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Effects of starvation on bacterial transport through porous media

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Abstract

A major problem preventing widespread implementation of microbial injection strategies for bioremediation and/or microbially enhanced oil recovery is the tendency of bacteria to strongly adhere to surfaces in the immediate vicinity of the injection point. Long term (weeks to months) nutrient starvation of bacteria prior to injection can decrease attachment and enhance transport through porous media. This paper summarizes results of starvation-enhanced transport experiments in sand columns of 30 cm, 3 m, and 16 m in length. The 16 m column experiments compared transport, breakthrough and distribution of adhered cells for starved and vegetative cultures of *Klebsiella oxytoca*, a copious biofilm producer. Results from these experiments were subsequently used to design and construct a field-scale biofilm barrier using starved *Pseudomonas fluorescens*. The 30 cm and 3 m sand columns experiments investigated starvation-enhanced transport of *Shewanella algae* BrY, a dissimilatory metal-reducing bacterium. In both cases the vegetative cells adsorbed onto the sand in higher numbers than the starved cells, especially near the entrance of the column. These results, taken together with studies cited in the literature, indicate that starved cells penetrate farther (i.e. higher breakthrough concentration) and adsorb more uniformly along the flow path than vegetative cells.

Keywords: Microbially enhanced oil recovery; Bioremediation; Biofilm barrier; Bacterial transport; Starvation; Permeable reactive barriers

1. Introduction

The movement of bacteria through porous media is a critical factor in microbially enhanced oil recovery operations (MEOR) as well as in the bioremediation of contaminated subsurface water supply systems. In MEOR, the injection and subsequent growth of bacteria has been suggested to promote plugging of subsurface formations for enhanced secondary oil recovery [12,37]. When *in situ* bioremediation is chosen as the remedial action, successful cleanup depends on the transport and distribution of the endogenous strains through the entire contaminant plume. When exogenous strains with specialized metabolic features are used, their transport from the injection source to and within the contaminant plume is a major factor determining the efficacy of the operation [56]. Alternatively, if *in situ* biofilm barriers are desired as a contaminant containment and remediation strategy, sufficient adhesion of introduced strains in the desired locale is required [11]. Bacterial transport phenomena are also important in assessing the potential movement of pathogens through aquifers [57].

Transport of bacteria through porous media is governed by aqueous phase convective movement coupled with attenuation by adhesion onto surfaces and straining or trapping in interstitial pores. Adhesion is commonly thought of as the main retarding factor, while straining is important only when the diameter of the particle exceeds 5% of the mean interstitial pore size [24,41]. Cell death (lysis caused by collision with solid surfaces) is also a major factor in closing a mass balance of transported cells [8]. Cell transport is also influenced by properties of the

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bacterial cells (e.g. surface charge, hydrophobicity, size, motility and physiological state), solution chemistry (e.g. ionic strength, pH, temperature), porous media properties, and interstitial fluid velocity. Bouwer et al., Mills, Lawrence and Hendry, and Harvey [5,22,34,42] provide excellent reviews of the factors influencing bacterial transport through porous media along with implications for field-scale applications.

Manipulating conditions in the subsurface to facilitate bacterial transport is problematic. For example, altering subsurface environmental conditions (e.g. lowering the ionic strength, changing pH or temperature of the groundwater) to facilitate bacterial transport may pose difficult problems both from an engineering as well as cost standpoint. pH and temperature are usually determined by the buffer capacity of the soil–groundwater system and temperature of the surrounding groundwater, respectively. Ionic strength could be lowered by using lower ionic strength water for injection but it might result in decreased viability of the injected bacteria and swelling of clayey soils, which could cause significant reductions in soil permeability and thus decreased bacterial transport. Altering the physiological properties of the bacterial inoculum (e.g. starvation) to facilitate transport may be more promising. In this article we focus exclusively on the effects of long-term (weeks to months) nutrient starvation on cell transport through porous media with an emphasis on case studies conducted at length scales relevant for field application.

2. Nutrient starvation

Long term starvation of bacteria results in radical size reduction and a rapid decrease in metabolic activity until the bacteria approach complete dormancy [28]. Starved bacteria can survive for years in the absence of nutrients but can be resuscitated relatively rapidly by the addition of suitable nutrients [4,28]. It is well known that a number of changes occur during long term starvation of non spore-forming bacteria. A large amount of RNA and protein appear to be degraded rapidly at the onset of starvation [27,47], however synthesis of new proteins appears to be required for long term survival of starving cells [19,23,26,29,48]. These proteins are believed to be part of a general stress response, often involving RpoS, and allow bacteria to become more stress resistant and more efficient scavengers [10,14,16,38,39,51–53,55,62,63]. Increased stress resistance is verified by the observation that starved cells survived better under a range of stress conditions compared to their vegetative counterparts [1,21,44–46] and that slow growing cells are better adapted for starvation stress survival than their fast growing counterparts [43].

3. Transport of starved cells

Starvation can significantly influence the attachment of bacteria to surfaces. Short term starvation of bacteria can result in an increased tendency to attach to surfaces [8,13,30] whereas long term starvation (weeks to months) may decrease bacterial attachment and enhance bacterial transport through porous media [5,18,31–33]. Results from these studies lead to the conclusion that long term starvation-induced changes in cell attachment characteristics result in enhanced porous media transport characteristics relative to cells in the vegetative (growing) state. However, it is important to note that these previous investigations usually relied on data mostly obtained from columns of 0.5 m length or less, thereby making extrapolation to field conditions difficult.

This paper examines starvation-enhanced microbial transport for columns of 30 cm, 3 m, and 16 m length. We begin by summarizing previously published material by the authors which, taken together, provide an example in which this research is taken from the laboratory to the field. The context here is the construction of an engineered subsurface biofilm barrier along with the laboratory studies which provided critical bacterial transport parameters used in the actual design of the field demonstration. This review will be followed by recent, previously unpublished results aimed in a similar way at the construction of permeable reactive barriers composed of dissimilatory metal-reducing bacteria (DMRB) for the treatment of metals and radionuclides in groundwater.

4. Enhanced transport of *Pseudomonas fluorescens* for construction of a subsurface biofilm barrier

The design, construction, and testing of a field-scale subsurface biofilm barrier for the containment and bioremediation of nitrate contaminated groundwater was reported by Cunningham et al. [11]. This demonstration project was conducted in an engineered outdoor facility consisting of a 42 m wide, 58 m long, 7 m deep PVC-lined test cell with an initial hydraulic conductivity of 4.2×10^{-2} cm/s. A flow field was established across the test cell by injecting water up-gradient while simultaneously pumping from an effluent well. A 10 m wide biofilm barrier was developed along the centerline of the test cell by injecting a starved inoculum of *P. fluorescens* strain CPC211a, followed by injection of a growth nutrient mixture composed of molasses, nitrate, and other additives.

The design of this demonstration project was based on starved bacterial transport data published by Sharp et al. [50]. Sharp et al. [50] conducted a series of experiments in 16 m long sand columns. Eight columns were used to compare transport, breakthrough and *in situ* biofilm production between starved and vegetative cultures of *Klebsiella oxytoca*, a copious biofilm producer (very similar to the CP211a strain used to construct the biofilm barrier). After inoculation, the columns were rinsed with sterile buffer to wash out unadsorbed cells in the interstitial spaces. The column effluent from the cell inoculation and the buffer rinse were collected and analyzed to determine the number of cells (starved or vegetative) that broke through each column. Results showed that almost 10% of the injected

starved cells broke through the 16 m columns, compared to less than 0.4% of the vegetative cells. After rinsing, two vegetative and two starved columns were destructively sampled and methods by Camper et al. [9] were used to determine the numbers of cells adsorbed along the column length (cells/g soil). It was found that the vegetative cells adsorbed onto the sand in much higher numbers than the starved cells, especially near the entrance of the column. These results indicated that starved cells penetrated farther (i.e. higher breakthrough concentration) and adsorbed more uniformly along the flow path than vegetative cells. Sharp et al. [50] also investigated the lag phase (i.e. time necessary for starved cells to become fully vegetative) after a molasses-based substrate was added to the starved cell columns. The duration of the lag phase increased with starvation time and appeared to reach a maximum of approximately 72 h at starvation times greater than 6 weeks.

These cell adsorption and lag phase observations provided the basis for establishing the nutrient injection flow rate for the biofilm barrier demonstration project. Starved *P. fluorescens* CPC211a were injected at a flow rate sufficient to inoculate the soil over a 10 m radius from each injection well. After starved bacteria had been injected, the flow rate for the subsequent injection of nutrients was established so as to develop biofilm which was uniformly distributed over a desired radius out from the point of injection.

Although Sharp et al.'s [50] results formed the basis for a successful field demonstration project [11], additional "long column" experiments are needed before cell starvation can be widely accepted as a viable method for enhancing bacterial transport in field-scale applications.

5. Enhanced transport of *Shewanella algae* BrY for construction of permeable reactive barriers

A recent investigation by Gerlach [17] provides additional insight into transport of starved vs. vegetative bacteria over longer length scales. This investigation looks at the potential for developing permeable reactive barriers (PRBs) *in situ* for the treatment of metal and radionuclides in groundwater. The use of PRBs has proven an effective technology for preventing off-site migration of chlorinated hydrocarbons, metals, and radionuclides [49,58,61]. One goal of this study was to provide an experimental database which can be used to design and construct PRBs using dissimilatory metal-reducing bacteria (DMRB). DMRB have demonstrated potential for the remediation of groundwater contaminated with chlorinated hydrocarbons, heavy metals, and radionuclides [3,40,60]. DMRB gain energy for growth by coupling the oxidation of organic compounds or hydrogen to the dissimilatory reduction of ferric iron [Fe(III)] and other oxidized metals. DMRB can enzymatically reduce a wide range of metal ions, including Fe(III), Cr(VI) and U(VI) [20,35,36].

The idea of utilizing indigenous or injected DMRB to establish PRBs in the subsurface is intriguing. Once delivered to iron-bearing formations, DMRB, could be stimulated to produce redox-reactive ferrous [Fe(II)] iron from ferric iron. The produced ferrous iron would then chemically react with chlorinated hydrocarbons, Cr(VI), U(VI), and other contaminants.

Long-term nutrient starvation as a strategy for the enhancement of bacterial transport through porous media was evaluated in this study using *S. algae* BrY as the model DMRB. *S. algae* BrY was isolated from sediments in the Great Bay Estuary in New Hampshire [7] and was chosen for this study because of its ability to reduce a wide variety of metals. Presented below are the results of transport experiments which quantitatively describe the transport of starved and vegetative cells of *S. algae* BrY through porous media.

6. Experimental approach

Experiments comparing the transport of starved and vegetative *S. algae* BrY were performed on two different scales in quartz sand columns. Short columns (30 cm length) were utilized to develop a test system for the relatively rapid assessment of bacterial transport in porous media. Larger scale experiments involving columns of 3 m length were utilized to test the applicability of the parameters derived from the short column experiments and to test the starvation transport enhancement strategy on a more field-relevant scale. The materials and methods used in these experiments are presented below.

7. Materials and methods

7.1. Strain and culturing methods

Cultures of *S. algae* BrY (formerly *S. alga* BrY [7]) were maintained on tryptic soy agar (40 g L^{-1}; Difco, Detroit, MI) at room temperature. Cells were aerobically grown to the late exponential, early stationary phase in tryptic soy broth (30 g L^{-1}; Difco, Detroit, MI) at room temperature for 15 h on a rotary shaker at 150 rpm. The cells were harvested by centrifugation (5860*g*, 20 min, 4 °C), washed twice in phosphate buffered saline solution (PBS; 8.5 g L^{-1} NaCl, 0.96 g L^{-1} K_2HPO_4, 0.61 g L^{-1} KH_2PO_4, pH 7.0), and resuspended in PBS. Washed *S. algae* BrY cells were either used directly (vegetative state) or starved by aseptically stirring on a magnetic stir plate at room temperature as described by Caccavo et al. [6]. Starved cells were harvested after 7 weeks of starvation by centrifugation and resuspended in PBS before incorporation into the transport experiments.

7.2. Transport studies

Inocula of *S. algae* BrY, starved or vegetative, were injected into porous media columns filled with 40 mesh quartz sand (Unimin Corp., Emmet, ID). The 30 cm and 3 m columns were constructed of commercially available

polyvinylchloride (PVC) pipe. All columns were equipped with sample ports at the influent, effluent, and along the flow path. The columns were packed wet to avoid the inclusion of air pockets and facilitate reproducible packing. The columns were operated in up-flow mode using a constant head tank or a peristaltic pump for 30 cm and 3 m long columns, respectively. The columns were disinfected previously to bacterial transport experiments by flushing with 2 pore volumes 0.5% NaOCl (dilute commercial bleach), 1 pore volume sterile PBS, 2 pore volumes 0.01 M sodium thiosulfate to neutralize residual chlorine, and 2 pore volumes sterile PBS. A commercial kit (HACH, Loveland, CO) was used to ensure residual chlorine concentrations below the detection limit (0.05 mg L^{-1}) in the effluent of the columns. Plate counts routinely performed on effluent samples after this treatment did not show any growth and growth other than the reddish/pinkish colonies characteristic for strain BrY was rarely observed during the transport experiments.

The pore volume and hydrodynamic dispersion coefficient of each column were determined utilizing Na–fluorescein (Aldrich, Milwaukee, WI) as a tracer and by fitting the experimental data to an analytical solution of the advection dispersion equation developed by Van Genuchten and Alves, [59]. The interstitial fluid velocity (v) in the bacterial transport experiments was calculated using the measured flow rate, the cross sectional area of the column, and the porosity, which was estimated from the tracer studies. The bacterial concentrations, expressed as percent of their influent concentrations in each experiment, were plotted as a function of the number of pore volumes eluted from the columns. The percent recovery was defined as the number of bacteria in the effluent of the column (integral of the effluent concentration over time) divided by the total number of bacteria injected into the column (integral of the influent concentration over time). During the transport experiments, the columns were inoculated with approximately two pore volumes of starved or vegetative cells of *S. algae* BrY suspended in PBS. The transport of bacteria was monitored by sampling the influent, effluent, and the pore water along the flow path of the columns for culturable cells. Aliquots (0.1 mL and 1 mL for 30 cm and 3 m columns, respectively) were taken using sterile, disposable 1 mL syringes equipped with 22 gauge needles. The needles were carefully inserted into the sand pack through butyl rubber septa held in place by tube clamps. The tip of the needles was positioned approximately in the center of the column and the needles were allowed to rest for approximately 1 min before withdrawing a sample. Dilutions in PBS were performed immediately and colony forming units were determined on tryptic soy agar (40 g L^{-1}; Difco, Detroit, MI; incubation for 24 h at 30 °C). The reported values are the arithmetic means of the three appropriate plate counts.

7.3. Porous media sampling

The distribution of sorbed cells throughout the columns was estimated at the end of the experiments by sectioning the columns and analyzing the sand cores for culturable cells after desorption from the sand. The 30 cm columns were divided into six sections of each five centimeter length while the 3 m long columns were sectioned into ten 30 cm long sections. The desorption of cells was accomplished using the desorption solution described by Camper et al. [9], which consists of Tris buffer (pH 7), peptone, zwittergent 3-12, and EGTA and was diluted 1:5 in PBS before use. An aliquot (5 ml) of this solution was added to 1 g of sand in a test tube, which was vortexed for 1 s. The test tube was placed on a horizontal shaker (150 rpm) for 30 min and then vortexed again for 3 s. The supernatant was sampled immediately after coarse particles had settled and plate counts on tryptic soy agar were performed after dilution in PBS.

8. Results and discussion

8.1. Short (30 cm) column experiments

Triplicate experiments utilizing 30 cm long columns were performed for both starved and vegetative cells. The dispersion coefficient and porosity which were estimated from tracer studies varied between 0.63 $cm^2\ min^{-1}$ and 2.83 $cm^2\ min^{-1}$, and 46% and 50%, respectively. These fairly high porosity estimates might be due to additional settling of the sand pack between the packing of the columns and the beginning of the tracer studies. Additional settling can cause the formation of free space in the effluent region of the column which would have been included into the porosity estimations. The interstitial fluid velocity varied between 1.84 cm min^{-1} and 3.09 cm min^{-1} and the average influent concentration between 6.86×10^7 CFU mL^{-1} and 2.65×10^8 CFU mL^{-1} (Table 1). These columns were operated in constant head mode. Variations in estimated interstitial fluid velocity are due to differences in the hydraulic conductivity resulting from the packing and the possibility of additional settling in the sand pack (discussed above).

Table 1
Summary of parameters for bacterial transport experiments in 30 cm porous media columns filled with 40 mesh quartz sand

D[a]	Θ[b]	v[c]	c_0[d]
Starved cells			
0.63	0.50	3.09	7.79×10^7
1.21	0.50	1.86	2.65×10^8
1.67	0.46	2.41	1.43×10^8
Vegetative cells			
2.83	0.48	2.51	8.75×10^7
1.98	0.50	2.27	6.86×10^7
2.32	0.50	1.84	9.84×10^7

[a] Dispersion coefficient [$cm^2\ min^{-1}$], estimated from tracer studies.
[b] Porosity, estimated from tracer studies.
[c] Interstitial fluid velocity [cm min^{-1}].
[d] Influent cell concentration [CFU mL^{-1}].

8.2. Breakthrough of bacteria

Breakthrough of starved and vegetative cells is plotted in Fig. 1. The solid lines were generated by fitting an analytical solution of the advection–dispersion equation [59] to the experimental data (Eq. (1)):

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} - v\frac{\partial c}{\partial x} - k_{\text{att}}c, \tag{1}$$

where c is the concentration of cells (cells mL^{-1} or CFU mL^{-1}), t is time (min), D the dispersion coefficient ($\text{cm}^2\ \text{min}^{-1}$), x the distance into the column (cm), v the average interstitial fluid velocity ($\text{cm}\ \text{min}^{-1}$), and k_{att} the first-order attachment coefficient (min^{-1}). The first-order coefficients were estimated by fitting an analytical solution of Eq. (1) given by Van Genuchten and Alves [59] to the effluent concentration profiles over time observed in the transport experiments.

As can be seen in Fig. 1, starved *S. algae* BrY were detected in the effluent of the columns at higher normalized concentrations than vegetative cells. Although the effluent cell concentrations appeared to still increase when the experiments were terminated after two pore volumes of bacterial cell suspension were injected, the average normalized breakthrough concentration for starved cells was significantly higher than for vegetative cells ($38 \pm 6\%$ compared to $13 \pm 1\%$). The higher breakthrough of starved cells is also reflected in greater recoveries in the effluent ($20 \pm 2\%$) compared to vegetative cells ($9 \pm 4\%$). A multiple regression analysis was performed to evaluate the effects of bacterial physiology (i.e. starved vs. vegetative cells), influent cell concentration, and interstitial fluid velocity on the percent recovery and normalized breakthrough concentration of *S. algae* BrY after transport through 30 cm quartz sand columns. The regression analysis revealed that, even after correcting for the potential influence of influent cell concentration and interstitial fluid velocity, the physiological state of the cells significantly influenced the transport behavior of cells in the column experiments. The resulting P-values of $P = 0.006$ and $P = 0.005$ for percent recovery in the effluent and normalized breakthrough concentration, respectively, clearly indicated the statistically significant difference between starved and vegetative cells of *S. algae* BrY.

8.3. Sorption of bacteria along the flow path

The distribution of sorbed cells throughout the 30 cm columns at the end of each experiment further supported the hypothesis that starved cells were better transported, less attenuated, and more homogeneously distributed throughout the columns. The number of cells desorbed per g of sand in each 5 cm section was normalized to the total number of cells injected to allow ready comparison between the different columns. It was evident that vegetative cells adsorbed in higher numbers than starved cells within the first few centimeters of the columns (Fig. 2). The relative number of vegetative cells (CFU) detected per g of sand decreased from $0.717\% \pm 0.241\%\ \text{g}^{-1}$ in the first 5 cm section to $0.363\% \pm 0.048\%\ \text{g}^{-1}$ (51% of sorbed bacteria detected in first 5 cm of column) at 15–20 cm into the column and then remained constant. The quantification limit of this method was approximately 1.5×10^4 CFU g^{-1} sand or approximately $0.0001\%\ \text{g}^{-1}$. Starved cells appeared to be more homogeneously distributed along the flow path. The normalized concentration of starved cells decreased only slightly from $0.322\% \pm 0.022\%\ \text{g}^{-1}$ within the first 5 cm of the column to $0.249\% \pm 0.040\%\ \text{g}^{-1}$ between 15 and 20 cm into the column and remained basically constant through the remainder the column. The large error bars obtained for sorbed vegetative cells in the influent region of the columns suggested a relatively high variability in the behavior of the three different vegetative cell cultures. Differences in the transport behavior of freshly cultivated cells were also noted by Johnson and Logan [25] who reported significant day to day variations in transport behavior of equivalently treated cell cultures.

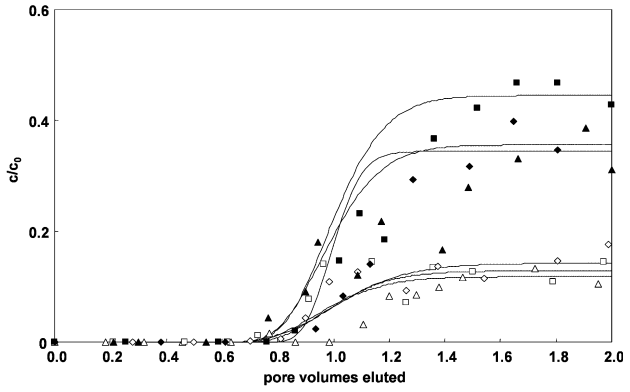

Fig. 1. Normalized breakthrough concentration (c/c_0) of starved (♦, ■, ▲) and vegetative (◇, □, △) cells of *Shewanella algae* BrY in the effluent of 30 cm long quartz sand columns. Solid lines were determined by fitting data to an analytical solution of the advection–dispersion equation [59].

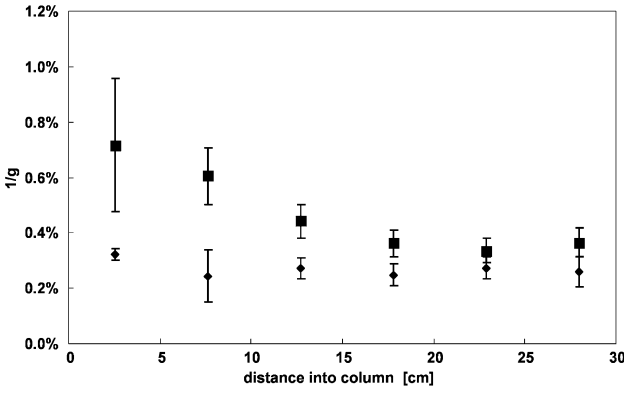

Fig. 2. Sorption of starved (♦) and vegetative (■) cells of *Shewanella algae* BrY to quartz sand along the flow path of 30 cm long columns. The sorbed cell concentrations were normalized to the total number of cells injected into each column. The averages of each three different columns and their corresponding standard errors of the mean are plotted.

The surface coverage of the quartz sand with cells was less than 0.5%. This estimate was established under the conservative assumption that all cells, which were not detected in the effluent of the columns, adhered to the first 5 cm of the porous media column. An equivalent surface area for vegetative cells was calculated using the cell dimensions (2.2 μm × 0.63 μm) given by Caccavo et al. [6]. The total surface area covered by vegetative cells was then compared to the total surface area of the quartz sand in the first five centimeters of the column ($15\ g \times 0.168\ m^2\ g^{-1}$, as determined by surface area analysis using nitrogen gas).

8.4. Recovery of bacteria from columns

The normalized effluent concentrations, percent recoveries, total recoveries of cells from the experimental systems, and the distribution of cells in the effluent, sorbed to sand, and present in the pore water are listed in Table 2. As mentioned above, the normalized breakthrough concentration and percent recoveries were significantly higher for starved cells than for vegetative cells. Total recoveries of cells from the experimental systems were limited using the desorption and plate count methods described. Between 47% and 78% of the total number of cells injected into each column were recovered by analyzing the effluent, the sand, and the pore water separately. No clear difference was observed between the ability to recover starved or vegetative cells (2-sample t-test $P = 0.319$). Such low and variable recoveries were also reported by other researchers who used plate counts for the enumeration of bacteria in porous media transport experiments [2,8,15,54]. Since a decay of cells in the feed reservoir was not observed (the commonly observed variations remained within 10% of the average influent concentration), the inability to close the mass balance might have been due to incomplete recovery of bacterial cells sorbed to the quartz sand. Furthermore, cells may have been injured during the desorption procedure and were thus not recoverable using the plate count method employed. Bacterial decay or a change of bacterial cells into the viable but not culturable state *during* the passage through the porous media column might have also caused incomplete recoveries. The relative distribution of the recovered cells to the three phases (cells in the effluent, sorbed to sand, and in the pore water) appeared to be significantly different for starved and vegetative cells. While $39 \pm 4\%$ of the recovered starved cells were detected in the effluent, only $15 \pm 3\%$ of the recovered vegetative cells were detected there (2-sample t-test $P = 0.003$). Relative recoveries of sorbed bacteria were higher for vegetative cells ($72 \pm 3\%$ compared to $43 \pm 9\%$ for starved cells; $P = 0.031$). No significant difference was observed for the relative number of bacteria recovered in the pore water ($18 \pm 5\%$ and $13 \pm 1\%$ for starved and vegetative cells, respectively; $P = 0.249$). The observation that a higher fraction of starved cells was observed in the effluent and a lower number was sorbed clearly shows that starved cells of *S. algae* BrY were better transported, less retarded, and more homogeneously distributed through porous media.

8.5. Large (3 m) column experiments

Intermediate scale experiments, utilizing 3 m long (2.54 cm diameter) columns were performed in duplicate to evaluate scale dependent effects on bacterial transport and to test the ability of the filtration model to predict bacterial transport at larger scales. A summary of the column parameters is given in Table 3.

Table 3
Summary of parameters for bacterial transport experiments in 3 m porous media columns filled with 40 mesh quartz sand

D[a]	Θ[b]	v[c]	c_0[d]
Starved cells			
3.85	0.49	3.98	2.57×10^7
1.98	0.47	3.89	1.40×10^8
Vegetative cells			
3.64	0.50	3.98	3.86×10^7
3.02	0.48	3.43	7.60×10^7

[a] Dispersion coefficient [$cm^2\ min^{-1}$], estimated from tracer studies.
[b] Porosity, estimated from tracer studies.
[c] Interstitial fluid velocity [$cm\ min^{-1}$].
[d] Influent cell concentration [$CFU\ mL^{-1}$].

Table 2
Normalized breakthrough concentrations, absolute, and relative recoveries of cells from 30 cm columns

Replicate	Normalized breakthrough concentration (%)	Total recovered (%)	Cells detected on sand (%)[a]	Cells detected in effluent (%)[a]	Cells detected in pore water (%)[a]
Starved cells					
1	34	47	19 (40)	18 (38)	11 (22)
2	45	53	20 (37)	22 (43)	11 (20)
3	35	55	29 (53)	20 (36)	6 (12)
Vegetative cells					
1	14	47	33 (71)	7 (15)	7 (14)
2	12	78	55 (70)	14 (18)	9 (12)
3	13	50	37 (75)	6 (12)	6 (13)

[a] Fractions given in parentheses are calculated based on the total recovery of cells from the system, i.e. the distribution of *recovered* cells to the three phases: sorbed (detected on sand), transported through column (detected in effluent), and present in pore water at the end of the experiment (detected in pore water).

8.6. Breakthrough of bacteria

The trends in concentrations of culturable cells in the effluent of the 3 m long columns were in agreement with the 30 cm column results (Fig. 3). However, the breakthrough occurred at lower concentrations since the bacteria had to pass through a longer column causing greater removal. The breakthrough of starved *S. algae* BrY cells occurred at higher concentrations ($10 \pm 3\%$) than for their vegetative counterparts ($0.5 \pm 0.7\%$) and the percent recoveries were $45 \pm 9\%$ for starved and $46 \pm 13\%$ for vegetative cells. Despite the obvious visual difference between the breakthrough curves of starved and vegetative cells, 2-sample *t*-tests indicated only a low statistical significance for the observed differences in the normalized breakthrough concentration ($P = 0.136$) and no difference in percent recovery ($P = 0.915$) of starved and vegetative cells. The low statistical significance was mainly due to the limited number of samples available ($n = 4$) and the high variability in normalized breakthrough concentrations and percent recovery for vegetative cells. It should also be noted that, like in the 30 cm columns, a steady state was possibly not completely achieved after two pore volumes in the 3 m long columns. The effluent cell concentrations appeared to still increase when the experiments were terminated after two pore volumes of bacterial cell suspension were injected.

8.7. Sorption of bacteria along the flow path

The distribution of sorbed cells throughout the columns at the end of each experiment (Fig. 4) showed the same trend as for the 30 cm column experiments. Vegetative cells appeared to attach in higher numbers to the quartz sand close to the injection point than starved cells. The normalized cell concentrations of vegetative cells decreased rapidly with distance into the column. The relative concentration of vegetative cells sorbed to sand between 120 cm and 150 cm into the column had dropped by approximately three orders of magnitude to $0.0009\%\ g^{-1}$ compared to $0.171\%\ g^{-1}$ in the first 30 cm of the column. The relative concentration of starved cells sorbed to sand between 120 cm and 150 cm into the column decreased by only approximately one order of magnitude to $0.0064\%\ g^{-1}$ compared to $0.07\%\ g^{-1}$ in the first 30 cm. The relative sorbed cell concentrations in the last 30 cm of the column (270–300 cm section) was 0.0003% and $0.0015\%\ g^{-1}$ for vegetative and starved cells, respectively, indicating that starved cells are being distributed more homogeneously throughout the column. As in the short column experiments, large standard errors were observed for sorbed vegetative cells, indicating higher culture to culture variability for vegetative cells. As in the 30 cm columns, the calculated surface coverage of the quartz sand with vegetative cells was less than 0.5%.

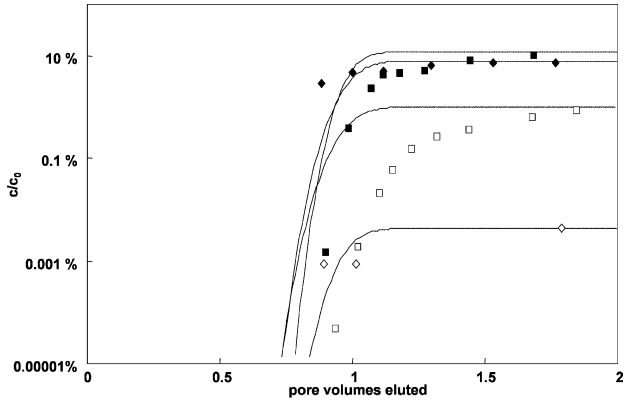

Fig. 3. Normalized breakthrough concentration of starved (♦, ■) and vegetative (◇, □) cells of *Shewanella algae* BrY in the effluent of 3 m long quartz sand columns. Solid lines were determined by fitting data to an analytical solution of the advection–dispersion equation [59].

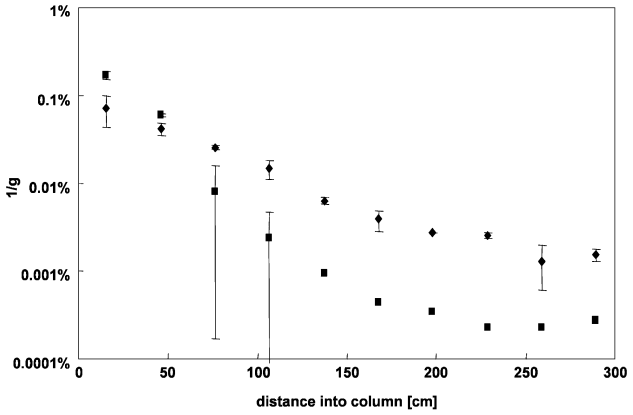

Fig. 4. Sorption of starved (♦) and vegetative (■) cells of *Shewanella algae* BrY to quartz sand along the flow path of 3 m long columns. The sorbed cell concentrations were normalized to the total number of cells injected into each column. The averages of each two different columns and their corresponding standard errors of the mean are plotted.

8.8. Recovery of bacteria from columns

Table 4 lists the normalized effluent concentrations, total recoveries of cells from the experimental systems, and the distribution of cells in the effluent, sorbed to sand, and present in the pore water. The total recoveries of cells from the 3 m long columns fall in the same range as for the 30 cm columns (45% and 46%). The relative distribution of recovered cells to the three phases (effluent, sorbed to the sand, and pore water) also followed the same pattern that was described for the 30 cm long columns. Due to the limited number of total samples ($n = 4$) and the high variability in the behavior of the vegetative cell cultures, significance levels of 2-sample *t*-tests remained low. The fraction of recovered vegetative cells sorbed to the quartz sand ($99 \pm 1\%$) was slightly higher than for starved cells ($89 \pm 4\%$) but not statistically significant ($P = 0.160$). This was balanced by a higher, though also not statistically significantly different, fraction of recovered starved cells in the effluent ($10 \pm 4\%$) compared to vegetative cells

Table 4
Normalized breakthrough concentrations, absolute, and relative recoveries of cells from 3 m columns

Replicate	Normalized breakthrough concentration (%)	Total recovered (%)	Cells detected on sand (%)[a]	Cells detected in effluent (%)[a]	Cells detected in pore water (%)[a]
Starved cells					
1	8	51	47 (91.5)	4 (7)	0.49 (0.97)
2	12	38	32.8 (86.3)	5 (13)	0.44 (1.15)
Vegetative cells					
1	0.004	37	37 (99.9)	0.0007 (0.002)	0.04 (0.11)
2	1.0	55	54 (98.7)	0.4 (0.7)	0.35 (0.63)

[a] Fractions given in parentheses are calculated based on the total recovery of cells from the system, i.e. the distribution of **recovered** cells to the three phases: sorbed (detected on sand), transported through column (detected in effluent), and present in pore water at the end of the experiment (detected in pore water).

($0.4 \pm 0.5\%$; $P = 0.163$). The difference between the fraction of recovered starved and vegetative cells detected in the pore water was also not statistically significant ($P = 0.222$).

9. Conclusions

A major problem preventing the widespread implementation of microbial injection strategies for bioremediation and/or microbially enhanced oil recovery is the tendency of bacteria to strongly adhere to the surfaces in the immediate vicinity of the injection point. Biofouling of these areas can lead to a failure of the injection system and limited dispersion of the bacterial inoculum in the subsurface. The use of starved bacteria, as documented here, might overcome this problem since starved bacteria typically adhere less, transport farther, and distribute more homogeneously through porous media than vegetative cells. The promising potential of starved cells for bioaugmentation strategies is indicated by higher recoveries of *S. algae* BrY in the effluent (2.6-fold higher for 30 cm columns, 25-fold higher for 3 m columns) and higher normalized breakthrough concentrations (2.9 and 19.9-fold). In addition to their improved transport behavior, starved cells are metabolically dormant. Their metabolic dormancy and lag phase before regaining full metabolic activity can allow for the simultaneous or subsequent injection of nutrients without the danger of immediately biofouling the employed injection system.

There is sufficient evidence to assume that transport enhancement by bacterial long term starvation is not limited to *K. oxytoca*, *P. fluorescens*, and *S. algae* BrY as reported here, but is a rather general starvation response across many bacterial strains [4,28,31]. Thus, the starvation transport enhancement strategy could be applicable to a large number of bacterial strains with a variety of metabolic capabilities. The exact reasons for starvation-enhanced transport behavior are not completely understood and are being investigated in our laboratories. If successfully developed, the starvation transport enhancement strategy can provide the practitioner with a versatile tool for the development of bioaugmentation technologies.

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