BIOFILM BARRIER FORMATION AND PERSISTENCE IN VARIABLE SATURATED ZONES

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ABSTRACT

A novel strategy for containment of contaminated groundwater consists of a biologically produced extracellular polysaccharide (EPS) barrier, i.e. biobarrier, within a subsurface formation. To bring this technology closer to field scale, a PVC column (12 inch [30.5 cm] diameter x 48 inches [121.9 cm] high) was constructed to examine the effects of thick biofilms in porous media under radial flow conditions. Bacteria (*Pseudomonas fluorescens*, a facultative anaerobe capable of denitrification) and nutrients were injected down a center injection well and allowed to flow radially outward through a sand-gravel mixture to the edge of the column. The bacteria were allowed to grow on the surface of the porous medium and produce thick biofilms that reduced the hydraulic conductivity of the soil. Nitrate was added as the electron acceptor to the column to facilitate denitrification during anaerobic growth. Bacterial growth created a reactive biobarrier that not only reduced the hydraulic conductivity of the soil, but also completely utilized nitrate and dissolved oxygen within 24 hours, creating anaerobic conditions throughout the column. Introduction of bacteria and nutrients above the static water level generated a biobarrier in the vadose zone. Creation of an elevated biobarrier in the field offers the potential for hydraulic containment of a dissolved contaminant plume.

Key words: *biobarrier, subsurface containment, bacterial transport, biofilm accumulation*

INTRODUCTION

More than half the population (52.5%) of the United States relies upon a groundwater source for drinking water (Fetter, 1993). The release of toxic chemicals from industrial and agricultural wastes is of major environmental concern, and successful containment and remediation are essential to protect downstream resources. Selective plugging of permeable strata is currently being explored as a means of preventing migration of groundwater contaminants from hazardous waste sites, and penetration of starved microorganisms into porous media and subsequent resuscitation by nutrient addition is a conceivable method for this subsurface plugging.

The uniform distribution of bacteria in porous media necessary to prevent contaminant migration is dependent on the successful transport of bacteria and nutrients through the ground. Injection of bacteria and/or nutrients into the subsurface may cause plugging near the injection well, limiting the distance bacteria can be transported through porous media (Shaw et al., 1985). Starving the bacteria results in a reduction in cell size and biofilm production that prevents plugging near the injection well and allows the bacteria to transport farther through porous media (MacLeod et al., 1988; Lappin-Scott et al., 1988; Lappin-Scott and Costerton, 1992; and Cusack et al., 1992). Ongoing Center for Biofilm Engineering (CBE) investigations using thick biofilms as a subsurface biobarrier have shown at the bench scale to be a promising alternative for the containment of hazardous waste plumes (Cunningham et al., 1991; Warwood et al., 1995; James et al., 1995; and

Cunningham et al., 1997). These laboratory experiments have demonstrated the effectiveness of injecting both bacteria and then nutrients into porous media, producing a thick, uniform biomass matrix capable of reducing aquifer permeability/hydraulic conductivity. Use of biobarriers in the field would involve injecting starved bacteria and then nutrients into a series of shallow wells. Sufficient amounts of nutrients would be added after bacterial inoculation to produce overlapping columns of soil in which pore space is virtually sealed by bacterial growth and EPS (extracellular polysaccharide) production (Costerton, 1994).

Traditional subsurface barrier technology such as slurry walls, funnel and gate, and hydraulic containment sheets are usually not very cost effective. Other technology, such as cryowall technology, is limited to small applications and cold climates due to the need for a continuous energy demand and a limited radius of influence. Biobarrier technology is believed to be very cost-effective to implement, maintain, and remove. In addition, biobarrier technology can be combined with bioremediation technologies to simultaneously degrade a contaminant while hindering its migration. Examples include funnel and gate technology, where strings of biobarriers are used to funnel a contaminant into a gate area where remediation of the contaminant can be performed on a localized scale. Also, a bacterial strain could be isolated from a hazardous waste site for its ability not only to form a stable biobarrier, but also to degrade the contaminant in question. This reactive biobarrier could degrade a contaminant while slowing its migration. Another type of reactive biobarrier creates anaerobic conditions in and downstream of the biobarrier through the activity of the bacterial species. The anaerobic environment created could be used to prevent the bacterial oxidation of sulfide minerals, such as acid-generating mine tailings.

To date, no formal field test of reactive or static biobarrier technology has been performed. The need to bring this technology toward field testing is imperative in determining the capabilities of biobarrier technology as a feasible alternative for the containment and bioremediation of contaminants from hazardous waste sites.

The research presented herein addresses several questions concerning the feasibility of this technology in the field. These questions include the following: Can a barrier be created above the static water table, thus preventing groundwater from short-circuiting above the established biobarrier (i.e. can a biobarrier be created in the vadose zone)? Can a biobarrier be created and maintained under radial flow conditions (field-scale flow conditions)? Can microbial activity within a biobarrier create anaerobic conditions within and downstream of a biobarrier? Can a biobarrier be used to degrade a contaminant while reducing its migration from a hazardous waste site?

MATERIALS AND METHODS

Bacterial Strain

The bacterial isolate used for this experiment was *Pseudomonas fluorescens*. This strain was isolated from a petroleum-contaminated hazardous waste site and chosen for its role in the intrinsic bioremediation of petroleum hydrocarbons, ability to denitrify, and ability to form a stable biobarrier.

Media and Inoculum Preparation

Forty liters (two pore volumes) of nutrient media and bacteria were injected into the column (36.5 L of molasses medium, 3 L of vegetative inoculation culture, and 0.5 L of starved inoculation culture). The molasses medium contained 10 g/L molasses, 0.123 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 1.0 g/L NaCl, 3.0 g/L $NaNO_3$, 2.0 g/L NH_4NO_3, and 0.05 g/L yeast extract. Molasses was determined to be a cost-effective carbon and energy source for the particular hazardous waste site where the *P. fluorescens* was isolated. Ammonia was used as the nitrogen source and nitrate was used as the electron acceptor to facilitate denitrification. The *P. fluorescens* vegetative batch culture was grown in the same molasses medium except for a 10-fold increase in the potassium concentration (1.23 g/L K_2HPO_4 and 0.4 g/L KH_2PO_4). The starved culture was prepared using a modified procedure described by James (1998).

Microscopic Direct Counts

Samples obtained from the constant head tank were first transferred to a fixative solution with a final concentration of 4% formaldehyde and stored at 4°C. The microscopic direct counts were performed using a DAPI staining technique with an Olympus BH2 fluorescence microscope (Kepner and Pratt, 1994). Total cell counts were averaged from at least ten microscopic fields (replicate fields were analyzed until a total of approximately 300 cells were counted).

HPLC (high performance liquid chromotogaphy)

Nitrate and nitrite concentrations were analyzed using HPLC. Samples obtained from the constant head tank were filtered through a 0.2 mm filter and stored at -70°C. The samples were then analyzed using the Dionex DX300 ion chromatography system with a 2 x 250 mm IonPac AS4-SC anion exchange column and a carbonate/bicarbonate eluent. Dionex OnGaurd-Ag filters were used to remove chloride from the samples, which interfered with the nitrite determinations.

Reactor Design

A 4-foot(1.22 m)-high, 1-foot(0.3 m)-diameter radial flow column was constructed from polyvinyl chloride (PVC) pipe material (Figure 1) and filled with a sand and gravel mixture obtained from a field site. A ¾-inch (1.9 cm) perforated injection well was installed in the center of the column to allow water, bacteria, and nutrients to be fed from a constant head tank above the column down through the center injection well and radially out into the soil. Piezometer ports were situated on the surface of the column and outer tub to monitor the water level both inside and outside the

column. Ports were located at 15 inches (38.1 cm), 29 inches (73.7 cm) and 43 inches (109.2 cm) above the bottom of the column. Water was pumped from the outer tub up to the constant head tank providing constant recirculation of the system.

Reactor Operation

The radial flow lysimeter was first characterized without bacteria or nutrient addition. This included monitoring the water level inside and outside the perforated PVC column with various flow rates to provide an indication of ambient water level fluctuations with various flow rates. Flow rates were determined using a graduated cylinder and stopwatch. Heterotrophic plate counts (HPC) using non-specific growth media (Bacto R2A Agar, Difco Laboratories) were used to determine background levels of the microorganisms present in the non-sterile soil.

After sufficient characterization was performed, the molasses-based nutrient media and bacteria were fed by gravity into the reactor from the constant head tank. Heterotrophic plate counts, microscopic direct counts, and HPLC (high performance liquid chromotogaphy) were performed at time t= 0, t= 6hr, t= 24hr, t= 48hr, t= 72hr, t= 5d, and t= 7d. Additional tests including Pseudomonas Isolation Agar (Difco Laboratories) plate counts and denitrifying MPNs (most probable numbers) (Gerhardt et al., 1994) were performed at time t=0, t=24hr, and t=7d.

The hydraulic conductivity (K) of the column was obtained by using Darcy's Law (Equation 1):

$$K = \frac{Q}{A}\left(\frac{\Delta L}{\Delta H}\right) \tag{1}$$

Where Q is the volume of water that travels through the column over time and A is the area perpendicular to flow (the cross sectional area of the PVC column). The length the water travels through the soil (ΔL) is the distance between the top of the soil in the column and the outer water tank. The change in hydraulic head (ΔH) was obtained by measuring the difference between standing head of water on top of the column and the outer water table. The center injection well was plugged with sand, bacteria, and nutrients, and perforations on the outside of the column were sealed with duct tape to minimize short-circuiting in the system.

RESULTS AND DISCUSSION

The column was inoculated with 40 liters (~ two pore volumes) of molasses media with starved and vegetative bacterial cultures. The addition of bacteria and nutrients to the radial flow column resulted in an increase in the water level inside the column (Figure 2). The initial column water level was five inches (12.7 cm) above the outer water table due to mounding effects around the injection well. The column water level was raised an additional 15 inches (38.1 cm) 24 hours after inoculation. The water level then remained constant throughout the experiment. It should be noted that perforations throughout the surface of the PVC column allowed water to flow freely out

of the column into the outer tub (Figure 1). These results indicate that injection of bacteria and nutrients in the vadose zone can create a perched biobarrier capable of reducing hydraulic flow over a saturated zone barrier.

The initial hydraulic conductivity of the sand and gravel column was approximately 4 cm/min. Twenty-four hours after nutrient addition, the hydraulic conductivity was measured to be 1 cm/min. Through bacterial inoculation and nutrient addition, a biobarrier was formed in the radial flow direction that reduced the hydraulic conductivity of the porous media by 75%.

The ambient microbial population before inoculation ($t \leq 0$ days) ranged from 1×10^6 to 1×10^7 cells/ml (Figure 3). Twenty-four hours after inoculation with *P. fluorescens* (2×10^7 cells/ml) and nutrient media, the bacterial population in the column increased approximately two orders of magnitude to 5×10^8 cells/ml and remained relatively constant throughout the experiment (Figure 3). Heterotrophic (R2A) and Pseudomonas Isolation Agar (PIA) plate counts were also performed on the column for the duration of the experiment (Figure 4). Results from the Pseudomonas Isolation Agar (PIA) plate counts show that the *Pseudomonas* species was not detected above 1×10^4 cfu (colony forming units)/ml before inoculation. Twenty-four hours after inoculation, the *Pseudomonas* population was 66% of the total population. This percentage decreased to 19% of the bacterial population one week after inoculation. Comparison of microscopic direct counts to HPC indicated that the majority of the cells in the column were culturable.

Denitrification is the process by which nitrate is converted to nitrite, which is then converted to nitrogen gas. To promote denitrification in the column, nitrate was used as the electron acceptor. Before inoculation there were minimal nitrate and nitrite in the column (1.2 mg/l and 2.1 mg/l, respectively). The concentration of nitrate added to the column during inoculation was 500 mg/l. Six hours after inoculation, the nitrate concentration decreased from 500 mg/l to 144 mg/l and the nitrite concentration increased slightly to 7.9 mg/l. Nitrate and nitrite were both completely utilized in the column after 24 hours. The decrease in nitrate and the increase then subsequent decrease in nitrite from the system show the creation and maintenance of a reactive biobarrier capable of degrading nitrate. Redox potential and dissolved oxygen (D.O.) concentration measurements were recorded throughout the column 24 hours after nutrient addition (-207 ± 8 mV and 0.07 ± 0.01 mg/l, respectively). These results also indicate that denitrification is occurring in an anaerobic environment.

CONCLUSION

The injection of bacteria and nutrients into porous media resulted in a reduction of the porous media permeability in field-scale flow conditions (radial flow direction). This experiment explained how short-circuiting over the top of an established biobarrier could be minimized through bacteria and nutrient injection in the vadose zone. In addition, bacterial growth created and maintained a

reactive biobarrier capable of completely utilizing a contaminant (nitrate) while creating anaerobic conditions in the system.

The potential of using bacteria injected into the subsurface as a means to contain and degrade contaminants in groundwater is a conceivable alternative to other subsurface containment technologies. The full potential for this technology depends on the success of a field-scale application. These results bring biobarrier technology closer to this goal.

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Figure 1. Radial flow column (4 feet [1.22m] high x 1 foot [0.3 m] diameter) filled with a sand-gravel mixture. Water, bacteria, and nutrients were fed from the constant head tank, down the center injection well, and radially out into the soil.

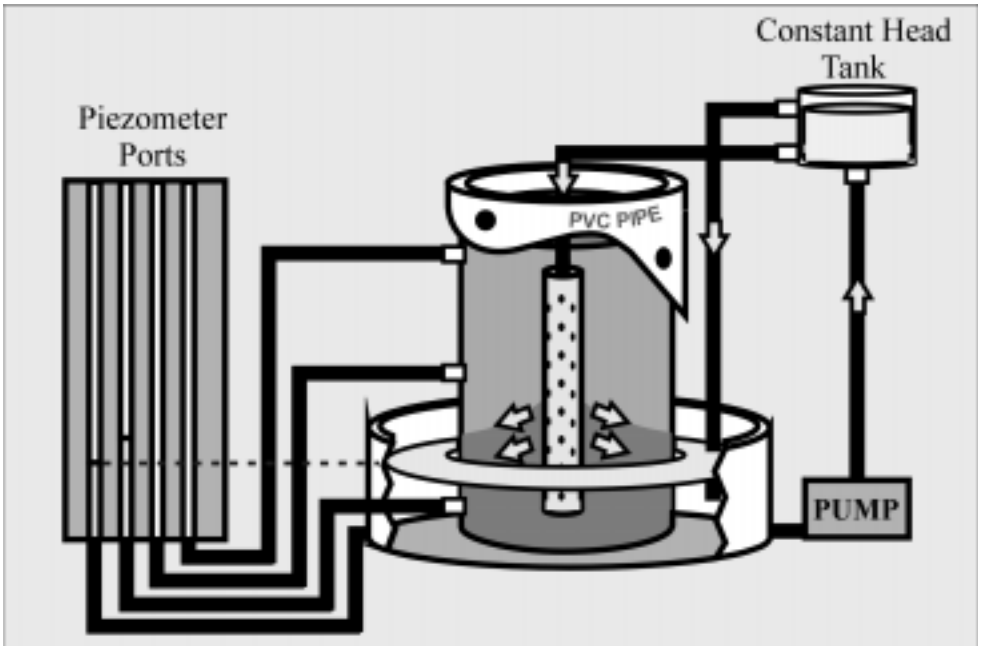

Figure 2. A biobarrier was generated in the vadose zone 24 hours after the introduction of bacteria and nutrients above the static water level. This vadose zone biobarrier remained stable throughout the experiment.

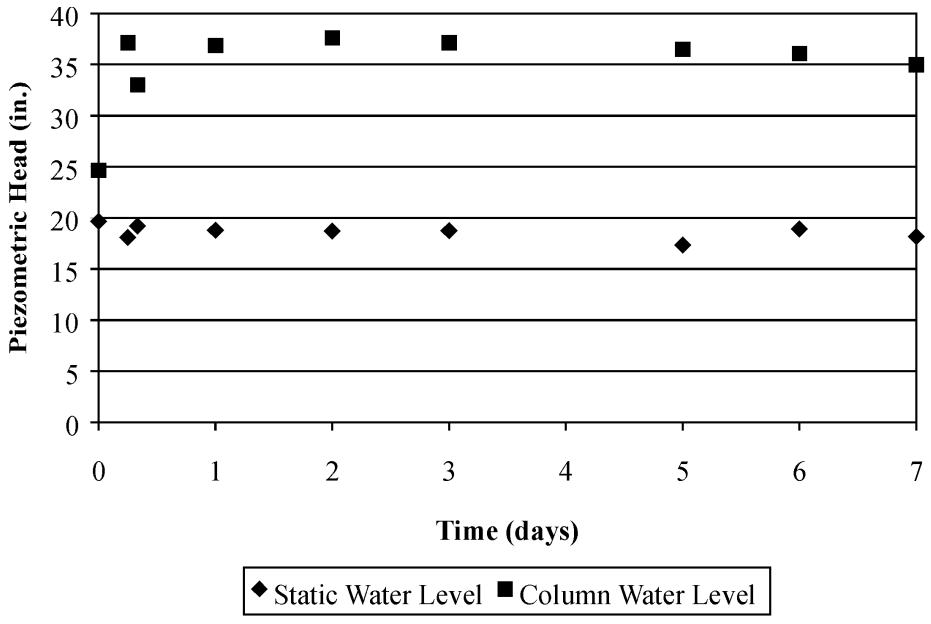

Figure 3. The microscopic direct counts show a two-orders-of-magnitude increase in the bacterial population 24 hours after the injection of bacteria and nutrients. This increase was maintained over time. Error bars indicate +/- the standard deviation.

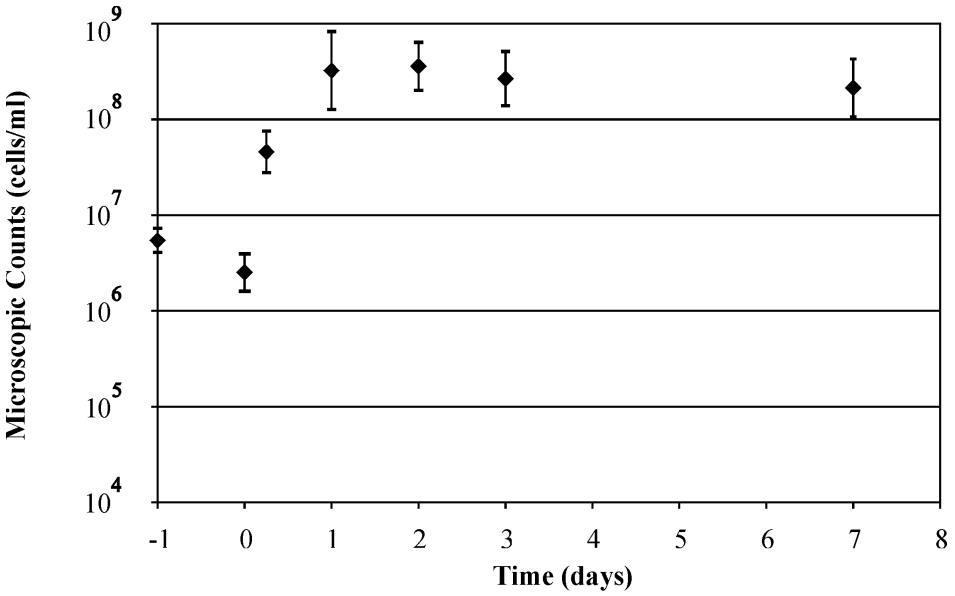

Figure 4. The heterotrophic (R2A) plate counts and Pseudomonas Isolation Agar (PIA) plate counts show more than a two-log increase in the bacterial population 24 hours after injection of bacteria and nutrients into the column. The *Pseudomonas* species was not detected above 10,000 cfu/ml before inoculation.