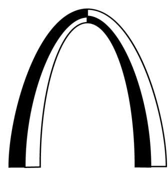

POPULATION DYNAMICS IN A MULTI-SPECIES BIOFILM FOR THE CREATION OF A REACTIVE BIOBARRIER

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ABSTRACT

A series of column experiments were performed using different bacterial inoculation scenarios to maximize the TCE-degrading potential of a multi-species biofilm. The single-species population dynamics of *Burkholderia cepacia* PR1 pTOM31c, a TCE-degrading microorganism unable to form a significant biofilm, were examined and compared to those of a dual-species population containing both *B. cepacia* and *Klebsiella oxytoca*, a strong biofilm-forming microorganism. These experiments were performed to determine if a dual-species TCE-degrading biofilm could be established in porous media. The results indicate that *B. cepacia* was able to persist throughout the porous media columns as part of a single- and dual-species population. The presence of *K. oxytoca* did not adversely affect *B. cepacia* in the porous media columns. The method of dual-species inoculation, *B. cepacia* before, after, or simultaneously with *K. oxytoca*, did not have a significant effect on the *B. cepacia* cell numbers.

Key words: *biofilms, biobarrier, multi-species, subsurface containment, trichloroethylene*

INTRODUCTION

The migration of contaminants from a hazardous waste site is of concern for the protection of downstream resources. An innovative solution to prevent contaminant migration in groundwater is the use of subsurface biofilm barriers (biobarriers). These biobarriers can manipulate soil hydraulic conductivity to allow for varying degrees of groundwater penetration, thus enabling the containment or diversion of a contaminant plume. These biobarriers can be applied in the field by injecting starved bacteria and then nutrients into a series of shallow wells. Sufficient amounts of nutrients would be added after bacterial inoculation to produce overlapping columns of soil in which the pore space is virtually sealed by bacterial growth and EPS (extracellular polysaccharide) production (Costerton, 1994). Research performed at the Center for Biofilm Engineering (CBE) has successfully demonstrated the feasibility of using thick biofilms as a subsurface biobarrier at the bench scale (Cunningham et al., 1991; Warwood et al., 1995; James et al., 1995b; Cunningham et al., 1997; and Komlos et al., 1998) and a field-scale demonstration is currently underway.

A developing, advanced application of the biobarrier technology is the use of reactive bacterial populations to produce reactive biobarriers. Reactive biobarrier technology offers a means of degrading a contaminant while simultaneously reducing its migration. Since recalcitrant compounds, such as trichlororethylene (TCE), show little natural attenuation, the development of a TCE reactive biobarrier could lead to a significant advancement in bioaugmentation technology. Reactive biobarriers can be developed in two ways: 1) using a single bacterial population to

reduce the soil hydraulic conductivity and simultaneously degrade the contaminant; and 2) using two species to develop a reactive barrier, one species to produce the biofilm and reduce the hydraulic conductivity, the other species to carry out the desired reaction(s). The single species method requires that the bacterial population produces copious amounts of biofilm while carrying out the desired reaction. The dual-species reactive biobarrier would offer the opportunity to separately select for microorganisms with specific characteristics (biofilm formation or contaminant degradation) and combine them into a single biofilm capable of performing multiple functions.

An understanding of how bacterial species interact in a multi-species biofilm is imperative for the successful implementation of a dual-species reactive, reduced-permeability biobarrier. It is important that one organism does not completely out-compete the other. Banks and Bryers (1991) and Sturman et al. (1994) reported that a microorganism's growth rate plays an important role in multi-species population dynamics in a biofilm, with the faster growing microorganism having a competitive advantage over the slower growing microorganism. However, Siebel and Characklis (1991) observed that factors other than growth rate may influence spatial distribution of cells and relative cell numbers in biofilms. It was concluded by James et al. (1995a) that multi-species interactions in a biofilm can be a function of the microorganisms present, the substratum the microorganisms are attaching to, as well as each microorganism's physiological parameters. For this reason it was important to perform preliminary experiments examining the feasibility of combining a TCE-degrading microorganism and a thick biofilm-forming microorganism in a dual-species environment in which both species are present in significant numbers.

The TCE-degrading organism used in this experiment is *Burkholderia cepacia* PR1-pTOM31c and it has been observed to not persist well in a porous media environment (Sharp, 1995). Banks and Bryers (1992) observed that a bacterial species deposits faster to a biofilm than to clean glass beads, even when the biofilm was that of another microorganism. Drury et al. (1993) also observed that biofilms are significantly porous for bacterial-sized beads to become entrapped and could provide a mechanism to envelop other bacterial cells. Therefore, the following questions were addressed: 1) Could a mucoid bacterial species provide a thick biofilm matrix enabling the non-mucoid TCE-degrading population to persist in a porous media environment? and 2) Could a particular method of dual-species inoculation increase the TCE-degrading population in a porous media environment? Power et al. (1998) observed that the method of inoculation could influence the survival and persistence of another bacterial culture introduced into a multi-species biofilm. This research set out to determine if the presence of a thick biofilm-forming bacterial species could provide a mechanism to increase the population numbers of a non-mucoid, degradative organism in a porous media environment. This research will also describe the effects of different inoculation scenarios on the population dynamics of a dual-species biofilm in a porous media environment.

MATERIALS AND METHODS

Bacterial Strains

The bacterial isolates used for this experiment were *Burkholderia cepacia* PR1-TOM31c and *Klebsiella oxytoca*. *B. cepacia* is an aerobic bacterium that can constituatively degrade TCE in cometabolic processes using the toluene ortho-monooxygenase (TOM) pathway (Shields and Reagin, 1992). The genetic information for the degradative pathway is located on the plasmid, pTOM31c. The plasmid also encodes for the resistance to the antibiotic, kanamycin, which was used for selection of *B. cepacia*. Sharp (1994) indicated that many attempts to grow a biofilm using a pure culture of *B. cepacia* on different types of porous media (glass beads, diatomaceous earth pellets, inert silica packing, and oyster shells) were unsuccessful. Because of its ability to degrade TCE and not form a thick biofilm, *B. cepacia* was chosen as the reactive, non-mucoid bacterial strain for this experiment.

K. oxytoca is a facultative anaerobic bacterium isolated from an oil field (MacLeod et al., 1988). Its ability to form thick biofilms made it an ideal candidate for use as the mucoid organism in the dual-species experiments. *K. oxytoca* was selected for utilizing its resistance to the antibiotic, streptomycin. Batch reactor studies indicate that *K. oxytoca* has a significantly faster growth rate than *B. cepacia*.

Bacterial Isolation and Characterization

Selective and non-selective nutrient agar plates were used to characterize the dual-species population dynamics. *B. cepacia* was selected for using either modified Luria-Bertani (LBG) agar plates amended with kanamycin (10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, and 17g Bacto-agar per liter of distilled water with 0.05g/L kanamycin added 45 minutes after autoclaving) or phenol agar plates amended with kanamycin (15g Bacto agar per liter of hydrocarbon minimal medium (HCMM2) with 94.1mg phenol and 50mg kanamycin added 45 minutes after autoclaving). HCMM2 media contains 2.84g of sodium sulfate (Na2SO4), 1.37g ammonium chloride (NH4Cl), 1.515g potassium phosphate monobasic (KH2PO4), 1.58g sodium phosphate dibasic (Na2HPO4), sodium hydroxide (NaOH) ~ pH 7.2, 0.01125g calcium chloride (CaCl2), and 0.0967g magnesium chloride (MgCl2) per liter of nano-pure water. *K. oxytoca* was selected for using Brain Heart Infusion (BHI) agar plates amended with streptomycin (4g BHI media and 15g Bacto agar per liter of distilled water with 0.1 g/L streptomycin sulfate added 45 minutes after autoclaving). R2A was used as the non-selective nutrient agar plates to determine total cell numbers and provide a total cell balance.

Inoculum Preparation

In order to prepare a viable, TCE-degrading culture of *B. cepacia* for inoculation into the batch reactor or porous media column, a *B. cepacia* colony was transferred from a phenol/kanamy-

cin agar plate to a LBG/kanamycin agar plate and incubated for 48 hours. A colony from the LBG/kanamycin plate is then transferred to 100ml LBG broth (without kanamycin and agar) and incubated for 18 hours at 36°C on a horizontal shaker (150 rpm). One ml of this culture is transferred to 100ml fresh LBG broth and incubated for 18 hours at 36°C on a horizontal shaker (150 rpm).

K. oxytoca was grown on a BHI agar plate amended with streptomycin. A colony was transferred to a 100ml LBG broth and incubated at 36°C on a horizontal shaker (150 rpm). After 18 hours, 1 ml is transferred to 100ml fresh LBG broth and incubated for 18 hours at 36°C on a horizontal shaker (150 rpm).

Dual-Species Batch Experiment

One ml of each growth culture (*B. cepacia and K. oxytoca*) was transferred to 100ml of LBG broth. The dual-species culture was incubated at 36°C on a horizontal shaker (150 rpm). After 18 hours, the concentration of each species was determined by spread agar plating on a) LBG with kanamycin (*B. cepacia*), b) BHI with streptomycin (*K. oxytoca*), and c) R2A (total).

Column Reactor Design and Operation

A series of 2.54 cm diameter, 25.4 cm long stainless steel columns were constructed and filled with 1mm glass beads (Figure 1). Metal screens were positioned on both ends of each column to contain the glass beads and the ends of the columns were sealed with rubber stoppers fitted with glass tubes which enabled connection to silicone tubing (size 14) leading to and from the column.

Continuous nutrient addition (1:100 diluted LBG broth) was supplied to the column using a peristaltic pump supplying flow at a rate of 1ml/min. Flow rates were determined using a graduated cylinder and stopwatch. Bacteria were added to the columns through syringe injection at the influent of the column and flow was stopped for 12 hours after inoculation to allow the microorganisms to attach initially to the glass beads. Flow breaks were situated upstream and downstream of the column to prevent bacterial contamination of the columns and nutrient reservoir. Effluent samples from each column were grown on selective and non-selective agar plates at various times throughout each experiment to indirectly monitor cell activity in the columns.

Methods of Inoculation

Using these columns, three experiments addressed the initial establishment of a reactive population in a dual-species biofilm. Experiment #1 involved the injection of two pore volumes (50ml) of *B. cepacia* culture into the column. After three days of nutrient addition, two pore volumes of *K. oxytoca* culture were inoculated into the column. Five days later, the column was destructively sampled to determine the number of the two bacterial populations throughout the column. These results were compared to experiment #2 *(K. oxytoca* culture was injected prior to *B. cepacia* culture) and experiment #3 (simultaneous injection) to determine which inoculation

method provided the highest fraction of the reactive population.

Sampling of Columns

Five days after the injection of the second bacterial species, the columns were destructively sampled and glass beads (~10 grams) were removed from the beginning, middle, and end of each column. The beads were placed in a test tube containing 15ml of Phosphate Buffered Saline (PBS) solution (8.7g of NaCl, 0.4g KH2PO4 and 1.23g K2HPO4 per liter of distilled water) and vortexed (bead-bashed) to remove the bacteria from the beads. Microscopic analysis of the glass beads indicated the removal of the majority of the bacteria from the bead surface (data not shown). The supernatant was removed and homogenized for 30 seconds to separate the biofilm cells. From this solution, the appropriate spread plate counts were performed.

RESULTS AND DISCUSSION

Results from the dual-species batch experiment showed that the *Burkholderia cepacia* population was out-competed by the faster growing *Klebsiella oxytoca* cells and contributed to less than one percent of the total dual-species population (Figure 2). The *B. cepacia* population in a dual-species culture was almost two orders-of-magnitude smaller than the *B. cepacia* population in a single-species culture. The presence of *K. oxytoca* had a detrimental effect on *B. cepacia* in the batch reactor.

The first set of column experiments was performed to determine if *K. oxytoca* would also out-compete *B. cepacia* in a biofilm growth environment. Two porous media column reactors (Figure 1) were set up in parallel and inoculated with either a single-species culture of *B. cepacia* or a dual-species culture of *B. cepacia* and *K. oxytoca*. Figure 3 shows that *B. cepacia* is able to persist in a porous media environment as a single culture and as part of a dual species biofilm. The concentration of *B. cepacia* in the dual-species column was comparable to that of *B. cepacia* in a single-species culture for the front and back of the column; however, an order-of-magnitude less was recorded in the middle of the column. The concentrations of *B. cepacia* ranged between 4.9 x 106 and 2.0 x 108 CFU (colony forming units)/g glass beads for both the single- and dual-species inoculations.

The next set of column experiments investigated the effects of different dual-species inoculation scenarios on the bacterial population distribution in a porous media environment. In the first inoculation scenario, *B. cepacia* was introduced into the column first and allowed to colonize the porous medium for three days before *K. oxytoca* was introduced. In the second inoculation scenario, *K. oxytoca* was introduced and allowed to colonize the column before *B. cepacia* was added. The third inoculation scenario involved the simultaneous injection of both cultures. Each column was fed nutrients for five days after the injection of the second culture and then destructively sampled. The results indicate that the method of inoculation did not have a significant effect on the reactive popula-

tion in a dual-species biofilm (Figure 4). The simultaneous inoculation provided the highest fraction of reactive bacteria averaged throughout the column. Using simultaneous inoculation, 59% of the column's total population consisted of *B. cepacia*, compared to 24% and 22% for the inoculation scenarios #1 and #2, respectively. This change in percent of reactive population is a result of fluctuations in the number of mucoid bacteria (data not shown). It should be noted that all of the inoculation scenarios for the porous media column provided significantly higher reactive population percentages than observed in the dual-species batch reactor experiments. In addition, the *B. cepacia* concentration was consistently lower in the middle of the column than at the influent and effluent of the column for the dual-species inoculations, while the *B. cepacia* concentration was highest at the influent and decreased throughout the column for the single-species column (Figures 3 and 4). No explanation for this observation can be given at this time.

CONCLUSION

A TCE-degrading organism (*Burkholderia cepacia*), that was out-competed by the faster growing, thick biofilm-forming organism (*Klebsiella oxytoca*) in a batch reactor, was able to persist and flourish in a biofilm environment as part of a single culture or in combination with *K. oxytoca*. The method of inoculation (reactive culture before, after, or simultaneously with the thick biofilm-forming culture) did not have a significant effect on the *B. cepacia* numbers throughout the porous media column. The presence of the mucoid bacterium, *K. oxytoca*, did neither significantly enhance nor inhibit the presence of the TCE-degrading organism in a biofilm. The described experiments, however, provided valuable information on how two bacteria interact in a porous media environment. The results presented will enable us to advance the reactive, reduced-permeability biobarrier research further.

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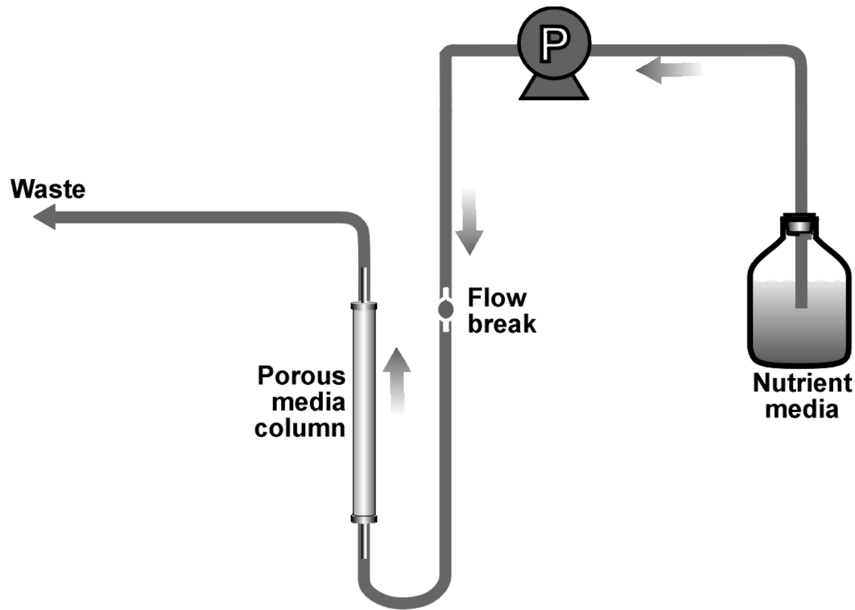

Figure 1. A constant-flow porous media column (25.4 cm long, 2.54 cm in diameter, filled with 1 mm glass beads) was inoculated with either a single-species bacterial population (reactive alone) or a dual-species bacterial population (reactive and mucoid) to determine if a reactive, non-mucoid bacterial population can exist in a porous media environment.

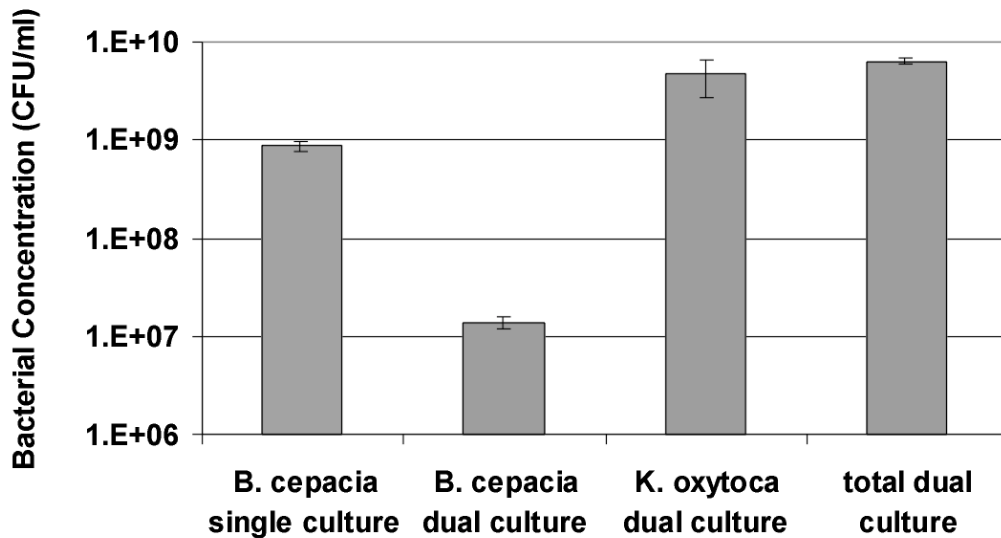

Figure 2. Population dynamics of *B. cepacia*, a TCE-degrading organism, in a single- and dual-species (with *K. oxytoca*, a mucoid organism) culture in a batch reactor. The presence of *K. oxytoca* significantly reduced the *B. cepacia* concentration compared to a mono-culture of *B. cepacia*.

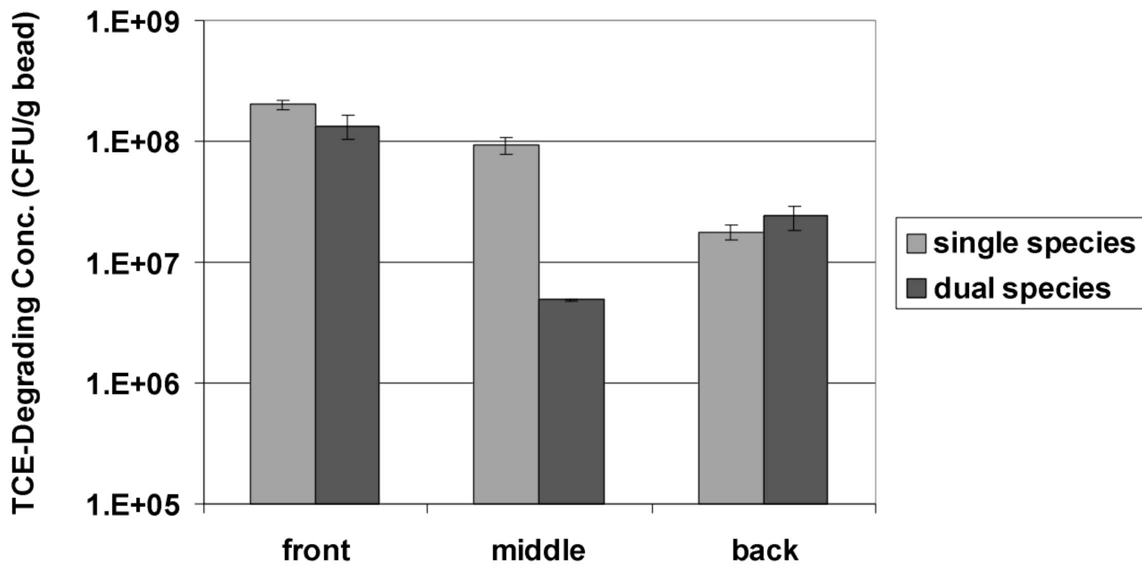

Figure 3. Population dynamics of the TCE-degrading organism (*B. cepacia*)throughout the porous media column. *B. cepacia* was able to persist in a porous media environment as a single culture and as part of a dual-species culture with *K. oxytoca*, the mucoid organism.

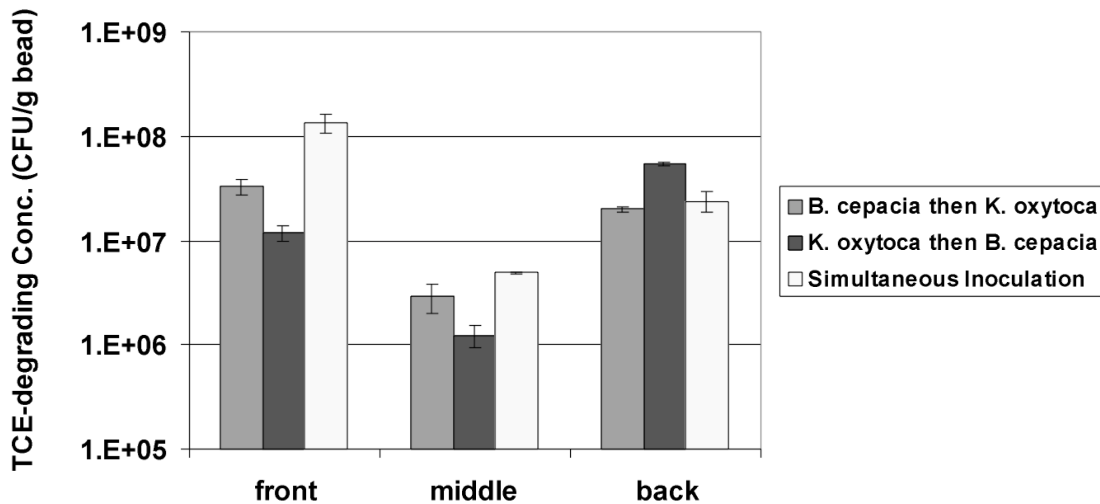

Figure 4. Results of different inoculation scenarios on the TCE-degrading (*B. cepacia)* bacteria's population dynamics in a porous media column. The method of inoculation, *B. cepacia* before, after, or simultaneously with *K. oxytoca* (mucoid organism), did not have a significan effect on the TCE-degrading bacteria's concentration througout the column.