µg/L and 51.7 µg/L, respectively). Chlorinated hydrocarbon concentrations in other nearby monitoring wells exhibited little or no change during the same time period. The TCE dechlorination daughter products *cis*-1,2-DCE and vinyl chloride were measured at concentrations of 430 µg/L and 190 µg/L, respectively, in well W-834-T2 by late 2009. Ethene, the final end product of the TCE dechlorination sequence, was detected at concentrations up to 970 µg/L (well W-834-T2) and 37 µg/L (well W-834-1825).

3.2 Materials and Methods

A polymerase chain reaction (PCR) technique was used to amplify the 16S rRNA gene and TCE degradation genes (reductive dehalogenase homologous, or rdh) from *Dehalococcoides* species present in groundwater samples collected from well W-834-T2 and well W-834-1825 during sampling events in April and August, 2009. The PCR methodology is described below.

3.2.1 Preparation of Genomic DNA

DNA sources for KB-1 were provided by SiREM Labs in two separate shipments from different culture batches. Both batches were prepared using the CTAB methodology, which entails freezing (with liquid N_2) and subsequently thawing cells to induce lysis, adding sodium dodecyl sulfate for organelle dissociation, adding proteinase K for cellular protein removal/digestion, adding CTAB (hexadecyltrimethylammonium bromide) to remove cell wall debris, polysaccharides, and remaining proteins by selective precipitation, and finally precipitating DNA

with isopropanol. A commercial DNA preparation kit (Promega-Wizard Genomic DNA purification kit) was also used for one of the aliquots of KB-1 culture.

3.2.2 PCR Methodology

PCR primer sets were designed for a "universal" bacterial 16S rRNA gene as well as a *Dehalococcoides*-specific 16S rRNA gene. TCE degrading primer sets were designed for rdh genes tceA, vcrA, and bvcA. These genes were included because these are the most common sets used to test for anaerobic TCE degradation. Also included were pceA (DET0318), DET1494, and DET1559, all primer sets targeting regions meant to be specific to *Dehalococcoides* ethenogenes 195. PCR protocol included addition of standard reagents and polymerase under a temperature cycling regime. PCR instrumentation included the Gene Amp® PCR System 9700 (PR Applied Biosystems).

PCR products were visualized in 2% agarose gels run with a 1X TAE (Tris Acetate-EDTA) buffer. For electrophoresis, gels were typically run at 80 volts for 60 min. Ethidium bromide was used to stain DNA products (0.5 μg/ml).

3.3 Results

The PCR analysis conducted under this study indicates that groundwater samples from both sampling events from wells W-834-T2 and W-834-1825 all appear to contain DNA associated with *Dehalococoides*, or at the very least an organism/organisms that amplify most of the rdh genes (Figure 3.1). To assess the consequences of this finding, it is important to consider that, in principle, there are three possible explanations for the detection and persistence of ethene in well W-834-T2, the monitoring well nearest the KB-1 injection well, W-834-1825:

1. A hydraulic connection exists between the two wells and KB-1 bacteria migrated to well W-834-T2 over a period of months, possibly much sooner.
2. KB-1 is active only in the immediate vicinity of well W-834-1825 as a consequence of limited subsurface mobility and only the reaction products migrated to well W-834-T2.
3. Complete dechlorination of TCE was achieved at well W-834-T2 by indigenous bacteria stimulated by the electron donor (Na-lactate) addition.

Past laboratory microcosm studies using Site 300 sediments have suggested that indigenous bacteria lack the enzymes necessary to subsequently transform the intermediate dechlorination daughter product *cis*-1,2-DCE to vinyl chloride and subsequently ethane, implying that the third explanation is unlikely. As such, the PCR analyses conducted in this current study imply that the first explanation represents the most likely case as *Dehalococoides* was found in the monitoring well some 7 meters away from the injection well. This discovery of KB-1 in the monitoring well demonstrates some degree of mobility for this organism in very fine-grained, low-permeability materials. Moreover, the detection of live organisms months after nutrient source cessation (Na-

lactate injection) supports their viability and productivity over time scales which may be relevant to groundwater remediation efforts.

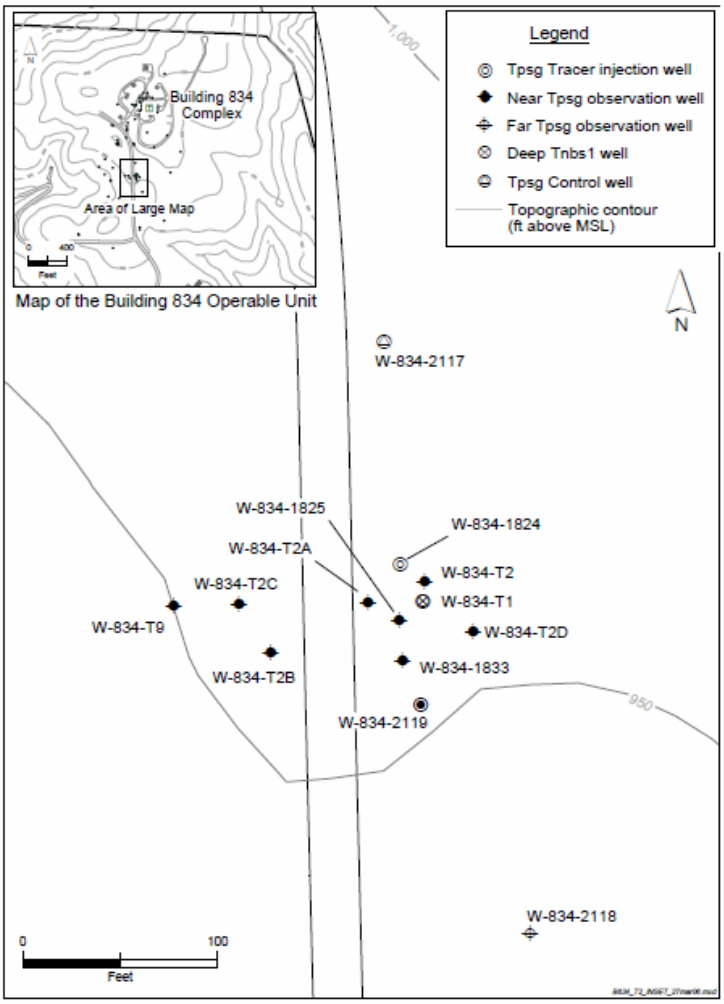

Figure 3.1. Map of the B834/T2 area at Site 300, indicating locations and types of wells used in the bioaugmentation treatability test.

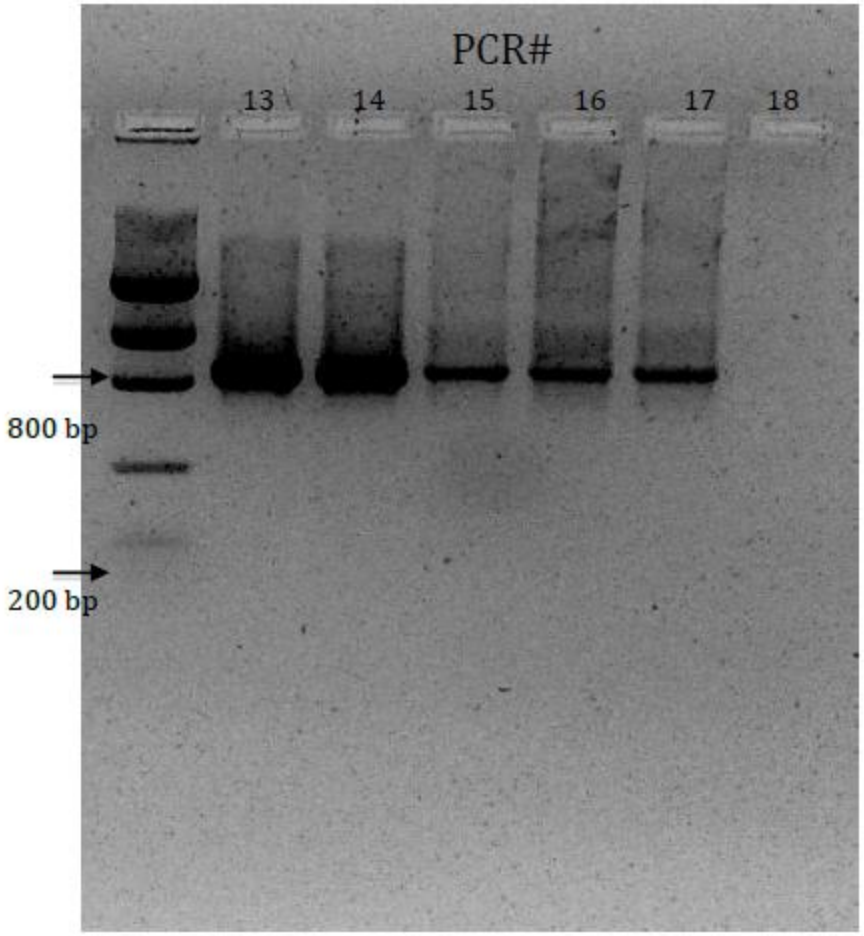

Figure 3.2. PCR products: template/primer set (left), *Bacillus Sterne* (16S.517.F-1406.R), along with *B. Sterne* (second sample), KB-1, W-834-1825, W-834-T2, no DNA; #13-18, respectively. Product size: 889 bp.

Acknowledgements

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