Effect of Substrate Concentration on Dual-Species Biofilm Population Densities of *Klebsiella oxytoca* and *Burkholderia cepacia* in Porous Media

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Abstract: The long-term operation of bioremediation technologies relies on the success of the contaminant-degrading microorganism(s) to compete for available resources with microorganisms already present in an aquifer or those that may contaminate a bioreactor. Though research has been performed studying the interaction of multiple species in batch and chemostat reactors, little work has been done looking at multi-species interactions in environments that more closely resemble field-scale applications. The research presented herein examined the interaction of *Burkholderia cepacia* PR1-pTOM$_{31c}$, an aerobic trichloroethylene (TCE)-degrading bacterium, with *Klebsiella oxytoca*, a facultative bacterium, in a flow-through porous media (PM) reactor. Growth characteristics and population distributions in PM were compared to previously reported values from batch and chemostat reactors. The faster growing organism in batch experiments (*K. oxytoca*) did not always have the greater population density in dual-species PM experiments. The biofilm population distribution was influenced by substrate concentration, with *B. cepacia* having a greater dual-species population density than *K. oxytoca* at a low (30 mg/L dissolved organic carbon [DOC]) substrate concentration and *K. oxytoca* having a greater population density at a high (700 mg/L DOC) substrate concentration. This change in species population distribution with change in substrate concentration, which was not observed in batch reactors, was also observed in chemostat reactors. Therefore, manipulation of substrate concentration enabled the control of species dominance to the advantage of the TCE degrading population in this dual-species PM system and may provide a mechanism to enhance bioremediation scenarios involving TCE or other contaminants of concern.
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Keywords: *Burkholderia cepacia*; dual-species; growth rate; *Klebsiella oxytoca*; porous media; substrate concentration

INTRODUCTION

Though research has been performed examining dual-species growth rates (Banks and Bryers, 1991; Siebel and Characklis, 1991; Sturman et al., 1994; Stewart et al., 1997) and dual-species population distributions (McEldowney and Fletcher, 1987; Cowan et al., 1991; Banks and Bryers, 1992; Sturman et al., 1994; Møller et al., 1997; Komlos et al., 2005) in biofilms on the surface of a well-mixed reactor, little research has been performed on dual-species biofilms in porous media (PM) environments. Therefore, knowledge of dual-species interactions in PM and how these interactions correlate with kinetics from batch cultures and biofilms grown in well-mixed reactors is essential in extrapolating data from these systems to field-relevant conditions. A well-mixed biofilm is created when the bulk fluid experiences completely mixed conditions, providing constant nutrient supply and waste removal. In the literature this is described as a chemostat, a completely mixed stirred tank reactor (CSTR), or a rotating disk reactor (RDR). In contrast, a PM biofilm grows on the surface of PM (soil, sediments, glass beads, etc.) and is subject to plug flow conditions. Both types of biofilms differ from batch cultures in that they can experience mass transfer limitation deep in the biofilm (Revsbech and Jørgensen, 1996), but in a PM system, mass transfer limitation could occur in the bulk fluid, which would have a negative effect on aerobic organisms if oxygen is utilized upgradient. A biofilm on the surface of a well-mixed reactor experiences significant shear from the mixing of the reactor while laminar flow conditions prevail near the interface of the bulk fluid and biofilm surface in a PM environment. The hydrodynamic differences between the two systems could influence the characteristics of the biofilms. Previous research has shown

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that growth rates of bacteria are negatively affected by biofilm thickness (Wentland et al., 1996) and biofilm cell density (Ellis et al., 2000). Growth rates may also be affected by the type of biofilm reactor, indicating that growth rates from a well-mixed reactor may vary significantly from those of bacteria in PM biofilms due to increased mass transfer limitations and hydrodynamic differences. Therefore, use of biofilm data from a well-mixed reactor to describe PM growth may not be applicable.

The two bacterial species in this research were an aerobic TCE-degrading bacterium (*Burkholderia cepacia* PR1-pTOM$_{31C}$) and a facultative bacterium capable of thick biofilm formation (*Klebsiella oxytoca*). *B. cepacia* has the ability to constitutively degrade trichloroethylene (TCE) via a cometabolic process using the toluene ortho-monooxygenase (TOM) pathway (Shields and Reagin, 1992) and therefore may be used in bioreactor or bioaugmentation scenarios for TCE biodegradation. *K. oxytoca* was chosen to be combined with *B. cepacia* because of its relatively high growth rate and ability to survive in aerobic and anaerobic environments; conditions that should be expected in most bioremediation scenarios. Understanding how *B. cepacia* can compete with *K. oxytoca* in PM may enable the enhancement of bioremediation technologies by providing insight as to how *B. cepacia* and other aerobic contaminant-degrading microorganisms can compete with indigenous organisms in aquifer settings or with organisms that may contaminate a bioreactor. Previous research has shown that the faster growing organism (*K. oxytoca*) out-competed the slower growing organism (*B. cepacia*) in batch cultures (Komlos et al., 2005). However, these two organisms can coexist in a biofilm on the surface of a well-mixed reactor, though the population distribution and species dominance were highly dependent on the substrate concentration (Komlos et al., 2005). In addition, batch and biofilm growth kinetics were unable to describe species dominance in a biofilm. The research presented herein (1) quantified the dual-species population densities and growth rates of *B. cepacia* and *K. oxytoca* in PM for various substrate concentrations and (2) compared the PM biofilm population densities and dual-species distribution trends to those observed when these two organisms were grown in a dual-species batch culture and a dual-species well-mixed system.

MATERIALS AND METHODS

Sources of Strains

The aerobic TCE-degrading bacterium, *Burkholderia cepacia* PR1-pTOM$_{31c}$, was supplied by Malcolm Shields, University of West Florida. The facultative bacterium, *Klebsiella oxytoca*, was isolated from water recovered with oil (produced water) in the shell production battery in Harmattan, Alberta, Canada and identified as *Klebsiella pneumoniae* (MacLeod et al., 1988). This environmental isolate was later reclassified as *Klebsiella oxytoca* (Cunningham et al., 1997).

Bacterial Isolation and Inoculum Preparation

Selective and non-selective nutrient agar plates were used to characterize the dual-species populations. *B. cepacia* was selected on modified Luria-Bertani (LBG) agar plates amended with kanamycin and *K. oxytoca* was selected on brain heart infusion (BHI) agar plates amended with streptomycin as described by Komlos et al. (2005). R2A was used as the non-selective nutrient agar to determine total cell numbers and provide a total cell balance. Viable, TCE-degrading cultures of *B. cepacia* or viable cultures of *K. oxytoca* were prepared as described by Komlos et al. (2005). Briefly, *B. cepacia* was successively grown from a frozen culture on a phenol/kanamycin agar plate incubated at 30°C for 48 h, an LBG/kanamycin agar plate incubated at 30°C for 24 h, and then finally in 100 mL LBG media (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g dextrose per liter of distilled water) for an incubation time of 18 h at 36°C on a horizontal shaker (150 rpm). One milliliter of this culture was transferred to 100 mL fresh LBG media and incubated for 18 h at 36°C on a horizontal shaker (150 rpm). A viable culture of *K. oxytoca* was prepared from a frozen culture by inoculating a BHI agar plate amended with streptomycin (incubated at 30°C for 24 h) followed by incubation (36°C, 150 rpm) in 100 mL LBG media for 18 h. One milliliter of this culture was transferred to 100 mL fresh LBG media and incubated for 18 h at 36°C on a horizontal shaker (150 rpm).

Porous Media Reactor

Each PM experiment used one of two glass chromaflex columns (Kimble Kontes, Vineland, NJ). Both columns were 30 cm long, 5 cm in diameter, and each was filled with 1 mm glass beads (BioSpec Products, Inc., Bartlesville, OK). Each column was identical except one column had three miniert sampling ports (Alltech, Deerfield, IL) along the length of the column (Fig. 1). A 1/4 inch Swaglok tee located just before the influent of the column served as an influent sampling port

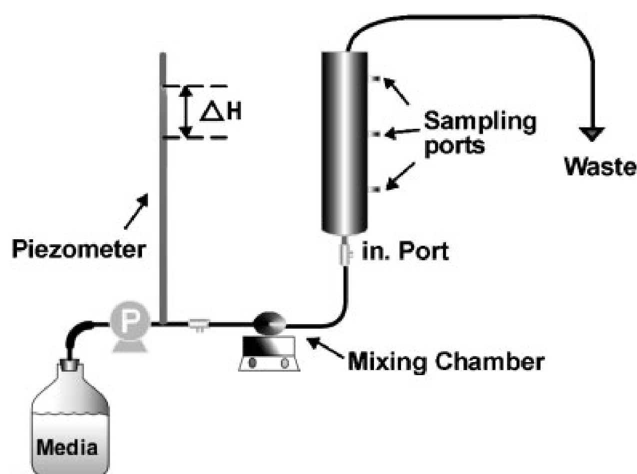


Figure 1. Porous media (PM) column setup.

and bacterial injection point. Oxygen gas was diffused into the feed media for all experiments to simulate a possible bioreactor scenario for aerobic TCE degradation. The column, tubing, and glass beads were disinfected prior to each experiment as described in Komlos et al. (2004). Briefly, the beads and tubing were rinsed with deionized (DI) water and autoclaved and the column was rinsed with 70% ethanol. The system was then completely assembled and disinfected with a 10% bleach solution.

Dual-species column experiments were performed for three different dilutions of LBG media (1:500, 1:100, or 1:10) resulting in target dissolved organic carbon (DOC) concentrations of 30, 70, or 700 mg/L, respectively. The source of carbon in LBG media is in the form of glucose, tryptone, and yeast extract. The 30 mg/L and 700 mg/L DOC experiments were performed in duplicate and the 70 mg/L DOC substrate concentration experiment was performed in triplicate. At the beginning of each experiment, 50 mL of *K. oxytoca* and 50 mL of *B. cepaci*a growth cultures were injected into the column and flow to the column began immediately after inoculation. The number 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. The feed media was pumped upflow through the column at a rate of 2.4 mL/min for 70 h. The hydraulic residence time and linear velocity were measured using fluorescein breakthrough curve analysis to be 100 min and 0.3 cm/min, respectively (data not shown). Effluent samples were periodically taken to monitor dissolved oxygen (DO) concentrations, DOC concentrations, and population densities over time. After 70 h, each dual-species column was taken off-line and destructively sampled as described by Komlos et al. (2004). Briefly, approximately 50 g of beads were removed from the entrance and exit of the column and put in separate test tubes. Fifteen milliliters of phosphate buffered saline (PBS) solution was added incrementally to each tube. 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 at 13,500 rpm using a Tekmar-Dohrmann tissue homogenizer and plated in triplicate on both selective (LBG with kanamycin for *B. cepacia* and BHI with streptomycin for *K. oxytoca*) and non-selective (R2A) agar plates to determine population densities throughout the column.

Specific Biofilm Growth Rate Analysis

Specific biofilm growth rates were calculated based on a method previously described by Van Der Wende et al. (1989) and Komlos et al. (2005). Briefly, specific biofilm growth rates were calculated by combining the mass balance of the suspended cell numbers exiting the reactor (Eq. 1a) with the mass balance of the biofilm cell numbers (Eq. 1b).

$$V\frac{dX_1}{dt} = Q(X_0 - X_1) + \mu_p X_1 V + r_d X_b A - b X_1 V \quad (1a)$$

$$A\frac{dX_b}{dt} = \mu_b X_b A - r_d X_b A - b X_b A \quad (1b)$$

Where:
μ_b, specific biofilm growth rate ($1/t_0$); V, volume of fluid (L^3); X_0, influent cell density (CFU/L^3); X_1, effluent (detached) cell density (CFU/L^3); Q, flow rate (L^3/t); μ_p, planktonic growth rate (1/t); r_d, biofilm detachment rate (1/t); A, biofilm surface area (L^2); X_b, biofilm cell density (CFU/L^2); b, endogenous decay coefficient (0.20/d (Droste, 1998)).

Under steady-state conditions, the cell concentration is assumed to be constant and the microorganisms leaving the column are due to shear of the biofilm. Steady-state conditions, combined with no cells added, allows Equations 1a and 1b to be combined to yield a solution for the biofilm growth rate (Eq. 2).

$$\mu_b = \frac{1}{X_b A} \times [(Q - \mu_p V + bV)X_1 + b X_b A] \quad (2)$$

Since there are relatively few planktonic cells in a PM reactor compared to attached cells, it was initially assumed that the effect of planktonic growth was negligible and that the biofilm growth rate could be described by Equation 3. The applicability of this assumption is addressed in the discussion of this manuscript.

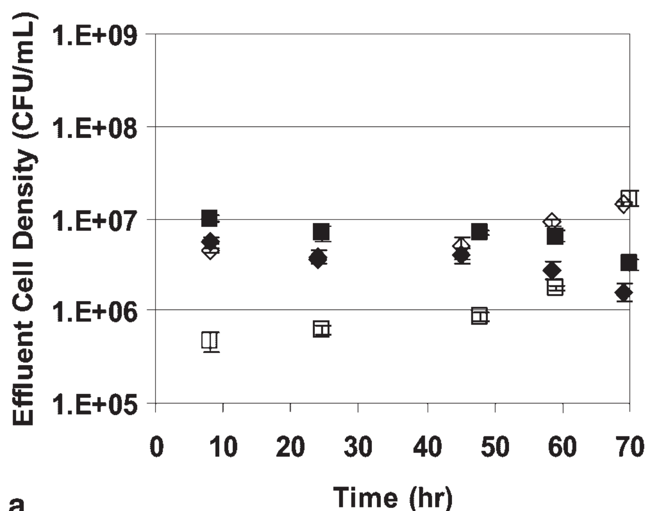
$$\mu_b = \frac{1}{X_b A} \times [(Q + bV)X_1 + b X_b A] \quad (3)$$

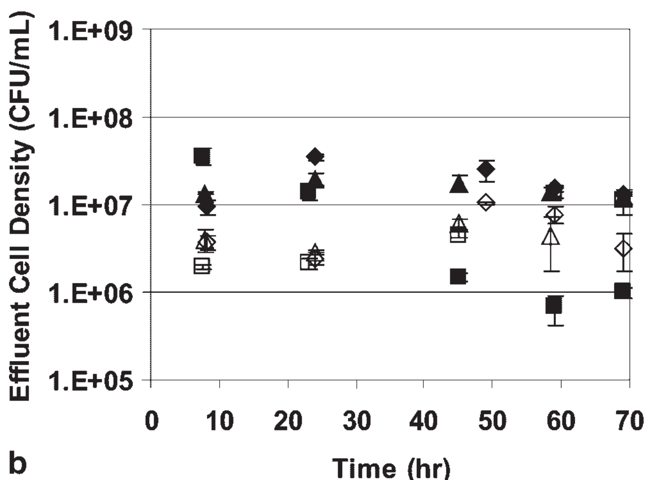
Other Analyses

DOC 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 µm syringe filter (Fisher Scientific, Pittsburgh, PA). Inorganic carbon was removed by adding two drops of 20% phosphoric acid into 2 mL of sample and subsequent air sparging for 6 min. DO was measured using an Accumet AP64 Series handheld DO meter (Fisher Scientific). The DO 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 a two sample (equal variance) *t*-test and data sets were considered significantly different if the *P*-values were lower than 0.05.

RESULTS

A series of continuous-flow PM column experiments were performed to quantify the interaction of *K. oxytoca* and *B. cepacia* over a range of substrate concentrations (approximately 30 mg/L, 70 mg/L, and 700 mg/L DOC). Effluent population densities, DO and DOC were monitored over time and biofilm population densities quantified after 70 h of column operation.





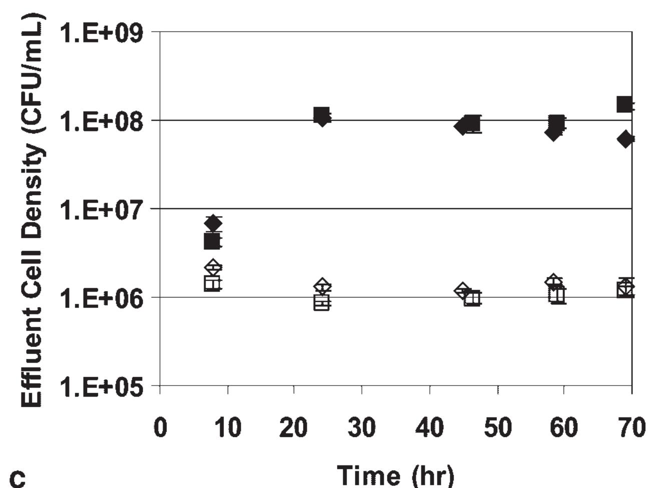


Figure 2. *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) effluent population densities for PM columns supplied the (**a**) 30 mg/L, (**b**) 70 mg/L, or (**c**) 700 mg/L DOC substrate concentrations. Data is average of triplicate plate counts (±SD).

Initially, each organism had comparable effluent population densities for all experiments with *K. oxytoca* higher than *B. cepacia* (Fig. 2a–c). For the 30 mg/L DOC substrate concentration experiments (Fig. 2a), effluent *K. oxytoca* remained relatively constant and *B. cepacia* gradually increased with time over 70 h to a level six times higher, and statistically greater (P-value < 0.035), than that of *K. oxytoca*. For the 70 mg/L DOC substrate concentration experiment, the population density of *K. oxytoca* was greater than *B. cepacia* for the first 25 h of the experiment but both organisms were comparable after 70 h (Fig. 2b). For the 700 mg/L DOC substrate concentration experiment, the effluent population density of *K. oxytoca* was two orders of magnitude greater than *B. cepacia* after 25 h of column operation with both populations remaining constant thereafter (Fig. 2c).

After 70 h of column operation, each column was taken off-line, glass beads removed from the entrance and exit of the column, and the bacteria quantified using selective and non-selective plating techniques (Table I). For the 30 mg/L DOC concentration experiment, the biofilm population density of each organism did not change significantly between the entrance and exit of the column with *B. cepacia* being greater than *K. oxytoca*. For the 70 mg/L DOC concentration experiment, the population density of *B. cepacia* was not significantly different than *K. oxytoca* at either the entrance or exit of the column, though a decrease in the population density of both organisms was measured (P-value < 0.04) over the length of the column. For the 700 mg/L DOC concentration experiment, the population density of *K. oxytoca* was higher than *B. cepacia* at both the entrance and exit (P-value < 0.02). *K. oxytoca* decreased two orders of magnitude from entrance to exit (P-value < 0.02) while *B. cepacia* increased (P-value < 0.02). While there were differences in the distribution of the two organisms within each substrate concentration, trends in the total population were seen. Total culturable bacteria (R2A plate counts) increased with increasing substrate concentration at the entrance of the column but remained constant at the exit of the column (Table I). Though the total culturable cell numbers were comparable between the entrance and exit of the column at the low (30 mg/L DOC) substrate concentration, there were almost two orders of magnitude more cells at the entrance of the column than the exit for the high (700 mg/L DOC) substrate concentration.

An average of the entrance and exit biofilm population density for each organism at each substrate concentration for replicate column experiments is shown in Figure 3. *B. cepacia* ($7.6 \pm 3.7 \times 10^7$ CFU/g bead, $n = 2$) was greater than *K. oxytoca* ($5.6 \pm 0.4 \times 10^6$ CFU/g bead, $n = 2$) for the 30 mg/L DOC concentration experiments. At 70 mg/L DOC, the average biofilm population density of *B. cepacia* ($2.1 \pm 0.9 \times 10^8$ CFU/g bead, $n = 3$) was comparable to *K. oxytoca* ($1.0 \pm 0.3 \times 10^8$ CFU/g bead, $n = 3$). An increase in substrate concentration to 700 mg/L resulted in a change in species dominance with the average biofilm population density of *K. oxytoca* ($1.9 \pm 0.5 \times 10^9$ CFU/g bead, $n = 2$)

Table I. Dual-species porous media (PM) population densities after 70 h of column operation from the entrance and exit of the column.

	K. oxytoca (CFU/g bead) $\times 10^8$		*B. cepacia* (CFU/g bead) $\times 10^8$		Total cells (CFU/g bead) $\times 10^8$	
DOC conc.	Entrance	Exit	Entrance	Exit	Entrance	Exit
30 mg/L	0.060 ± 0.027	0.052 ± 0.019	0.34 ± 0.04	1.2 ± 0.8	0.46 ± 0.07	1.1 ± 0.5
70 mg/L	1.9 ± 0.6	0.051 ± 0.022	3.7 ± 2.1	0.61 ± 0.76	5.8 ± 3.0	0.49 ± 0.44
700 mg/L	39 ± 10	0.32 ± 0.02	0.023 ± 0.007	0.11 ± 0.02	39 ± 6.8	0.48 ± 0.02

Values are an average of duplicate (30 mg/L and 700 mg/L experiments) or triplicate (70 mg/L experiments) column experiments $\pm$ SD.

greater than *B. cepacia* ($6.5 \pm 1.4 \times 10^6$ CFU/g bead, $n = 2$). It is important to note that cell densities from the beginning and end of the column may not be the same as the cell density in the middle of the column. However, analysis of *K. oxytoca* and *B. cepacia* biofilm data after TCE addition to each column (Table II of Komlos et al., 2004) showed no statistical difference between the average using data from the beginning and end of the column compared to the average of the beginning, middle, and end of the column. This supports the use of an average of the cell densities from the beginning and end of the column to be used as the representative cell density throughout the column.

The effluent and biofilm fraction of each organism to the total population (*B. cepacia* + *K. oxytoca*) was calculated using data after 70 h of column operation (Table II). The effluent fraction for each organism was similar to the biofilm fraction at each substrate concentration, thus showing that the same trends of species dominance measured in the biofilm were also measured in the effluent. This also suggests that the cells exiting the column are representative of the suspended biomass throughout the column.

PM growth rates were calculated using Equation 3 and the effluent population densities and biofilm population densities (normalized to a per surface area basis) after 70 h of column operation (Table III). All growth rates ranged from 0.01/h to 0.09/h. *K. oxytoca* biofilm growth rates were greater than *B. cepacia* at the lower (30 mg/L and 70 mg/L) DOC concentration experiments and less than *B. cepacia* for the high (700 mg/L) DOC concentration experiments.

As the substrate concentration increased, the amount of carbon utilized in the column (Table IV) also increased, though a significant amount of the initial DOC (65%–90%) was measured in the effluent. Cell yields from growth at each substrate concentration were calculated (Table IV). Under steady-state conditions, the suspended cell numbers are a function of those produced due to both planktonic and biofilm growth. Table II indicates that the effluent population is representative of the suspended population throughout the column, and hence the net number of cells produced over time. Therefore, the total culturable (R2A) cells exiting the column can be compared to the DOC consumed between the influent and effluent of the column to estimate the cell yield from growth on different dilutions of LBG media. An increase in DOC concentration from 30 mg/L to 700 mg/L resulted in a decrease in cell yield from 0.55 to 0.33 g cell/g DOC.

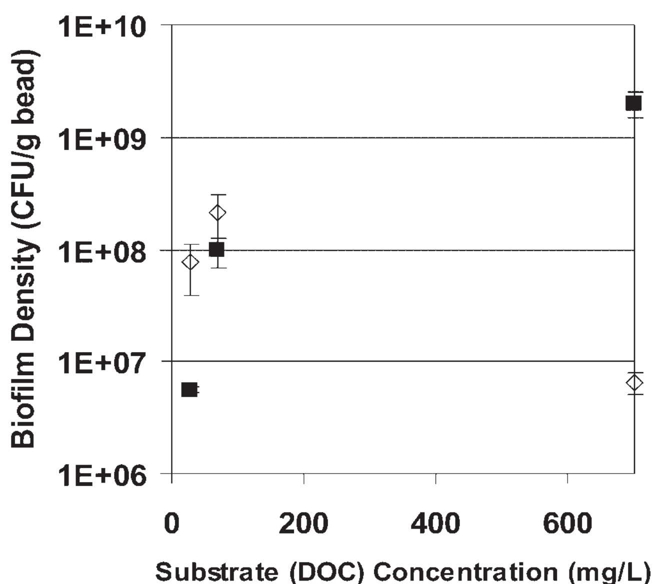


Figure 3. *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) PM biofilm population densities averaged from the beginning and end of the column after 70 h for the three substrate concentrations. Values are an average of separate experiments ($\pm$SD).

Table II. Effluent biomass fraction and biofilm biomass fraction for *K. oxytoca* and *B. cepacia* in the PM reactor after 70 h of column operation.

Substrate concentration	Effluent fraction		Biofilm fraction	
	K. oxytoca	*B. cepacia*	*K. oxytoca*	*B. cepacia*
30 mg/L DOC	0.13 ± 0.04	0.87 ± 0.04	0.08 ± 0.03	0.92 ± 0.03
70 mg/L DOC	0.45 ± 0.36	0.55 ± 0.36	0.33 ± 0.13	0.67 ± 0.13
700 mg/L DOC	0.99 ± 0.01	0.01 ± 0.01	1.00 ± 0.00	0.00 ± 0.00

Fractions are an average ($\pm$SD) of data from duplicate (30 mg/L and 700 mg/L experiments) or triplicate (70 mg/L experiments) column experiments.

Table III. Specific biofilm growth rates (1/h) of *K. oxytoca* and *B. cepacia* when grown in a dual-species PM system supplied three different substrate concentrations.

Substrate concentration	*K. oxytoca*	*B. cepacia*
30 mg/L DOC	0.086 ± 0.032 ($n = 2$)	0.049 ± 0.016 ($n = 2$)
70 mg/L DOC	0.025 ± 0.016 ($n = 3$)	0.017 ± 0.006 ($n = 3$)
700 mg/L DOC	0.017 ± 0.003 ($n = 2$)	0.044 ± 0.005 ($n = 2$)

Values are an average of replicate column experiments $\pm$ SD. (*n* indicates number replicate experiments).

Table IV. Dissolved organic carbon (DOC) utilized, total (R2A) effluent cell density, and resulting cell yields for the PM (PM) reactor experiments.

Target DOC conc.	DOC utilized between influent and effluent of column (mg/L)	(%)	Effluent cell density (CFU/mL) $\times 10^7$	Cell yield[a] (g cell/g DOC)
30 mg/L	9.1 ($n = 1$)	32.8 ($n = 1$)	1.8 ($n = 1$)	0.55 ($n = 1$)
70 mg/L	19.3 ± 4.3 ($n = 2$)	25.7 ± 3.3 ($n = 2$)	2.4 ± 0.3 ($n = 2$)	0.36 ± 0.12 ($n = 2$)
700 mg/L	77.7 ± 28.7 ($n = 2$)	12.8 ± 3.6 ($n = 2$)	9.6 ± 5.4($n = 2$)	0.33 ± 0.07 ($n = 2$)

Data is from the final sample before destructive sampling at $t = 70$ h. Values are the average from separate experiments ($\pm$SD).
[a]One cell $= 2.8 \times 10^{-13}$ g (for *E. coli*) (Madigan et al., 1997).

DO was measured at the effluent of the column for all three substrate concentrations (Fig. 4). A constant supply of oxygen to the feed media enabled supersaturated effluent DO concentrations at the beginning of the experiment (13.8 ± 1.0 mg/L, $n = 5$). Non-limiting DO concentrations were recorded only from the column fed the lowest substrate concentration (30 mg/L DOC). The effluent DO concentration at the 30 mg/L DOC concentration experiments appears to decrease with time which, along with the slight increase in effluent cell numbers with time (Fig. 2), indicated that steady-state conditions were not reached for the lowest substrate concentration.

DISCUSSION

The interaction of *K. oxytoca* and *B. cepacia* in a PM flow-through column was examined for three different substrate concentrations (approximately 30, 70, and 700 mg/L DOC) by comparing effluent populations over 70 h (Fig. 2) and biofilm population densities (Table I) obtained after 70 h. These results show that after 70 h, *K. oxytoca* was the dominant organism at the high (700 mg/L DOC) substrate concentration and *B. cepacia* dominated at the low (30 mg/L DOC) substrate concentration. The 70 mg/L DOC substrate concentration showed no significant difference in the populations of the two organisms. One of the goals of this research was to determine if trends in population distribution/

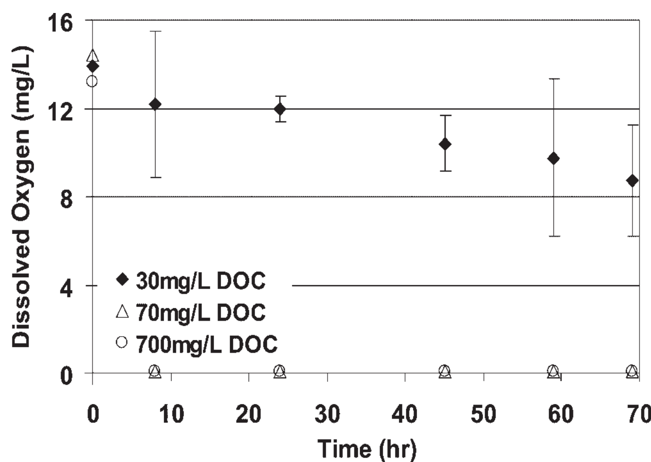


Figure 4. Effluent dissolved oxygen (DO) concentration over time for the 30 mg/L, 70 mg/L, and 700 mg/L DOC column experiments. Data is average of duplicate experiments and error bars are $\pm$ SD.

dominance observed in planktonic batch cultures and in biofilms grown on the surface of well-mixed reactors would compare to more field-relevant (PM) bioremediation scenarios. Previous research has been done using *K. oxytoca* and *B. cepacia* in batch and rotating disk (well-mixed) reactors under the same substrate conditions (Komlos et al., 2005). Results from the batch experiments showed that for the two substrate concentrations tested (70 mg/L and 700 mg/L DOC), the population density of *K. oxytoca* was two to three orders of magnitude higher than *B. cepacia*, which compares well with the PM results at the 700 mg/L substrate concentration but contradicts those at 70 mg/L. However, the results from the dual-species biofilm grown in a well-mixed (rotating disk) reactor are in agreement with the population dominance trends that are observed in the PM reactor; *B. cepacia* was greater than *K. oxytoca* at the low (30 mg/L DOC) substrate concentration and *K. oxytoca* greater than *B. capacia* at the high (700 mg/L DOC) substrate concentration (the two organisms were comparable at the 70 mg/L for both biofilm reactors). Varying substrate concentration provided a mechanism to control the population density and fraction of total population of *K. oxytoca* and *B. cepacia* in both biofilm systems.

The effluent and biofilm data for the PM reactor and RDR are presented in Table V with the average PM biofilm population densities of each microorganism normalized to a per surface area basis for comparison with the biofilm data from the RDR. In all situations, the PM effluent population density was either similar to or greater than (but within an order of magnitude of) the RDR effluent population density. Though more planktonic growth would be expected in a well-mixed reactor compared to a plug flow reactor, the similarities in the effluent population densities may be attributed to additional factors including (i) a detention time in the PM reactor (100 min) that was three times longer than the detention time of the RDR (33 min), (ii) a larger pore volume in the PM reactor (236 mL) compared to the RDR (180 mL), and (iii) a greater surface area for biofilm growth (and detachment) in the PM reactor (510 cm^3) than the RDR (253 cm^3). Comparison of the biofilm populations between the RDR and PM reactors showed no significant difference in the *K. oxytoca* biofilm population density at each substrate concentration. Biofilm *B. cepacia* was greater (P-value < 0.03) in the RDR than the PM reactor for the two lower substrate concentrations. The change from a completely mixed (RDR) reactor to a plug flow PM reactor appeared

Table V. Comparison of dual-species effluent and biofilm biomass densities from the rotating disk reactor (RDR) experiments (Komlos et al., 2005) and PM column experiments (this study).

	K. oxytoca		*B. cepacia*	
DOC conc.	RDR	PM	RDR	PM
	Effluent population densities (CFU/mL) $\times 10^7$			
30 mg/L	0.06 ± 0.04 (9)	0.24 ± 0.11 (2)	0.53 ± 0.22 (9)	1.5 ± 0.2 (2)
70 mg/L	0.44 ± 0.24 (8)	0.85 ± 0.66 (3)	2.3 ± 1.7 (8)	0.89 ± 0.50 (3)
700 mg/L	4.5 ± 2.1 (12)	10 ± 5.8 (2)	0.18 ± 0.16 (12)	0.12 ± 0.01 (2)
	Biofilm population densities (CFU/cm^2) $\times 10^7$			
30 mg/L	0.57 ± 1.03 (5)	0.03 ± 0.002 (2)	7.7 ± 3.8 (5)	0.39 ± 0.19 (2)
70 mg/L	0.78 ± 0.45 (4)	0.51 ± 0.16 (3)	7.6 ± 4.2 (4)	1.1 ± 0.5 (3)
700 mg/L	15 ± 3.0 (6)	10 ± 2.7 (2)	1.4 ± 1.1 (6)	0.03 ± 0.01 (2)

RDR data is the average ($\pm$SD) of steady-state sample points. PM data is from the time of destructive sampling ($t = 70$ h) and is the average ($\pm$SD) of replicate experiments.
() denotes sample number

to disadvantage the aerobic *B. cepacia* even at the lowest substrate concentration (30 mg/L DOC) where significant DO was measured at the effluent (Fig. 4). However, even with this disadvantage, *B. cepacia* still had a greater population density than *K. oxytoca* at the low substrate concentration.

The effect of substrate concentration on total culturable effluent and biofilm cell numbers was examined for the RDR and PM reactors. The effluent concentrations for each microorganism were multiplied by the pore volume of each reactor and summed together to obtain the total culturable effluent (suspended) population for each reactor and substrate concentration (Table VI). The same was done for the biofilm populations, except they were multiplied by the surface area of the reactor. An increase in substrate concentration from 30 mg/L DOC to 700 mg/L DOC increased the total effluent population by less than an order of magnitude for both reactors. Under these same conditions in the RDR, the total biofilm population increased by only a factor of two, while in the PR system the difference was 24 fold higher at the 700 mg/L concentration. Though the biofilm concentration (CFU/cm^2) for the PM column experiments was either less than or comparable to the biofilm concentration in the well-mixed (RDR) reactors (Table V), the PM column had a higher total biofilm population (CFU) for all three substrate concentrations than the RDR (Table VI) due, in part, to the increased surface area and longer detention time available for colonization in the PM environment. In addition, lower shear stresses in a PM reactor compared to an RDR will influence biofilm thickness and may have also contributed to the higher biofilm populations in the PM reactor. The majority of the cells for both reactors were attached to the surface with the biofilm comprising 80%–95% of the total amount in the RDR and 94%–99% in the PM columns (Table VI).

The PM growth rates calculated using Equation 3 were summarized in Figure 5 along with previously published dual-species growth rates for *K. oxytoca* and *B. cepacia* for batch and RDR experiments (Komlos et al., 2005). All PM growth rates were between 0.01/h and 0.09/h, which are lower than those measured from the batch and RDR experiments (Fig. 5). Recall that planktonic growth was initially neglected in calculating the biofilm growth rates in the PM columns because (1) the suspended population is only a small percentage (6% or less) of the total biomass in the column (Table VI) and (2) the lack of information as to the correct planktonic growth rate to use under these conditions. At the beginning of the PM column, the planktonic organisms may grow at a rate similar to that measured in a batch experiment where nutrients are in excess. However, as the plug flow of nutrients and oxygen are utilized over the length of the column, the growth rate of the planktonic organisms is likely to decrease further into the column. Though batch growth rates are available for these two organisms in dual species, it is unknown if these rates can be applied to the

Table VI. Total effluent and biofilm biomass densities (and fraction of total biomass) from the RDR experiments (Komlos et al., 2005) and PM column experiments (this study).

	Effluent biomass (CFU) $\times 10^{10}$		Biofilm biomass (CFU) $\times 10^{10}$		% total biomass (effluent/biofilm)	
DOC conc.	RDR	PM	RDR	PM	RDR	PM
30 mg/L	0.11 ± 0.04	0.40 ± 0.03	2.1 ± 0.9	6.3 ± 2.1	4.8%/95.2%	6.0%/94.0%
70 mg/L	0.50 ± 0.30	0.55 ± 0.07	2.1 ± 1.1	25 ± 11	19.0%/81.0%	2.1%/97.9%
700 mg/L	0.85 ± 0.38	2.3 ± 1.3	4.0 ± 0.8	157 ± 27	17.4%/82.6%	1.4%/98.6%

PM data is non-selective (R2A) plate counts from the time of destructive sampling ($t = 70$ h) and is the average of duplicate (30 mg/L and 700 mg/L experiments) or triplicate (70 mg/L experiments) column experiments $\pm$ SD. RDR data is the average ($\pm$SD) of the sum of *B. cepacia* and *K. oxytoca* population densities for each sample point (sample number for each substrate concentration correspond to RDR data in Table IV).

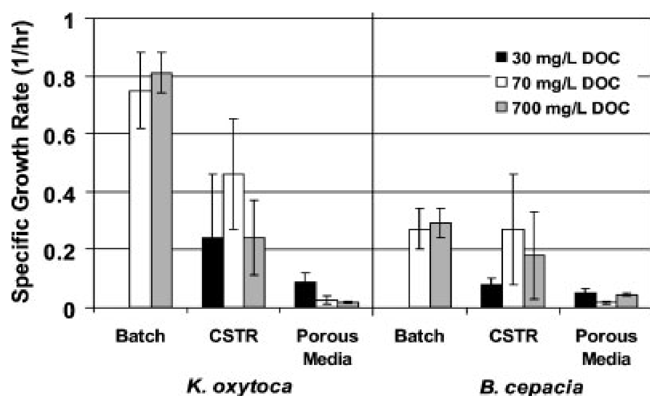


Figure 5. Specific growth rates (1/h) of *K. oxytoca* and *B. cepacia* grown together in dual-species batch, completely mixed stirred tank reactor (CSTR), and PM systems. Batch and CSTR growth rates are from Komlos et al. (2005). PM growth rates are from Table II.

suspended cultures of a PM reactor. If suspended growth in the PM was not ignored and Equation 2 was used to calculate the specific biofilm growth rate in PM, the resulting biofilm growth rates would be even smaller than that calculated using Equation 3 due to the negative effect of the suspended growth term on the biofilm equation. One possible explanation for the lower biofilm growth rates in the PM reactors is oxygen and/or substrate limitation along the length of the column. Significant DOC was measured in the effluent of all PM column experiments (Table IV), indicating that carbon was not a limiting nutrient. The lack of oxygen in the effluent of columns supplied the two higher substrate concentrations strongly suggests that the lower growth rates in the PM reactor compared to the RDR was related to oxygen limitation. Though oxygen was not limiting in the pore water of columns supplied the lowest substrate concentration (30 mg/L), the decrease in growth rate in PM compared to a RDR may have been attributed to diffusion limitation of oxygen into the biofilm. Another possibility for lower measured growth rates in PM compared to a RDR is that a fraction of the biofilm cells were present but inactive in the column. This would explain the detection of the aerobic *B. cepacia* in biofilms at the end of the PM columns where DO was depleted. It should be noted that the growth rate equation is dependent on the assumption that the system is at steady state. For the 70 mg/L DOC and 700 mg/L DOC columns experiments, the lack of change in the effluent population data over time after 45 h (70 mg/L DOC experiments) and after 25 h (700 mg/L DOC experiments) supports this assumption (Fig. 2a and b). However, the effluent population densities of *B. cepacia* appear to increase slightly toward the end of the 30 mg/L DOC column experiments suggesting that the system may have not reached steady-state conditions.

DO limitation caused by increasing the substrate concentration appeared to play an important role in the population distribution of the aerobic organism (*B. cepacia*) and the facultative organism (*K. oxytoca*) in a dual-species PM biofilm. There was 13.5 ± 0.7 mg/L of DO in the effluent of the column prior to the inoculation of bacteria, but measurable DO at the effluent (8.7 mg O_2/L) was recorded only at the lowest (30 mg/L DOC) substrate concentration. The depletion of oxygen correlated with a shift in population dominance from *B. cepacia* at the lower substrate concentration to *K. oxytoca* at the high substrate concentration. The DO data correlated with an aerobic TCE degradation experiment performed afterward (Komlos et al., 2004). Aerobic TCE degradation by *B. cepacia* occurred throughout the column only for the lowest substrate concentration. For the 70 mg/L DOC column experiment, TCE degradation was measured within the first 6 cm (but not in the remaining 24 cm) of the column. TCE degradation was not detected in the columns supplied the highest substrate concentration (700 mg/L DOC). Though there was only a finite amount of oxygen available, the amount of DOC utilized over the length of the PM column increased with increasing substrate concentration (Table IV), suggesting facultative respiration by *K. oxytoca* once oxygen was depleted along the length of the column and/or a result of oxygen diffusion limitations into the biofilm where oxygen was present in the pore water. Though limiting oxygen concentrations appeared to have caused the shift in the population dynamics with the aerobic organism (*B. cepacia*) dominating the dual-species population at the low substrate concentration and the facultative organism (*K. oxytoca*) dominating the dual-species population at the high substrate concentration, the aerobic organism was still present in significant numbers throughout the column fed even the highest substrate concentration (Table I).

In conclusion, substrate concentration played an important role in the dual-species biofilm population distribution of *B. cepacia* and *K. oxytoca* in a PM biofilm. *B. cepacia* dominated the dual-species biofilm at low (30 mg/L) DOC concentrations when DO concentrations were not limiting. Increasing the substrate concentration resulted in DO limitation and resulted in *K. oxytoca* dominating the PM reactor. This trend in species dominance with change in substrate concentration correlated with the population distribution of these two organisms in a biofilm on the surface of a well-mixed reactor but did not correlate with the dual-species distribution in batch cultures. Therefore, for the conditions set forth herein, the dual-species distribution observed in a well-mixed reactor could be used to predict which organism would dominate the dual-species culture in a more field-relevant (PM) system. As previously observed for a dual-species biofilm grown in a well-mixed reactor (Komlos et al., 2005), the specific biofilm growth rates of each organism did not correlate with which organism was the dominant species in the dual-species PM culture. Though the dual-species population trends observed in both biofilm systems were similar, the specific biofilm growth rates for both organisms were measurably lower in the PM reactor compared to the well-mixed reactor, which suggests that biofilms from different reactor geometries behave differently and the use of dual-species biofilm growth rates measured from a well-mixed reactor would not accurately predict dual-species bacterial biofilm growth in a PM system. Finally, manipulation of the substrate concentration played an

important role in not only controlling the TCE-degrading bacterial population in a biofilm but also in enhancing TCE degradation in the dual-species PM reactor (Komlos et al., 2004), which may be of value for the implementation of TCE or other contaminant degrading bioremediation scenarios.

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