Biofilm Barriers to Contain and Degrade Dissolved Trichloroethylene

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Biologically produced subsurface barriers (i.e., biofilm barriers) are a viable technology for controlling contaminant migration from hazardous waste sites. Biofilm barriers are created through the injection of bacteria and selective growth medium into a series of wells downstream of a contaminant plume. Adequate substrate addition enables the bacteria to grow and form thick biofilms capable of uniform plugging of the subsurface. This technology has been successful in significantly reducing porous media permeability in bench-scale and field-scale applications. The research presented herein expands on current biofilm barrier technology by examining the feasibility of using a biofilm barrier to not only control contaminant migration through permeability reduction, but also facilitate contaminant biodegradation. The experimental scenario involved the creation of a dual-species biofilm matrix: one organism to reduce porous media permeability through thick biofilm formation and another organism to degrade a contaminant, in this case trichloroethylene (TCE). Porous medium column experiments demonstrated that a dual-species biofilm barrier can be created and that growth medium concentration was a very important variable in controlling simultaneous TCE degradation and permeability reduction. © 2004 American Institute of Chemical Engineers Environ Prog, 23: 69–77, 2004

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INTRODUCTION

Biofilm barriers are an attractive alternative to traditional subsurface barrier technologies (slurry walls, grout curtains, sheet pilings) because they do not require excavation, may utilize indigenous organisms, require minimal maintenance, have no depth limitations, and are cost effective to install and maintain [1]. Figure 1 is a schematic of an application of a biofilm barrier used in a funnel and gate type technology. Field-scale biofilm barriers are created by injecting bacteria, selected for their ability to produce copious amounts of thick biofilms, into a series of injection wells. Sufficient amounts of a selective growth medium are added after bacterial inoculation to produce overlapping columns of soil in which pore space is virtually sealed by bacterial growth and thick biofilm production. Research has shown, however, that the injection of bacteria and/or growth medium into the subsurface may cause premature plugging near the injection well, limiting the distance over which bacteria can be transported through porous media [2]. Using starved cells as the inoculum prevents plugging near the injection well and allows the bacteria to transport further through porous media [3]. Subsequent resuscitation of the starved cells in situ has been shown to produce uniform plugging of the porous medium on a large scale (>50-ft radius from the injection well) [1]. Bench-scale research has shown the effectiveness of biofilm barriers to reduce porous medium permeability under various field-relevant conditions such as nutrient starvation [4], heavy metal and organic solvent challenge [4], and vadose zone biobarrier formation [5]. A field demonstration of biofilm barrier technology resulted in hydraulic conductivity reduction of greater than 99%

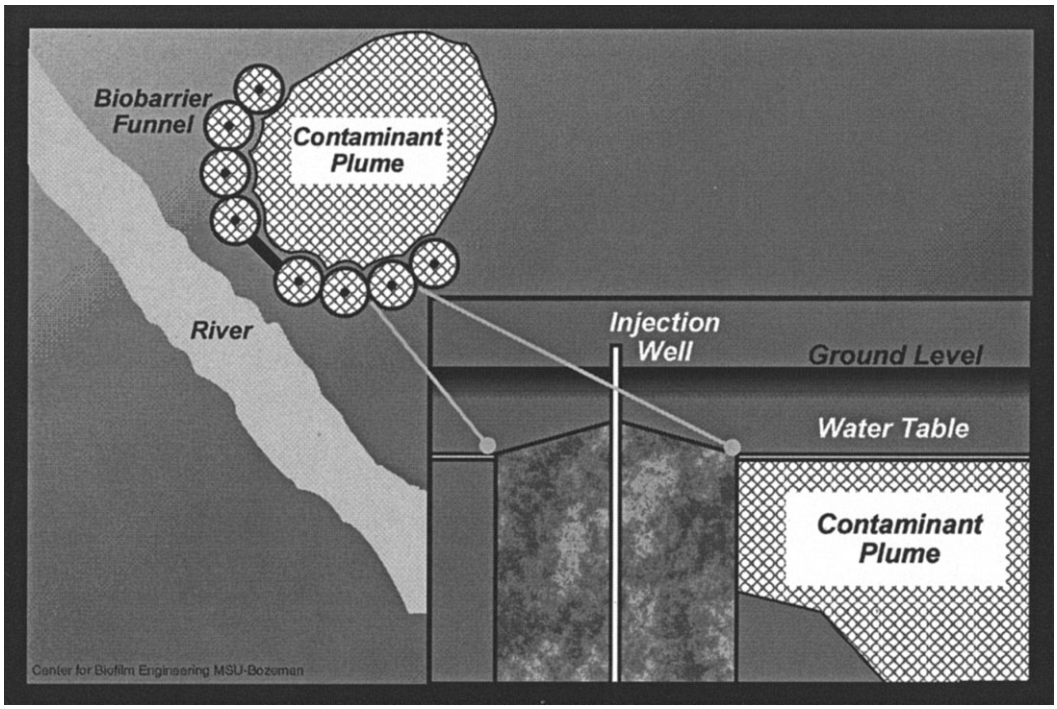

Figure 1. Schematic of a field-scale biofilm barrier used to funnel a contaminant plume into a localized treatment zone.

along the length of the 56-m barrier [1, 6]. In addition, this reduction in hydraulic conductivity was maintained over a 2-year period with little supplemental growth medium.

The purpose of this research was to expand on current biofilm barrier technology by creating a biofilm barrier that not only controls the migration of a contaminant (in this case dissolved trichloroethylene) but also biologically degrades it. Trichloroethylene (TCE) was selected as the model contaminant because it is a suspected carcinogen whose increased production and presence in groundwater is of major concern. A field-scale application of biofilm barrier technology would involve screening indigenous organisms at the site for the desired activities (thick biofilm formation, contaminant degradation) as well as compatibility (if more than one organism is desired for the bioaugmentation scenario) and thus the organism(s) chosen would be site specific. Few organisms are known to aerobically mineralize TCE. One such organism is *Burkholderia cepacia* PR1-pTOM$_{31c}$, an aerobic bacterium that can constitutively degrade TCE via a cometabolic process using the toluene ortho-monooxygenase (TOM) pathway [7]. The genetic information for the TCE degradative pathway is located on the plasmid, TOM$_{31c}$. The plasmid also encodes for the resistance to the antibiotic kanamycin [8]. Unfortunately, *B. cepacia* PR1-pTOM$_{31c}$ is unable to form a stable biofilm capable of reducing porous medium permeability [9]. Therefore, to create a TCE-degrading, permeability-reducing biofilm barrier, *B. cepacia* PR1-pTOM$_{31c}$ would need to be combined with another organism capable of thick biofilm production. The thick biofilm forming organism chosen for this pairing was *Klebsiella oxytoca*. *K. oxytoca* is a highly mucoid, facultative anaerobic bacterium that is a documented thick biofilm forming organism which has been used in previous biofilm barrier studies [4]. *K. oxytoca* was also chosen for its resistance to the antibiotic streptomycin, which, combined with *B. cepacia*'s resistance to the antibiotic kanamycin, enabled the use of selective plating techniques to differentiate each population in dual-species cultures.

A series of experiments was performed to determine which factors influence the interaction of *B. cepacia* and *K. oxytoca* in a dual-species biofilm. The results conclude that these two organisms can coexist and that the method of inoculation did not significantly affect the population distribution in a porous medium reactor [10]. Additional experiments identified substrate concentration as an important variable in controlling the population distribution of both organisms in dual-species biofilms [11]. The research presented herein was performed to determine whether varying substrate concentration could also be used to maximize the activities of *B. cepacia* (TCE degradation) and *K. oxytoca* (permeability reduction) in a porous medium column, thus creating a dual-species TCE-degrading/reduced permeability biofilm barrier.

MATERIALS AND METHODS

Sources of Strains

The aerobic TCE-degrading bacterium, *B. cepacia* PR1-pTOM$_{31c}$, was supplied by Malcolm Shields (University of West Florida). The thick biofilm forming bacterium, *K. oxytoca*, was isolated from water recovered with oil (produced water) in the Shell production battery in Harmattan, Alberta, Canada and identified as

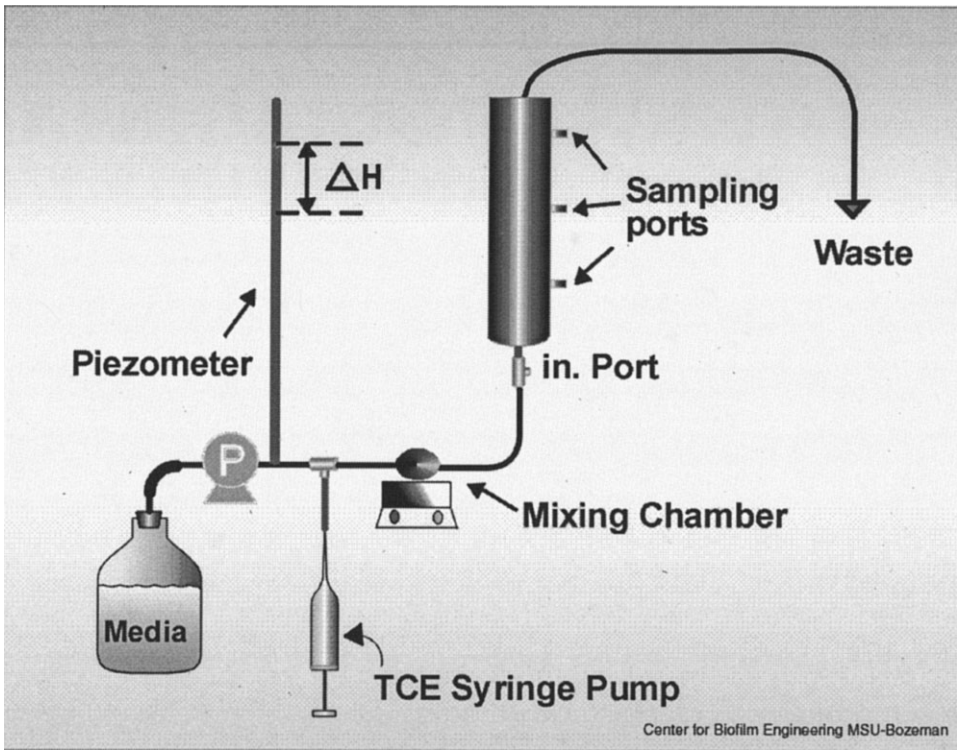

Figure 2. Porous medium column with sampling ports.

Klebsiella pneumoniae [3]. This environmental isolate was later reclassified as *K. oxytoca* [4].

Selective and Nonselective Plating Techniques

B. cepacia was selected on either modified Luria–Bertani (LBG) agar plates or phenol agar plates. LBG agar plates contain 10 g tryptone (Becton–Dickinson), 5 g yeast extract (Becton–Dickinson), 5 g NaCl, 1 g dextrose (Becton–Dickinson), and 17 g Bacto-agar (Becton–Dickinson) per liter of distilled water with 0.05 g/L kanamycin (Sigma Chemical Co.) added after autoclaving. Phenol agar plates contain 15 g Bacto-agar per liter of hydrocarbon minimal medium (HCMM2) with 94.1 mg/L phenol (J.T. Baker Chemical Co., Phillipsburg, NJ) and 0.05 g/L kanamycin added after autoclaving. HCMM2 medium contains 2.84 g Na_2SO_4, 1.37 g NH_4Cl, 1.515 g KH_2PO_4, 1.58 g Na_2HPO_4, NaOH, ~pH 7.2, 0.01125 g $CaCl_2$, and 0.0967 g $MgCl_2$ per liter of nanopure water. All chemicals for HCMM2 medium were purchased from Fisher Scientific. *K. oxytoca* was selected on brain heart infusion (BHI) agar plates (4 g BHI medium (Becton–Dickinson) and 15 g Bacto-agar per liter of distilled water) amended with 0.1 g/L filter sterilized streptomycin sulfate (Fisher Scientific) after autoclaving. R2A (Becton–Dickinson) was used as the nonselective nutrient agar to determine total cell numbers and provide a total cell balance.

Inoculum Preparation

To prepare a viable, TCE-degrading culture of *B. cepacia* for inoculation into the reactors, a loopfull of *B. cepacia* was transferred from a frozen culture (−70°C in 2% peptone (Becton–Dickinson), 20% glycerin (Fisher Scientific)) to a phenol/kanamycin agar plate and incubated at 30°C for 48 h. A colony from the phenol/kanamycin plate was transferred to a modified LBG agar plate (amended with kanamycin) and incubated at 30°C for 24 h. A colony from the LBG/kanamycin plate was transferred to 100 ml LBG broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g dextrose per liter of distilled water) and incubated for 18 h at 36°C on a horizontal shaker (150 rpm). One milliliter of this culture was transferred to 100 ml fresh LBG broth and incubated for 18 h at 36°C on a horizontal shaker (150 rpm). *K. oxytoca* was transferred from a frozen culture (−70°C in 2% peptone, 20% glycerin) and incubated at 30°C for 24 h on a BHI agar plate amended with streptomycin. A colony was transferred to 100 ml LBG broth and incubated at 36°C on a horizontal shaker (150 rpm). After 18 h, 1 ml was transferred to 100 ml fresh LBG broth and incubated for 18 h at 36°C on a horizontal shaker (150 rpm).

Reactor Design

Each experiment used one of two glass Chromaflex columns (Kimble Kontes, Vineland, NJ). Both columns were 30 cm long and 5 cm in diameter and each was filled with 1 mm glass beads (BioSpec Products, Inc., Bartlesville, OK). Each column was identical except for one column that had three miniert sampling ports (Alltech, Deerfield, IL) along the length of the column (Figure 2). A Kd Scientific syringe pump (Fisher Scientific) supplied TCE from a Hamilton gas-tight 10-ml syringe (Fisher Scientific) to a 1/4 inch Swaglok tee connected to the main flow line. Another Swaglok tee located between the TCE injection point and the influ-

ent of the column served as an influent sampling port and bacterial injection point. Between the TCE injection point and influent sampling point was an in-line mixing chamber constructed from a glass flow break with a stir bar inside. Teflon tubing (0.64 cm) (Cole-Parmer Instrument Co.) was attached to both ends of the flow break and connected to the Swagelok tees. Oxygen gas was diffused into the feed medium for all experiments.

Column Preparation and Characterization

Prior to each experiment, the column was rinsed with 70% ethanol. Beads and tubing were rinsed with deionized (DI) water and autoclaved. The system was completely assembled and 1 liter of a 10% bleach solution was pumped through the reactor for ~3 h followed by 1 liter of 38 g/L sodium thiosulfate (Fisher Scientific) for ~3 h. Eight to 10 liter of autoclaved DI water was then rinsed through the column for ~24 h.

Conservative tracer studies were performed on both columns (with and without sampling ports) by pumping 0.3 mM fluorescein (Sigma Chemical Co.) from the syringe pump at a rate of 0.1 ml/h to the main flow line (Q = 2.4 ml/min) and measuring influent and effluent fluorescein concentrations over time. Samples were analyzed by measuring the absorbance using a Genesys 5 spectrophotometer (Spectronic Instruments) at a wavelength of 490 nm. Abiotic TCE breakthrough curves were performed prior to inoculation of each column by continuously pumping (0.16 ml/min) water saturated with TCE (TCE solubility = 1100 mg/L at 25°C) via syringe pump to the column for 9 h and measuring TCE concentrations from the influent and effluent of the column over time.

Column Operation

At the beginning of the experiment, 50 ml of *B. cepacia* growth culture (single-species experiment) or 50 ml of both *K. oxytoca*'s and *B. cepacia*'s growth cultures (dual-species experiments) was injected into the column. The mass of *B. cepacia* and *K. oxytoca* inoculated into each column was $(5.7 \pm 0.87) \times 10^{10}$ CFU and $(1.6 \pm 0.34) \times 10^{11}$ CFU, respectively. Different dilutions of LBG medium, resulting in 30, 70, or 700 mg/L initial dissolved organic carbon (DOC), were pumped through the system at a rate of 2.4 ml/min for 70 h. Effluent samples were periodically taken to monitor dissolved oxygen concentrations, dissolved organic carbon concentrations, and population densities over time. After 70 h, each dual-species column was taken off-line and approximately 5 g of beads was removed from the beginning and end of the column and put in separate test tubes. Fifteen milliliters of phosphate-buffered saline solution (8.7 g NaCl, 0.4 g KH_2PO_4, 1.23 g K_2HPO_4 per liter distilled water) was added incrementally to each tube (5 ml, 5 ml, 3 ml, 2 ml). After each addition, the test tube was vortexed for 1 min to detach the bacteria from the beads and the supernatant was poured off. Microscopic analysis revealed that this "bead bashing" procedure removed virtually all of the biofilm from the beads. The resulting supernatants were homogenized and plated in triplicate on both selective and nonselective plates to determine population dynamics throughout the column. The column was put back on-line and 3 pore volumes were run through the column before the TCE experiment was begun. TCE addition to the column consisted of water saturated with TCE being continuously pumped (0.16 ml/h) from the syringe pump into the column for 12 (single-species experiment) or 9 h (dual-species experiment) and TCE concentrations from the influent and effluent were measured. TCE concentrations from the sampling ports were monitored periodically using a 1-ml gas-tight syringe (SGE Chromatography Supplies) with a 11.5-cm-long 22-gauge needle (SGE Chromatography Supplies). At the end of each single- and dual-species TCE experiment the column was taken off-line and drained and approximately 5 g of beads was removed from the beginning, middle, and end of the column. The bacteria were desorbed from the glass beads and quantified using the procedure described above.

TCE Analysis

One-half milliliter of sample was combined with 0.5 ml of hexane (Fisher Scientific) in a 2-ml glass vial (Fisher Scientific) and closed with a Teflon/silicone/Teflon screw cap (Fisher Scientific). The vial was shaken at 150 rpm for 10 min and 2 μL of the hexane phase was injected into a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 2.44 m × 2 mm ID glass column packed with 1% SP1000 on 60/80 Carbopak B (Supelco) and a Tracor 700A Hall Electrolytic Conductivity Detector (Finnigan MATT, Austin, TX). The injector and detector temperatures on the gas chromatograph were 200 and 250°C, respectively. The Hall reactor temperature was 900°C and the Hall electrolyte was 1-propanol (Fisher Scientific). The oven temperature was initially 150°C for 1 min and increased at a rate of 10°C/min to a final temperature of 200°C for 2 min. The carrier gas was helium (29 ml/min) and the reactor gas was H_2 (20 ml/min).

A specific TCE degradation rate was calculated by inputting the steady-state influent and effluent TCE concentrations from a TCE breakthrough curve into Eq. (1)

$$k = \frac{(\mathrm{TCE}_{\mathrm{in}} - \mathrm{TCE}_{\mathrm{out}}) * Q}{X_b * W_b}, \qquad (1)$$

where k is the specific TCE degradation rate (mass TCE/time/biomass) and Q is the flow rate (L^3/t) through the column. The total weight (W_b) of 1 mm beads in a packed column was 771 g. X_b is the bacterial population of the TCE-degrading organism (*B. cepacia*) desorbed from the glass beads collected at the end of the TCE breakthrough curve. To compare single- and dual-species TCE degradation rates to literature values, *B. cepacia*'s densities were converted from a population basis (CFU) to a weight basis (gram biomass) using a standard curve that compared plate counts to protein concentrations for *B. cepacia* grown in a batch culture with LBG medium at different population densities (data not shown).

Hydraulic Conductivity

The hydraulic conductivity (K) of the column was monitored over time using Darcy's Law (Eq. 2):

$$K = -\frac{Q}{A}\left(\frac{\Delta L}{\Delta H}\right), \quad (2)$$

where Q is the volume of water that travels through the column over time, A is the area perpendicular to flow (the cross-sectional area of the column), and ΔL is the length of the column. The change in hydraulic head (ΔH) was obtained by measuring the difference between the water level inside the influent piezometer port and height of the effluent tubing (open to the atmosphere) (Figure 2).

Other Analysis

Total protein was analyzed using the BCA protein assay kit (Pierce, Rockland, IL). Samples were rinsed to remove any remaining dissolved protein through centrifugation at 7660*g* for 20 min, supernatant was poured off, and the biomass pellet was resuspended in phosphate-buffered saline solution. Dissolved organic carbon was measured using a Dohrmann DC 80 carbon analyzer (Tekmar Dohrmann, Cincinnati, OH). Particulate organic carbon was removed from the samples using a 0.2-ìm syringe filter (Fisher Scientific). Inorganic carbon was removed by adding two drops of 20% phosphoric acid into 2 ml of sample and subsequent air sparging for 6 min. Dissolved oxygen was measured using an Accumet AP64 Series handheld dissolved oxygen meter (Fisher Scientific). The dissolved oxygen meter was fitted to a flow-through system made from a 40-ml centrifuge tube with an O-ring. Inlet and outlet ports were tapped in the side. The flow-through system was designed to attach to the influent and effluent tubing of the column. Statistical analysis was performed using the statistical software Minitab 13.20 (Minitab, Inc., State College, PA) and data sets were considered significantly different if the P values were less than 0.05.

RESULTS

To determine *B. cepacia*'s single-species TCE degradation potential in the column and to measure the effect of the chosen TCE concentration on the TCE degradation pathway, a pure culture of *B. cepacia* was inoculated into the porous medium column and supplied with 30 mg/L DOC substrate concentration for 82 h, with a constant concentration of TCE supplied to the column for the final 12.5 h of the experiment. The steady-state influent and effluent TCE concentrations during the period of TCE injection to the column were 1.07 mg/L (SD=0.11 mg/L, n=8) and 0.23 mg/L (SD=0.05 mg/L, n=8), respectively. Abiotic TCE breakthrough curves prior to bacterial inoculation showed no loss of TCE throughout the column (data not shown). Thus, a column inoculated with a pure culture of *B. cepacia* and supplied with 30 mg/L DOC substrate concentration removed 79.1% of the TCE supplied to the system. The specific TCE degradation rate calculated for *B. cepacia* in single species for the 30 mg/L DOC substrate concentration was 0.029 μg TCE/min/mg protein. To determine whether the TCE degrading pathway of *B. cepacia* was affected by the TCE concentration, the column was destructively sampled after TCE addition and the total amount of viable cells (measured using R2A plates) was compared to the population of cells with an active TCE degrading pathway (measured using LBG/kanamycin plates). An average of the plate counts from the beginning, middle, and end of the column reveals that the population of cells with an active TCE degrading pathway ($1.9 \times 10^8 \pm 1.2 \times 10^8$ CFU/g glass beads) comprised the majority of the total cell population ($2.3 \times 10^8 \pm 1.6 \times 10^8$ CFU/g glass beads).

Cultures of both *B. cepacia* and *K. oxytoca* were then inoculated into the column and a substrate concentration of 30, 70, or 700 mg/L DOC was supplied to the system. After approximately 70 h of bacterial growth, TCE breakthrough curves were performed on the dual-species columns (Figure 3). Probabilities were calculated by comparing the last four steady-state influent and effluent data points from duplicate experiments. All three substrate concentrations had similar steady-state influent TCE concentrations (1.15 ± 0.05 mg/L). These biotic influent TCE concentrations also compared well to abiotic TCE breakthrough curves performed prior to bacterial inoculation (data not shown). The dual-species TCE breakthrough curves for the 30 mg/L DOC substrate concentration experiments (Figure 3A), however, show a steady-state effluent TCE concentration that is significantly lower ($P < 0.001$) than the steady-state influent TCE concentration. The dual-species TCE breakthrough curves for the 70 mg/L DOC substrate concentration experiments (Figure 3B) also show an effluent steady-state TCE concentration that is significantly lower (P value < 0.001) than the influent steady-state TCE concentration but not as low as the steady-state effluent concentration in the 30 mg/L DOC substrate concentration TCE breakthrough curves (Figure 3A); more TCE was removed at the lower substrate concentration experiment. No measurable removal of TCE was observed between the influent and effluent of the 700 mg/L DOC substrate concentration columns (Figure 3C). A percentage removal was obtained by taking the steady-state average of the difference between the influent TCE concentration and the corresponding effluent TCE concentration after 1 pore volume of flow (Table 1). The greatest amount of TCE degradation occurred in the low substrate concentration and increasing the substrate concentration caused a decrease in both the TCE degradation and the specific TCE degradation rate (Table 1). Sorption of TCE to biomass in the column was assumed to be negligible because no removal of TCE was observed at the high substrate concentration (where the highest population densities, mostly *K. oxytoca*, were observed).

Dissolved organic carbon was monitored from the influent and effluent of the column over time to quantify substrate utilization in the porous medium columns. The amount of DOC utilized at steady state for a column fed 30, 70, and 700 mg/L DOC was 9.4 ± 1.1 mg/L (n=3), 21.4 ± 8.0 mg/L (n=3), and 99.1 ± 1.4

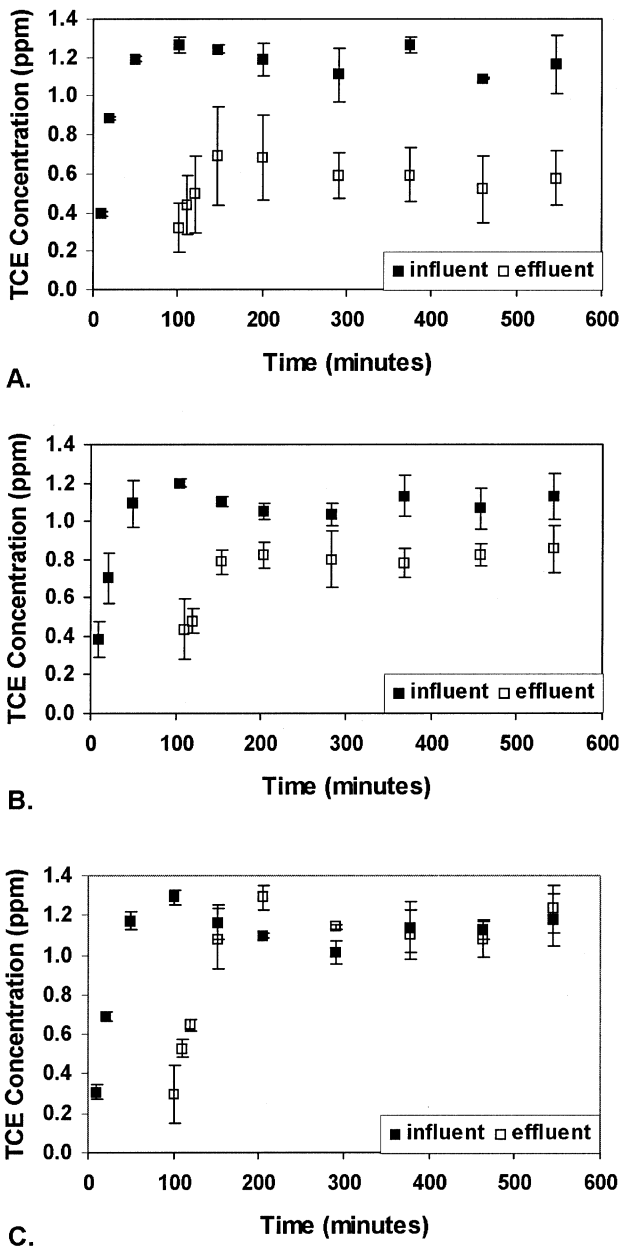

Figure 3. TCE breakthrough curve for the dual-species column experiments administered (A) 30 mg/L DOC, (B) 70 mg/L DOC, and (C) 700 mg/L DOC substrate concentration. Values are the average of duplicate experiments and error bars are ±SD.

mg/L (n=2), respectively. A significant amount of DOC (>65%) remained in the effluent of each column after 70 h of reactor operation.

The biofilm population density of each organism in the dual-species culture was quantified after 9 h of TCE addition (Table 2). *B. cepacia* had a higher population density than *K. oxytoca* throughout the column at the 30 mg/L DOC substrate concentration, with the population density of both organisms remaining relatively constant with distance. For the 70 mg/L DOC substrate concentration experiments, the population density of both organisms decreased from the entrance to the exit of the column. The population density of *B. cepacia* was comparable to that of *K. oxytoca* at the beginning of the column and was greater than that of *K. oxytoca* in the middle and end of the column. For the 700 mg/L substrate concentration experiments, *K. oxytoca* had a higher population density than *B. cepacia* throughout the column. Therefore, increasing the substrate concentration caused a shift in the population distribution, with *B. cepacia* (the TCE degrading organism) being the dominant organism at the low substrate concentration and *K. oxytoca* (the thick biofilm forming organism) being the dominant organism at the high substrate concentration.

The column with sampling ports was used to measure the fate of TCE over the length of the column for the three substrate concentrations (Figure 4). The 700 mg/L DOC substrate concentration experiments show a constant TCE concentration over the length of the column, indicating no loss of TCE in the column (Figure 4C). The 70 mg/L DOC substrate concentration experiments show a decrease in TCE concentration from the influent sampling port to port 1 (6 cm into column) but then remains constant (Figure 4B) while the 30 mg/L DOC substrate concentrations show a decreasing TCE concentration over the length of the column (Figure 4A). Effluent dissolved oxygen concentrations below detection were recorded throughout the duration of the 70 mg/L DOC and 700 mg/L DOC substrate concentration experiments while effluent dissolved oxygen concentrations greater than 6 mg/L were recorded for the duration of the low substrate concentration experiments (30 mg/L dissolved organic carbon). Oxygen present throughout the column allowed for a continual decrease of TCE throughout the column by the aerobic TCE-degrading organism (*B. cepacia*) while the oxygen could have become limited before the first sam-

Table 1. Dual-species TCE breakthrough curve data.

Substrate concentration (mg/L DOC)	Steady-state influent TCE concentration (mg/L)	Steady-state effluent TCE concentration (mg/L)	TCE removed (%)	Specific TCE degradation rate*
30	1.19 ± 0.04	0.59 ± 0.19	48.8 ± 16.5	0.024 ± 0.009
70	1.10 ± 0.05	0.81 ± 0.11	26.9 ± 6.9	0.014 ± 0.002
700	1.15 ± 0.02	1.16 ± 0.09	−0.5 ± 5.4	−0.0002 ± 0.004

Note: Values are the average of replicate experiments ± the standard deviation.
*Micrograms of TCE per minute per milligram of protein.

Table 2. Dual-species porous medium population densities after the TCE addition was stopped.

Substrate conc. (DOC)	*K. oxytoca* (CFU/g bead) × 10^8			*B. cepacia* (CFU/g bead) × 10^8		
	Entrance	**Middle**	**Exit**	**Entrance**	**Middle**	**Exit**
30 mg/L	0.03 ± 0.04	0.04 ± 0.05	0.02 ± 0.02	0.73 ± 0.26	1.03 ± 0.09	0.37 ± 0.16
70 mg/L	0.58 ± 0.08	0.04 ± 0.02	0.02 ± 0.008	0.48 ± 0.21	0.31 ± 0.06	0.08 ± 0.04
700 mg/L	5.37 ± 0.04	0.41 ± 0.39	0.17 ± 0.06	0.06 ± 0.01	0.16 ± 0.04	0.08 ± 0.10

Note: Values are an average of duplicate experiments ± SD.

pling port at the 70 mg/L DOC substrate concentration experiment, resulting in no additional TCE degradation further into the column. Lower TCE concentrations were measured in the center of the column compared to the sides of the column (Figure 4A) for the 30 mg/L DOC substrate concentration experiment. This could be due to preferential flow along the side of the column or lower TCE degradation activity near the side of the column compared to the middle (thus higher TCE concentrations). Lower TCE degradation activity could have been attributed to lower TCE-degrading populations or limiting dissolved oxygen concentrations near the side of the column.

The hydraulic conductivity of the porous medium column was monitored over time for each substrate concentration (Figure 5). An increase in substrate concentration resulted in an increased reduction in hydraulic conductivity. The initial hydraulic conductivities for all three experiments ranged from 0.85 to 0.95 cm/min and reached steady-state conditions after approximately 20 h. At the 30 mg/L DOC substrate concentration, a steady-state hydraulic conductivity of 0.79 cm/min was recorded. This resulted in a hydraulic conductivity reduction of less than 10%. The steady-state hydraulic conductivity was 0.52 cm/min during the 70 mg/L DOC substrate concentration experiment, which resulted in a hydraulic conductivity reduction of 39%. An order of magnitude increase in substrate concentration (70 to 700 mg/L DOC) resulted in a steady-state hydraulic conductivity of 0.19 cm/min, which resulted in a hydraulic conductivity reduction of 79%.

DISCUSSION

The results of the *B. cepacia* pure culture and dual-species TCE breakthrough curves for the 30 mg/L DOC substrate concentration show that the dual-species columns removed 48.8% of the TCE compared to 79.1% removal in the column with a pure culture of *B. cepacia*. In addition, the population of *B. cepacia* in a pure culture column was greater than the *B. cepacia* biofilm population in a column inoculated with both *B. cepacia* and *K. oxytoca* and supplied the same substrate and TCE concentration. Therefore, *K. oxytoca* had a negative affect on *B. cepacia*'s ability to degrade TCE. Competition for surface sites on the porous medium and increased diffusion limitation of oxygen into the dual-species biofilm (now at least partly consisting of mucoid EPS produced by *K. oxytoca*) are possible reasons for the decreased TCE degradation in the dual-species column compared to *B. cepacia* alone. Dissolved oxy-

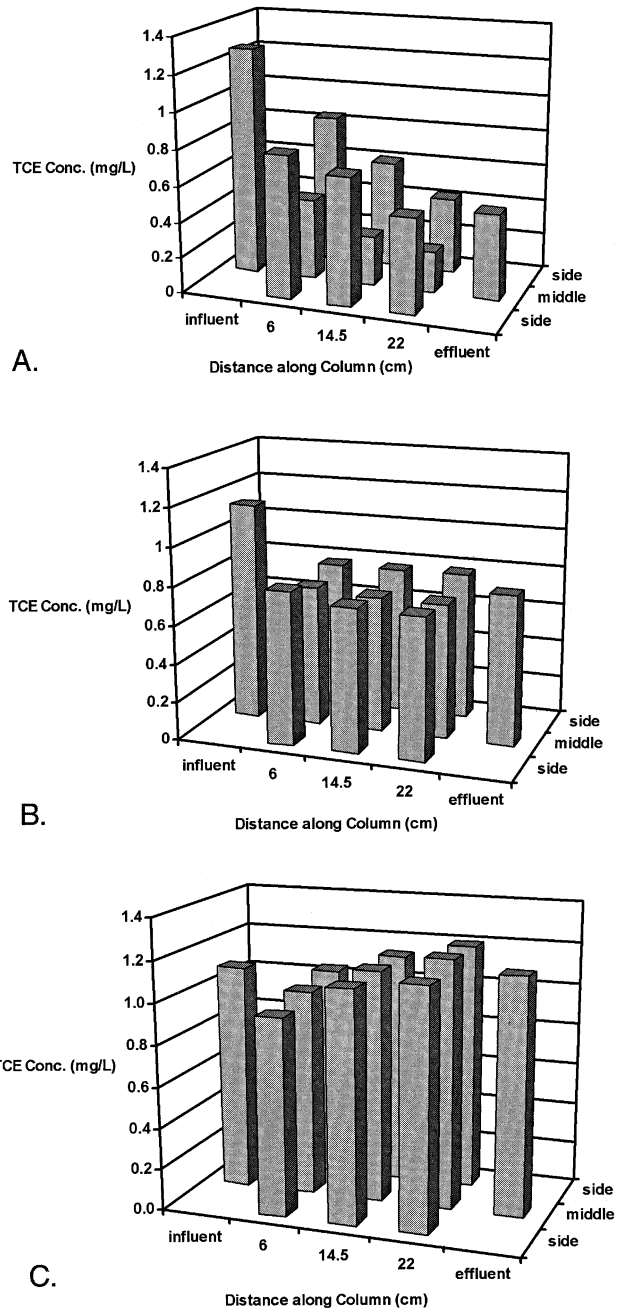

Figure 4. TCE concentrations over the length of the 30-cm-long column for dual-species experiments supplied with (A) 30 mg/L DOC, (B) 70 mg/L DOC, or (C) 700 mg/L DOC substrate concentration.

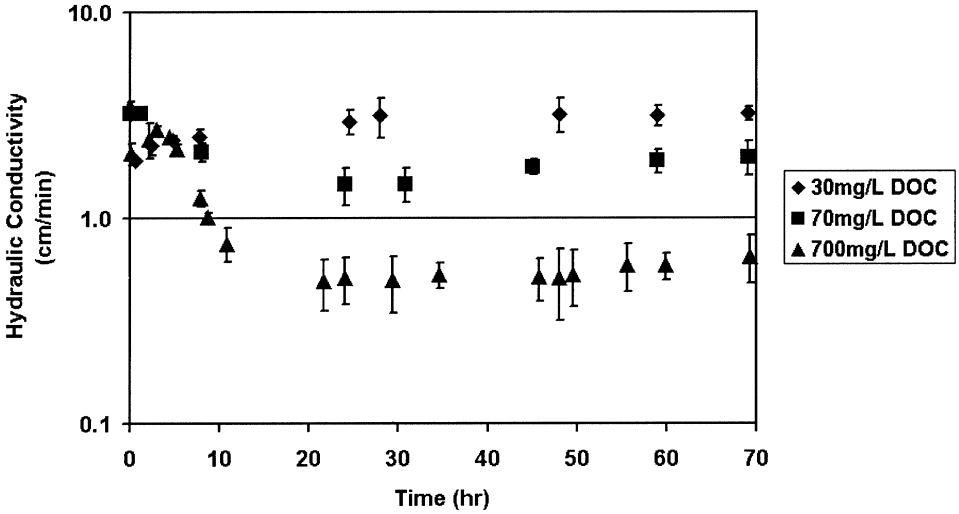

Figure 5. Hydraulic conductivity over time. Values are the average of duplicate experiments and error bars are ±SD.

gen was present in the effluent of the dual-species experiment administered 30 mg/L DOC, but localized regions of oxygen depletion caused by thick biofilm formation by *K. oxytoca* may have decreased the extent of aerobic TCE degradation by *B. cepacia* (although it should be noted that the thick biofilm formation was not significant enough to reduce the hydraulic conductivity). The specific TCE degradation rate of *B. cepacia* in a pure culture (0.029 μg TCE/min/mg protein), and in combination with *K. oxytoca* (0.024 μg TCE/min/mg protein), was similar for the 30 mg/L DOC substrate concentration experiments, suggesting that the presence of *K. oxytoca* did not affect *B. cepacia's* specific TCE degradation rate. A decrease in substrate concentration resulted in an increase in *B. cepacia's* specific TCE degradation rate in the dual-species experiments (Table 1). Biofilm-specific TCE degradation rates in porous medium were lower than batch TCE degradation rates for *B. cepacia* $PR1_{31c}$ (0.76 μg TCE/min/mg protein) reported by Sharp et al. [12] for the same TCE concentration (1.2 mg/L). Lower TCE degradation activity in biofilm cultures compared to batch cultures has been previously reported [13]. One possible reason for the lower rates in porous media is overestimation of the TCE degrading population. The specific TCE degradation rate equation (Eq. 1) assumes that the entire *B. cepacia* population of the column was actively degrading TCE. Oxygen utilization along the length of the column (observed in the 70 and 700 mg/L DOC substrate concentration experiments) as well as diffusion limitation of TCE and oxygen into the biofilm on the surface of the glass beads could also have limited the number of *B. cepacia* cells actively degrading TCE. Lower specific TCE degradation rates corresponded with significantly lower specific growth rates in porous medium compared to batch cultures (data not shown).

Substrate concentration played a very important role in controlling TCE degradation in dual-species porous medium columns. Decreasing the substrate concentration caused an increase in TCE degradation. Approximately twice as much TCE was removed from the column supplied with 30 mg/L DOC compared to 70 mg/L DOC (Table 1), which corresponded with a TCE-degrading population that was higher at the lower (30 mg/L DOC) substrate concentration experiment. The increase in TCE degradation at the lower substrate concentration was also attributed to oxygen limitation. The constitutive degradation of TCE by *B. cepacia's* TOM pathway requires oxygen and the negative affect of oxygen utilization with respect to *B. cepacia's* ability to degrade TCE has been documented [14]. Nonlimiting effluent dissolved oxygen concentrations were only present in columns supplied with the lowest substrate concentration (30 mg/L DOC).

The initial hydraulic conductivities for all three experiments ranged from 0.85 to 0.95 cm/min, which is a hydraulic conductivity typical of clean sand [15]. The steady-state hydraulic conductivity decreased with increasing substrate concentrations. This decrease in hydraulic conductivity corresponded with an increase in *K. oxytoca* population density. An increase in substrate concentration also caused oxygen-limiting conditions. *K. oxytoca* is a facultative organism and can survive without oxygen through fermentation [16]. This change in metabolism could influence the type or magnitude of mucoid biofilm produced. Therefore, permeability reduction appears to be a function of *K. oxytoca's* population density and possibly a function of *K. oxytoca's* metabolic changes from oxygen stress. The steady-state hydraulic conductivity from the 700 mg/L DOC substrate concentration experiment (0.19 cm/min) was comparable to that found in literature using *K. oxytoca* administered a similar carbon concentration [4].

The use of biological barriers to reduce soil permeability was shown to be effective in field-scale applications. This research demonstrated the feasibility of creating a dual-species biofilm barrier that can not only reduce porous medium permeability, but also simultaneously degrade TCE. In addition, the importance of substrate concentration on both permeability reduction

and TCE degradation provides insight into the possible scenario of both organisms being used for a field-scale biofilm barrier. As the bacteria and growth medium are injected down a well and pumped out through the soil, the growth medium would be diluted due to mixing with upstream groundwater, thus creating a system with varying substrate concentrations. Higher substrate concentrations a certain radius of influence from the injection well would provide adequate permeability reduction between injection wells by the thick biofilm-forming organisms (provided the wells were spaced close enough to each other), while the lower substrate concentrations upstream of the well (along with dissolved oxygen in the upstream groundwater) could provide an environment for TCE degradation to occur. This research provides the framework for a possible TCE-degrading, reduced-permeability biofilm barrier in the field, which would be a significant advancement in bioaugmentation technology.

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