# EVALUATION OF SUBSURFACE BIOBARRIER FORMATION AND PERSISTENCE

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## ABSTRACT

The effectiveness of microbial biobarriers for reducing the hydraulic conductivity of porous media was evaluated using cylindrical columns (6" diameter x 36" length) packed with sand. The columns were inoculated with starved cell suspensions of Klebsiella pneumoniae. Resuscitation of starved bacteria resulted in a uniform increase in cell numbers throughout the column and a corresponding decrease in hydraulic conductivity. Reductions in hydraulic conductivity were compared for columns packed with F70 and F110 sand (initial permeabilities 27 and 5.3 Darcies, respectively). Although the F70 sand columns had an initial hydraulic conductivity approximately an order of magnitude higher than the F110 sand columns, biobarrier formation resulted in stabilization at a similar minimum hydraulic conductivity. Challenge of an established biobarrier with starvation conditions resulted in a loss of biobarrier integrity after five to ten days. However, the biobarrier rapidly reformed following reintroduction of nutrients. Souring of the columns also resulted in a loss of biobarrier effectiveness. Overall, the results of this study suggest that biobarrier technology is a feasible method for the manipulation of permeability and mass transport properties of porous media.

## KEY WORDS

biobarrier, porous media, plugging, starved bacteria, bioremediation

## INTRODUCTION

One of the major environmental problems facing society today is the contamination of ground water resources by toxic chemicals from industrial and agricultural wastes. Ground water supplies over 50% of the fresh water used in the United States and this increases to over 90% of supplies in rural America [1]. In situ bioremediation technologies are a promising approach for the successful clean-up of contaminated aquifers, but these technologies require considerable development. One such technology is the formation of microbial biobarriers to manipulate the permeability and mass transport properties of porous media. This approach has the potential to reduce the migration of contaminants from hazardous waste sites and also be coupled with contaminant biodegradation or biosequestration.

Subsurface biobarrier formation has been studied in relation to secondary oil recovery, where microbial plugging near the well bore has a deleterious effect by reducing the injection rate during waterflood operations [2]. Conversely, the addition of bacteria and/or nutrients to model subsurface formations resulted in selective plugging of high permeability zones which enhanced secondary oil recovery from zones of lower permeability [3-6]. Biobarrier formation is

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governed by general processes involved in biofilm accumulation, such as microbial cell adsorption, desorption, growth on surfaces, detachment and filtration [7].

Research into the microbial plugging of porous media has identified several important factors for successful biobarrier formation in laboratory-scale systems. The injection of bacteria and/or nutrients into core systems typically results in plugging near the core inlet [5, 8]. This shallow-plugging phenomenon was mitigated through the use of starved bacteria for injection [9, 10]. Starvation of bacteria results in a survival response that includes phenotypical changes in cell morphology and physiology [11]. Changes in bacterial morphology during starvation often include a reduction in cell size [12-14], which may enhance the transport of these bacteria through porous media [9, 10]. Furthermore, the lag-time associated with starvation recovery [11] may increase nutrient penetration through porous media by temporarily reducing consumption near the injection site. This delayed response has been enhanced through resuscitation of the starved bacterial inoculum with less easily utilizable nutrient sources, such as citrate [10].

The growth of injected bacteria results in significant reductions in the permeability of porous media [5, 8-10]. An important factor in this permeability reduction seems to be the production of extracellular polysaccharides (EPS) by bacteria [2, 9, 15]. These extracellular polymers likely aid permeability reduction by several mechanisms, such as enhanced cell retention (i.e., biofilm stability) and enhanced accumulation of organic and inorganic matter by filtration.

Although biobarrier technology is a promising bioremediation strategy, several important questions must be answered for successful scale-up to a field-relevant scale. Previous biobarrier research has utilized bench-scale reactors and, often, sterilized porous media. Although this approach was important in demonstrating the role of microbial growth in porous media plugging, indigenous microbial communities may have significant effects on biobarrier formation. Furthermore, little is understood of the factors influencing long-term biobarrier stability and the resistance of these barriers to typical ground water contaminants. The following study utilized relatively large scale reactors (three-foot packed-sand columns) and non-sterile conditions as a step towards scale-up. Biobarrier formation was compared in columns of different initial permeabilities (27 and 5.3 Darcies). The columns were equipped with a series of piezometers to determine changes in hydraulic conductivity along the length of the flow path. The persistence of biobarriers was examined by challenging the established barriers with starvation conditions. The results of this study confirm the hypothesis that biofilm formation in porous media results in a minimum hydraulic conductivity that is independent of initial hydraulic conductivity [16]. Furthermore, the creation of an effective hydraulic barrier throughout the length of the columns suggests biobarriers are a viable technology for the containment of ground water contaminants, and further scale-up research is warranted.

## MATERIALS AND METHODS

### Test chamber design

Test chambers consisted of three-foot lengths of clear polyvinyl-chloride (PVC) pipe 6 inches in diameter, with a wall thickness of ¼ inches. A cone-shaped inlet and outlet were used to provide uniform flow transition. The chamber walls were coated with PVC adhesive and sand to limit bypass flow along the chamber walls. The columns were packed with F70 or F110 sand (U.S. Silica Co., F70: 65% 297-210 µm sieve size, F110: 69% 149-74 µm) resulting in initial permeabilities of 27 and 5.3 Darcies, respectively. The chamber inlet and outlet were layered with large gravel,

pea gravel and course sand to prevent settling of sand into the inlet tubing and wash-out of sand from the outlet tubing. The chamber was fitted with four sample ports, spaced exponentially along the column at distances of 8.5, 18.2, 38.0 and 76.6 cm from the column inlet, to allow pressure measurements and withdrawal of fluid samples. Pressure was measured using piezometers located at each sample port. Flow of solutions through the chambers was provided by maintaining a 10 liter constant-head tank approximately 6 feet above the column inlet. Flow rates were determined using a graduated cylinder and a stopwatch. Porosity was determined by measuring the displacement of a known volume of water by a volume of settled saturated sand.

The hydraulic conductivity (K) of the column between each sample port was calculated using Darcy's law,

$$K = Q/A\ (dh/dl)$$

where Q is the flow-rate, A is the cross-sectional area of the column, and (dh/dl) is the piezometric gradient.

## Inoculum preparation

The inoculum for column plugging consisted of starved cells of streptomycin-resistant environmental isolate of Klebsiella pneumoniae suspended in a phosphate-buffered saline (PBS) solution [9]. Cells were grown to stationary phase in sodium citrate medium (SCM: $Na_3C_6H_5O_7{\cdot}2H_2O$ 7.36 g, $(NH_4)_2SO_4$ 3.30 g, $KH_2PO_4$ 7.30 g, $K_2HPO_4$ 9.22 g, $MgSO_4$ 0.12 g, $FeCl_3$ 0.0041 g, $H_2O$ 1 L, pH 7.2), harvested by centrifugation (10,000 x g, 10 min, 4 C), washed twice in phosphate buffered saline (PBS: NaCl 8.5 g, $KH_2PO_4$ 0.61 g, $K_2HPO_4$ 0.96 g, $H_2O$ 1 L, pH 7.2), suspended in an equal volume of PBS to initiate starvation, and incubated for twenty to forty days in a stirred-flask. The starvation suspensions were monitored weekly for viable cells (CFU $ml^{-1}$) and mean bacterial size. Initially, the cultures contained 8.3±0.6 x $10^8$ viable cells $ml^{-1}$ with a mean cell size of approximately 3.4 by 1.1 µm. Starvation resulted in a decrease in both viable cell counts and mean cell size. After three weeks of starvation, cell densities stabilized to 5.8±2.4 x $10^6$ CFU $ml^{-1}$, with a mean cell size of approximately 1.0 by 0.5 µm. The starved bacterial suspensions were filtered with a #1 Whatman filter to remove aggregates and diluted tenfold immediately before inoculation into the columns.

## Inoculation and resuscitation of starved bacteria

The columns were prepared for inoculation by flushing with two pore-volumes (PV) of PBS. Two PV of bacterial suspension were then added to the columns. Following inoculation, the columns were flushed with two PV of SCM and incubated for 48 hours to resuscitate the starved bacteria. The columns were then perfused daily with SCM until the permeability became constant, after which the columns were continuously supplied with SCM. The biobarriers were challenged by replacing the SCM in the constant-head tank with either deionized water or deionized water containing 0.21 g $L^{-1}$ $MgSO_4$ and 0.0041 g $L^{-1}$ $FeCl_3$.

## Sample analysis

Bacterial populations were estimated by heterotrophic plate-counts (HPC), streptomycin-resistant HPC, and acridine orange direct counts (AODC). HPC were performed using 10%-strength brain-heart infusion agar (BHA: 4.0 g brain-heart infusion broth, 15 g agar, 1 L distilled water. Difco, USA) after serial dilution with PBS. For streptomycin-resistant HPC, 0.1 g $L^{-1}$ streptomycin (Sigma, USA) was added to BHA. Samples for AODC were preserved with formalin (final concentration 4%) and stored at 4°C. These samples were then filtered with a black 0.2 µm filter

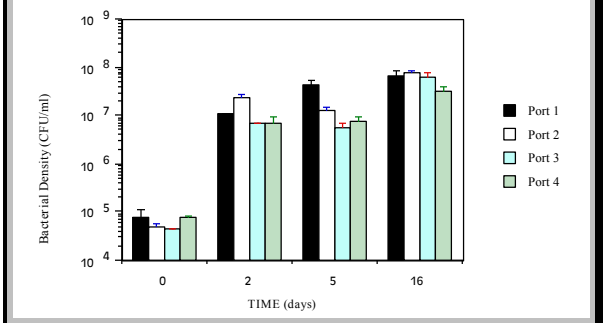



Figure 1. Typical increase in bacterial density throughout a packed-sand column during nutrient resuscitation with SCM. The column was inoculated with a starved cell suspension of Klebsiella pneumoniae containing 8.4 x $10^6$ CFU $ml^{-1}$. Note the even distribution of cells throughout the length of the column at each time interval.

(Porotechtics, USA), stained with 100 µg $ml^{-1}$ acridine orange (Sigma, USA), and examined by epifluorescent microscopy at a total magnification of 1250X. Total cell counts were averaged from at least ten microscopic fields (replicate fields were analyzed until a total of approximately 300 cells were counted).

The concentrations of medium components (citrate, phosphate and sulfate) were determined by ion chromatography (IC) using a Dionex DX300 ion chromatography system equipped with an Ionpac AG11 guard column, an Ionpac AS11 analytical column, an ASRS-I self-regenerating suppressor, and a conductivity detector (Dionex, Sunnyvale, CA). Samples were eluted with a 21 mM NaOH solution at a flow rate of 1.0 ml $min^{-1}$.

# RESULTS

## Inoculation of columns

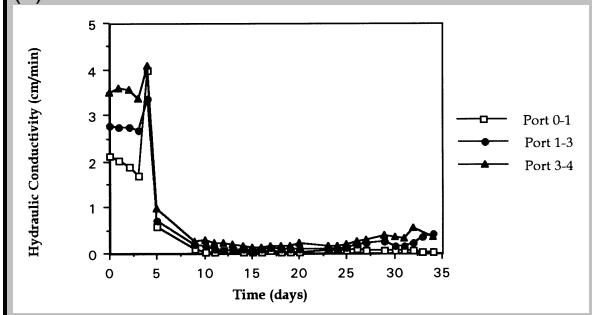
Columns were inoculated with two pore-volumes of starved K. pneumoniae suspension. The inoculum cell-densities were approximately an order of magnitude higher than the initial effluent cell-densities (2.5±1.7 x $10^6$ and 1.4±0.6 x $10^5$ CFU $ml^{-1}$, respectively). After two PV of inoculation, the effluent cell density was 4.0±2.5 x $10^5$ CFU $ml^{-1}$, which was not significantly higher than the initial effluent cell-density. However, the number of streptomycin-resistant cells in the column effluent increased from <1 x $10^3$ CFU $ml^{-1}$ initially, to 1.1 x $10^5$ CFU $ml^{-1}$ after two PV of inoculation. This indicates that although inoculated cell recovery was less than 10%, the streptomycin-resistant inoculum comprised a significant proportion of the culturable microbial community in the column effluent.

Following the inoculation of starved bacteria, the columns were treated with two pore volumes of sodium citrate medium. This treatment resulted in effluent concentrations of nutrient (citrate) and buffer (phosphate) that were 69±8% and 77±12% of the influent concentration, respectively. Analysis of fluid samples taken throughout the column revealed that viable cells (Figure 1) and nutrient medium components (data not shown) were uniformly distributed throughout the column.

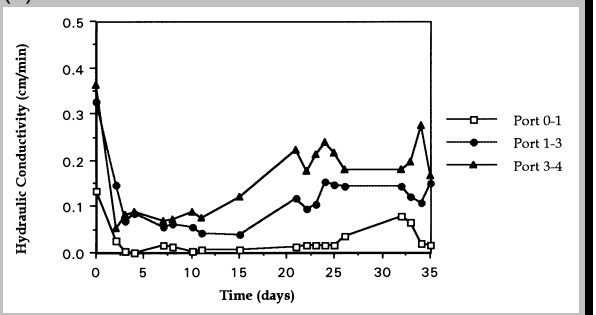
## Nutrient resuscitation

Resuscitation led to a uniform increase in bacterial numbers throughout the column (Figure 1). Examination of colony morphotypes on HPC enumeration plates suggested that the use of SCM for resuscitation led to a selective advantage for the colonization success of the inoculated K. pneumoniae population over the culturable indigenous bacterial population. After inoculation, the plates contained a variety of colony morphotypes in addition to the distinctive mucoid colonies of K. pneumoniae, while, following resuscitation, colony morphotypes were predominantly consistent with those of K. pneumoniae. Colonization success was further suggested by comparison of HPC and AODC cell counts (population culturability). Initially, approximately 6% of the microbial population in the column effluent were culturable. Following 16 days of nutrient resuscitation, nearly 100% of the population were culturable.

(a)




(b)




Figures 2a and 2b . Reduction in hydraulic conductivity during biobarrier formation in columns packed with F70 (a) and F110 sand (b). Even though the column containing F110 sand had a lower initial conductivity, columns packed with each type of sand reached a similar minimum hydraulic conductivity. Columns demonstrated a gradient of permeability reduction, with the greatest reduction in sections nearest the column inlet.

## Reduction in hydraulic conductivity

Nutrient resuscitation of the starved bacterial inoculum resulted in a reduction in hydraulic conductivity throughout the length of the columns, which became relatively stable after ten to twenty days. Typical reductions of hydraulic conductivity during biobarrier formation in columns packed with F70 and F110 sand are shown in Figure 2. The reduction from initial hydraulic conductivity was greater for the F70 columns, which had a higher initial conductivity than the F110 columns; however, a similar stable minimum hydraulic conductivity was reached regardless of the initial conductivity (Table 1). Stable hydraulic conductivity reductions were accompanied by a relatively constant number of culturable cells in the column effluent that were similar for both F70 and F110 columns ($6.9\pm1.2 \times 10^7$ and $6.4\pm4.1 \times 10^7$ CFU $ml^{-1}$, respectively). Hydraulic conductivity reduction occurred in a gradient through the column, with the greatest reduction nearest the column inlet.

## Starvation challenge

The persistence of an established biobar-

| Column | Sand | Section* | Hydraulic conductivity (cm/min) Initial | Days 20-35 | % Reduction |
|---|---|---|---|---|---|
| A | F70 | 1 | 1.94 | 0.12±0.03 | 94 |
| A | F70 | 2 | 1.95 | 0.23±0.07 | 88 |
| B | F70 | 1 | 2.76 | 0.20±0.10 | 93 |
| B | F70 | 2 | 3.49 | 0.29±0.11 | 92 |
| C | F110 | 1 | 0.33 | 0.13±0.02 | 61 |
| C | F110 | 2 | 0.34 | 0.21±0.03 | 38 |
| D | F110 | 1 | 0.30 | 0.19±0.03 | 37 |
| D | F110 | 2 | 0.44 | 0.24±0.16 | 45 |

* Section 1; ports 1-3: Section 2; ports 3-4.

Table 1. Average hydraulic conductivites following biobarrier formation.

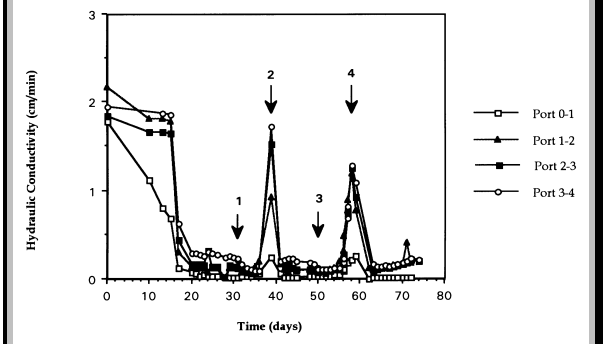



Figure 3. Hydraulic conductivity during biobarrier formation and challenge with deionized water from days 31-38 (arrows 1-2) and mineral solution from days 50-61 (arrows 3-4). The conductivity remained constant for several days following the challenge and then began to increase. This increase in hydraulic conductivity was more pronounced in column sections farther from the inlet (port 0). Restoration of nutrient medium resulted in a rapid reduction in conductivity to values equivalent with those before the challenge.

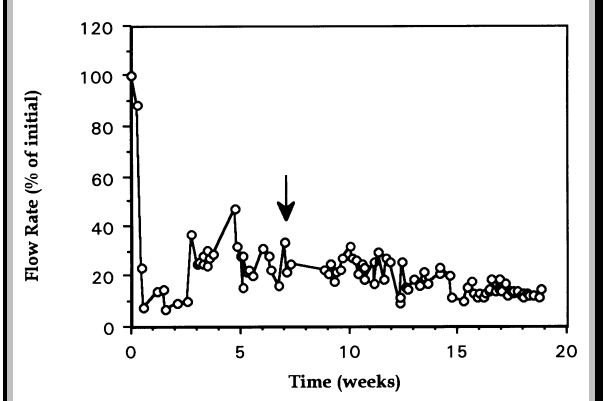



Figure 4. Long-term stability of a biobarrier formed in an F110 sand column. The biobarrier reduced flow to less than 40% of the initial rate. Periodic fluctuations in the flow-rate of the columns correspond to fouling and cleaning of the nutrient supply system (see text). The influent medium was amended on day 55 (arrow) to reduce the concentration of sulfate, which resulted in more stable flow rates.

rier subjected to starvation conditions was examined by changing the influent medium from nutrient medium to deionized water (Challenge 1) or a mineral solution (Challenge 2). The hydraulic conductivity remained relatively constant for the first five days of Challenge 1 and the first ten days of Challenge 2, and then rapidly increased in column sections farthest from the inlet (Figure 3). Following challenges, reintroduction of nutrient medium resulted in a rapid reformation of the biobarrier throughout the column (Figure 3). The effluent culturable cell density decreased from $9.4\pm0.9 \times 10^7$ to $7.0\pm3.0 \times 10^6$ CFU $ml^{-1}$ during starvation challenge.

### Biobarrier maintenance

Long-term maintenance of biobarriers was complicated by the fouling of the nutrient supply lines, head tank and reservoir tank due to microbial back-growth. Fouling was accompanied by the formation of a black precipitate in the columns, a strong $H_2S$-like odor in the column effluent, and an increase in hydraulic conductivity. This situation was remedied by sanitizing the nutrient delivery with a bleach solution, followed by treatment with sodium thiosulfate to neutralize residual chlorine, rinsing with deionized water, and refilling the system with sterile medium. The sequential fouling and cleaning of the nutrient supply system resulted in periodic fluctuations in the flow-rate of the columns (Figure 4). Initially it was assumed that the increase in hydraulic conductivity associated with fouling was due to nutrient limitation (e.g., oxygen). However, amendment of the nutrient medium to reduce the sulfate concentration resulted in biobarriers that had a relatively stable hydraulic conductivity even when the nutrient supply system was fouled (Figure 4) and a lack of the $H_2S$-like odor in the column effluent.

## DISCUSSION

### Inoculation and resuscitation of starved bacteria

The columns were inoculated with starved Klebsiella pneumoniae cell suspensions. Although the inoculum cell concentrations were approximately an order of magnitude higher then the initial column effluent culturable cell concentrations, inoculation with two pore-volumes of starved bacteria did

not significantly increase the culturable cell concentrations in the column effluent. This indicates that the effluent cell recovery was less than 10%. Low recovery of inoculated bacteria in the effluent of porous media reactors has often been observed [17-20]. Although these low recoveries may result from the retention of bacteria within the porous media, cell death may also be significant [17]. Nonetheless, the number of streptomycin-resistant cells in the column effluent increased significantly, indicating that the streptomycin-resistant K. pneumoniae formed a significant proportion of the culturable bacterial community in the column effluent.

Inoculation of columns was followed by the addition of two pore-volumes of nutrient. Streptomycin-resistant plate-counts throughout the column after this treatment indicated that the starved bacterial inoculum was uniformly distributed throughout the column. This result agrees with previous reports of efficient transport of starved bacterial suspensions through porous media [9, 10].

Nutrient addition to inoculated columns resulted in an increase in the K. pneumoniae population. This increase was relatively uniform in distribution throughout the columns and corresponded with a reduction in hydraulic conductivity. However, there was a tendency for greater conductivity reduction towards the column inlet. These results are likely due to higher nutrient concentrations at the column inlet and suggest there is a maximum porous media length that can be plugged using a microbial biobarrier.

## Reduction in hydraulic conductivity

Biobarrier formation resulted in a reduction in hydraulic conductivity throughout the length of columns packed with F70 and F110 sand. Although the initial hydraulic conductivities of columns packed with each sand type were different, biobarrier formation resulted in similar hydraulic conductivities for both types of sand. Thus, a minimum hydraulic conductivity was reached regardless of the initial permeability of the matrix, resulting in a greater reduction of hydraulic conductivity in the more permeable matrix (F70 sand). These results are consistent with observations of preferential plugging of high permeability zones [5, 6] and the hypothesis that biofilm thickness stabilizes to preserve a minimum permeability [16]. Observations of biofilms that developed in flow cells in situ using confocal scanning laser microscopy revealed arrays of channels through the biofilm which may facilitate nutrient transport and/or waste diffusion [21-23]. Similar advective flux channels probably exist in porous media reactor biofilms. The minimum hydraulic conductivity observed in this study likely reflects a minimum flow through these channels required for adequate nutrient and/or waste diffusion to maintain the biobarrier.

## Starvation challenge

Challenge of established biobarriers with starvation conditions resulted in an increase of hydraulic conductivity after five to ten days. These results suggest established biobarriers require nutrient supply for maintenance. Increased conductivity was more pronounced towards the column outlet. This may be the result of the biobarrier near the column inlet having a better initial nutrient supply than the deeper sections of the column, resulting in a healthier and more stress-resistant microbial population. The rapid reduction in hydraulic conductivity following alleviation of the starvation challenge suggests that the biobarrier population remained in the column under starvation conditions but was ineffective in maintaining reduced conductivity.

### Effect of souring on biobarrier stability

The increase in hydraulic conductivity associated with fouling of the nutrient supply system in the presence of sulfate and the $H_2S$-like odor emanating from the column suggests that the activity of sulfate-reducing bacteria had a deleterious effect on biobarrier integrity. It is unclear from this study whether this effect was indirect (e.g., nutrient competition) or direct (e.g., inhibitory effects of $H_2S$ on the biobarrier-forming bacteria or channeling caused by gas production). Because microbial plugging of porous media is often dependent on carbohydrate production [2, 9, 15], the activity of sulfate-reducing bacteria likely affects the carbohydrate-producing bacterial species in the biobarrier. Few biobarrier studies have assessed the role of interactions between indigenous microbial communities and inoculated populations. However, such interactions may be an important factor under field-relevant conditions.

## CONCLUSIONS

Microbial biobarriers were effective for reducing hydraulic conductivity throughout the length of the three-foot sand-packed columns. A stable minimum hydraulic conductivity was reached regardless of the initial permeability of the matrix. This phenomenon likely reflects a minimum flow through the biofilm that is required for the transport of nutrients and metabolic wastes. Starvation conditions had a deleterious effect on biobarrier effectiveness, suggesting that nutrient supply is required for biobarrier maintenance. Long-term biobarrier maintenance also required control of souring. Overall, these results indicate the hydrodynamic properties of porous media can be manipulated using microbial biobarriers and these barriers may be an effective technology for the containment of ground water contaminants.

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