Microbial Ecology

Interaction of *Klebsiella oxytoca* and *Burkholderia cepacia* in Dual-Species Batch Cultures and Biofilms as a Function of Growth Rate and Substrate Concentration

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Abstract

Dual-species microbial interactions have been extensively reported for batch and continuous culture environments. However, little research has been performed on dual-species interaction in a biofilm. This research examined the effects of growth rate and substrate concentration on dual-species population densities in batch and biofilm reactors. In addition, the feasibility of using batch reactor kinetics to describe dual-species biofilm interactions was explored. The scope of the research was directed toward creating a dual-species biofilm for the biodegradation of trichloroethylene, but the findings are a significant contribution to the study of dual-species interactions in general. The two bacterial species used were *Burkholderia cepacia* PR1-pTOM$_{31c}$, an aerobic organism capable of constitutively mineralizing trichloroethylene (TCE), and *Klebsiella oxytoca*, a highly mucoid, facultative anaerobic organism. The substrate concentrations used were different dilutions of a nutrient-rich medium resulting in dissolved organic carbon (DOC) concentrations on the order of 30, 70, and 700 mg/L. Presented herein are single- and dual-species population densities and growth rates for these two organisms grown in batch and continuous-flow biofilm reactors. In batch reactors, planktonic growth rates predicted dual-species planktonic species dominance, with the faster-growing organism (*K. oxytoca*) outcompeting the slower-growing organism (*B. cepacia*). In a dual-species biofilm, however, dual-species planktonic growth rates did not predict which organism would have the higher dual-species biofilm population density. The relative fraction of each organism in a dual-species biofilm did correlate with substrate concentration, with *B. cepacia* having a greater proportional density in the dual-species culture with *K. oxytoca* at low (30 and 70 mg/L DOC) substrate concentrations and *K. oxytoca* having a greater dual-species population density at a high (700 mg/L DOC) substrate concentration. Results from this research demonstrate the effectiveness of using substrate concentration to control population density in this dual-species biofilm.

Introduction

Biofilm models often use planktonic cell kinetics to predict biofilm growth rates [1, 12, 20, 23, 25]. However, measurable differences in growth rate [7] and activity [13, 15, 26] between suspended and attached bacteria have been reported. In addition, these studies of growth kinetics and activity of suspended and attached cells used either monocultures [1, 13, 15, 20, 26] or undefined mixed cultures [7, 23, 25]. The research presented herein examined whether planktonic or biofilm specific growth rates determine species dominance in a dual-species biofilm. In addition, the effect of substrate concentration on the fraction of each organism in a dual-species biofilm was explored.

The scope of the dual-species interactions was to improve trichloroethylene (TCE) biodegradation using *Burkholderia cepacia* PR1-pTOM$_{31C}$ in a biofilm, but the findings may be applied to other industrial, environmental, and medical systems where multiple organisms are present. *B. cepacia* is an aerobic bacterium that can constitutively degrade trichloroethylene (TCE) via a co-metabolic process using the toluene ortho-monooxygenase (TOM) pathway [28]. Possible uses of *B. cepacia*, or other contaminant-degrading organisms, include bioreactor or bioaugmentation technologies. During a

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long-term bioreactor operation, the interaction of *B. cepacia* with another organism is highly probable because of contamination, and during a bioaugmentation scheme where *B. cepacia* is introduced into the subsurface, the interaction of *B. cepacia* with indigenous organisms is unavoidable. Therefore, it is important to examine different aspects of these interactions to maximize the TCE-degrading population and subsequent TCE-degrading capabilities. This research set out to explore one possible interaction: that of the aerobic TCE-degrading *B. cepacia* with the facultative, thick-biofilm-forming *Klebsiella oxytoca*. *K. oxytoca* was chosen because of its relatively high growth rate and ability to survive in both aerobic and anaerobic environments: conditions that should be expected in many bioremediation scenarios. In addition, *K. oxytoca* was chosen for its resistance to the antibiotic streptomycin which, combined with the resistance of *B. cepacia* to the antibiotic kanamycin [27], enabled the use of selective plating techniques to differentiate each population in dual-species cultures.

The mechanisms that control microbial interactions in multispecies biofilms are not fully understood. Research has demonstrated that many different dual-species scenarios can occur. An established biofilm can have a beneficial [2, 8], detrimental [4, 31], or neutral [19] effect on the growth and maintenance of an inoculated organism. Likewise, an inoculated organism can have a positive [10] or negative [21] effect on the growth and survival of organisms already present in a biofilm. Therefore it is essential to explore which factors may influence the interaction of the organisms in question in order to understand and manipulate the population densities. Banks and Bryers [3] and Sturman et al. [31] reported that a microorganism's growth rate plays an important role in multispecies population dynamics in a biofilm, with the faster-growing microorganism having a competitive advantage over the slower-growing microorganism. However, Siebel and Characklis [29] and Stewart et al. [30] observed that the organism with the higher growth rate was not necessarily present in the highest population density, suggesting that factors other than growth rate may influence spatial distribution and relative cell numbers in biofilms. In addition to growth rate, research has shown that substrate concentration plays an important role in biofilm growth rate [24], population size [5], and population distribution [6, 9, 14, 18] of single-and dual-species cultures. Houtmeyers et al. [14] and Marsh et al. [18] observed that certain organisms were isolated at higher frequencies at high substrate concentrations, while other organisms were isolated at higher frequencies at lower substrate concentrations. Camper et al. [6] observed that slower-growing organisms survived in higher numbers at lower substrate concentrations. This suggests a possible link between substrate concentration and an organism's growth rate in determining species dominance in a multispecies culture.

To determine which factors influence the interaction of *B. cepacia* and *K. oxytoca* in a dual-species biofilm, a series of batch and continuous-flow biofilm reactor experiments were performed. Earlier results concluded that these two organisms can coexist in a biofilm and that the order of inoculation did not significantly affect the relative fraction of each organism in a porous media reactor [16]. The research presented herein (1) examined whether planktonic or biofilm growth rates can describe the dual-species interaction of these two organisms in batch and biofilm reactors and (2) explored the effect of substrate concentration on species dominance in a biofilm.

Methods

Sources of Strains. The aerobic TCE-degrading bacterium *Burkholderia cepacia* PR1-pTOM$_{31c}$ was supplied by Malcolm Shields, University of West Florida. The thick-biofilm-forming 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* [17]. This environmental isolate was later reclassified as *Klebsiella oxytoca* [11].

Bacterial Isolation and Characterization. Selective and nonselective nutrient agar plates were used to characterize the dual-species populations. *B. cepacia* was selected on either modified Luria-Bertani (LBG) agar plates or phenol agar plates. Modified Luria-Bertani agar plates contained 10 g tryptone (Becton Dickinson, Sparks, MD), 5 g yeast extract (Becton Dickinson), 5 g NaCl (Fisher Scientific, Pittsburgh, PA), 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., St. Louis, MO) added after autoclaving and incubation for 45 min in a 55°C water bath. Phenol agar plates contained 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 and incubation for 45 min in a 55°C water bath. 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 $\sim$ pH 7.2, 0.01125 g $CaCl_2$, and 0.0967 g $MgCl_2$ per liter of nanopure water. All chemicals for HCMM2 media 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 Bactoagar per liter of distilled water] amended with 0.1 g/L filter sterilized streptomycin sulfate (Fisher Scientific) added 45 min 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. A viable, TCE-degrading culture of *B. cepacia* was prepared by inoculating a phenol/kanamycin agar plate with *B. cepacia* from a frozen culture [−70°C in 2% peptone (Becton Dickinson), 20% glycerin (Fisher Scientific)] using a sterilized inoculation loop. The phenol/kanamycin agar plate was incubated at 30°C for 48 h. A colony from the phenol/kanamycin plate was transferred to an LBG/kanamycin agar plate 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 mL 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). To prepare a viable culture of *K. oxytoca*, a loopfull of *K. oxytoca* was transferred from a frozen culture (−70°C in 2% peptone, 20% glycerin) to a BHI agar plate amended with streptomycin and incubated at 30°C for 24 h. 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).

Planktonic (Batch) Experiments. The nutrient media used for batch and biofilm reactor experiments were different dilutions (either 1:100 or 1:10) of LBG broth resulting in dissolved organic carbon concentrations of 70 mg/L and 700 mg/L, respectively. For the single-species experiments, 50 μL of growth culture containing either *B. cepacia* or *K. oxytoca* was transferred to 500 mL of nutrient media in a 1-L Erlenmeyer flask. For the dual-species experiments, 50 μL of *B. cepacia* growth culture and 50 μL of *K. oxytoca* growth culture were transferred simultaneously to the same flask. The flasks were sealed with aluminum foil and autoclaved prior to inoculation. After inoculation, the flasks were incubated at 20–25°C and provided constant aeration via a horizontal shaker (150 rpm). Samples were taken over time and plated in triplicate on agar plates: (a) LBG with kanamycin (*B. cepacia*), (b) BHI with streptomycin (*K. oxytoca*), and (c) R2A (total). The plate count data was used to obtain each organism's population density over time and subsequent growth rate for the two substrate concentrations in single- and dual-species cultures.

Biofilm Experiments. A rotating-disk reactor (RDR) system was used to determine the biofilm growth rates and steady-state biofilm population densities of the two organisms in single and dual species cultures for varying substrate concentrations. A rotating-disk reactor

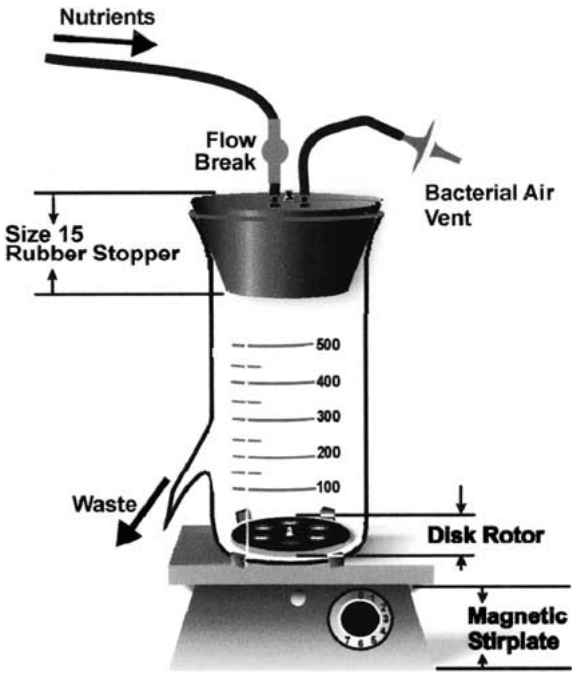

Figure 1. Rotating-disk reactor.

is a one liter glass beaker containing a magnetically driven rotor with six 1.27-cm-diameter biofilm test-surface coupons (Fig. 1). A drain spout on the side of the RDR provided a constant volume of 180 mL and a wetted surface area (including rotating disk) of 253 cm^2. A complete description of an RDR can be found in Zelver et al. [34]. Continuous nutrient addition was supplied to the reactors using a peristaltic pump at an average rate of 5.5 mL/min resulting in a detention time of 33 min. To minimize suspended cell growth in the completely mixed reactors, the hydraulic detention time (0.55 h) was designed to be less than the cell doubling time (0.86 h) of the faster-growing organism (*K. oxytoca*) calculated using the planktonic growth rate data obtained from the batch experiments. Flow rates were established using a graduated cylinder and stopwatch. Flow breaks were situated upstream of the reactor to prevent bacterial contamination of the nutrient reservoir.

Two mL of concentrated growth culture ($1.6 \times 10^{10} \pm 6.4 \times 10^9$ CFU/mL and $9.5 \times 10^9 \pm 2.4 \times 10^8$ CFU/mL for *K. oxytoca* and *B. cepacia*, respectively) were added through syringe injection to each rotating-disk reactor containing 180 mL of medium and left in batch mode for 1 h to allow the microorganisms to attach initially to the surface. After 1 h, nutrient medium was pumped through the reactor at the desired flow rate. The system ran for 3 h to wash out the high concentration of inoculated cells in suspension before sampling began (therefore, $t = 0$ was 4 h after inoculation). Effluent samples were taken periodically and grown on selective agar plates (plated in triplicate). In an ideal completely mixed stirred tank reactor (CSTR), the effluent bacterial population density is

equal to the completely mixed suspended population. For each substrate concentration and inoculation scenario (single or dual species), rotating-disk reactor experiments were performed for various lengths of time (18 h, 48 h, and 5 days). At the end of the predetermined time period, each reactor was taken offline and destructively sampled to remove the bacteria from the disks using a procedure described by Zelver et al. [34]. Triplicate measurements of the biomass density were obtained during the destructive sampling procedure by physical scraping of the biomass from the disks into three separate test tubes containing PBS solution (two disks were scraped into each tube). The cells were homogenized at 13,500 rpm for 30 s to disrupt biomass clumps and then grown on selective agar plates (plated in triplicate). The single-species experiments were performed at diluted LBG media concentrations resulting in influent DOC substrate concentrations of 70 and 700 mg/L. The dual-species experiments were performed at influent DOC substrate concentration of 30, 70, and 700 mg/L.

Growth Rate Analysis. Planktonic growth rates were calculated using linear regression analysis of the log values of the plate count data from the batch experiments as a function of time when the rate of growth remained constant: i.e., exponential growth phase. Specific biofilm growth rates were calculated using a method previously described by Van Der Wende et al. [32]. Briefly, the specific biofilm growth rate was calculated by combining the mass balance of the planktonic biomass originating from detached biofilm in the suspended region of the rotating-disk reactor with the mass balance of the biofilm biomass on the total surface area of the rotating-disk reactor and solving for steady-state, yielding Equation (1). It was assumed that the biomass density on the 1.27-cm polycarbonate disks was similar to the biomass density on the wetted glass and rotor surface of the reactor, thus enabling an estimate of the total biomass to be obtained.

$$\mu_b = \frac{1}{A} \cdot \frac{X_1}{X_b}(Q - \mu_p V) \qquad (1)$$

where

μ_b = specific biofilm growth rate (1/t)
V = volume of fluid (L^3)
X_1 = effluent (detached) cell density (CFU/L^3)
Q = flow rate (L^3/t)
μ_p = planktonic growth rate (1/t)
A = biofilm surface area (L^2)
X_b = biofilm cell density (CFU/L^2)

Other Analyses. 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-μm 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 six minutes. Dissolved oxygen (D.O.) was measured using an Accumet AP64 Series handheld dissolved oxygen meter (Fisher Scientific). 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

Planktonic (Batch) Experiments. Planktonic (batch) growth curves were performed for *K. oxytoca* and *B. cepacia* grown as single species or combined together in a dual culture (Fig. 2). Each batch reactor contained a diluted LBG media resulting in an initial dissolved organic carbon (DOC) concentration of either 70 mg/L (Fig. 2A, B, C) or 700 mg/L (Fig. 2D, E, F). For all experiments, the initial population density of *K. oxytoca* and *B. cepacia* was $4.1 \pm 2.7 \times 10^5$ CFU/mL ($n = 5$) and $2.1 \pm 1.2 \times 10^5$ CFU/mL ($n = 5$), respectively. All growth curves show the organism(s) entering stationary phase of growth except for *B. cepacia* in single species supplied an initial substrate concentration of 700 mg/L DOC (Fig. 2E). A separate single-species 700 mg/L DOC batch experiment determined that the population density of *B. cepacia* after 48 h of growth was 6.8×10^8 CFU/mL. The maximum population density of *B. cepacia* measured in the 70 mg/L DOC substrate concentration batch experiments was 6.7×10^7 CFU/mL (Fig. 2B), and the maximum population density of *K. oxytoca* measured in the 70 mg/L and 700 mg/L DOC substrate concentration batch experiments was 1.2×10^8 CFU/mL (Fig. 2A) and 1.1×10^9 CFU/mL (Fig. 2D), respectively. In single species, both organisms reached similar maximum population densities for each substrate concentration (Fig. 2A, B, D, E), though more time was needed for *B. cepacia* to reach its maximum population density due to a longer lag period and slower rate of growth (Fig. 2B, E). An order-of-magnitude increase in substrate concentration resulted in an order-of-magnitude increase in maximum population density for each organism in single species.

In a dual-species culture, both organisms entered stationary phase at approximately the same time (15 h) for both substrate concentrations (Fig. 2C, F). The maximum population densities of *K. oxytoca* in the dual-species culture for the 70 and 700 mg/L DOC experiments were 1.5×10^8 CFU/mL and 1.2×10^9 CFU/mL, respectively, which correlated with the maximum population density of *K. oxytoca* in single-species cultures at the same substrate concentrations. The maximum population densities of *B. cepacia* in a dual-species culture

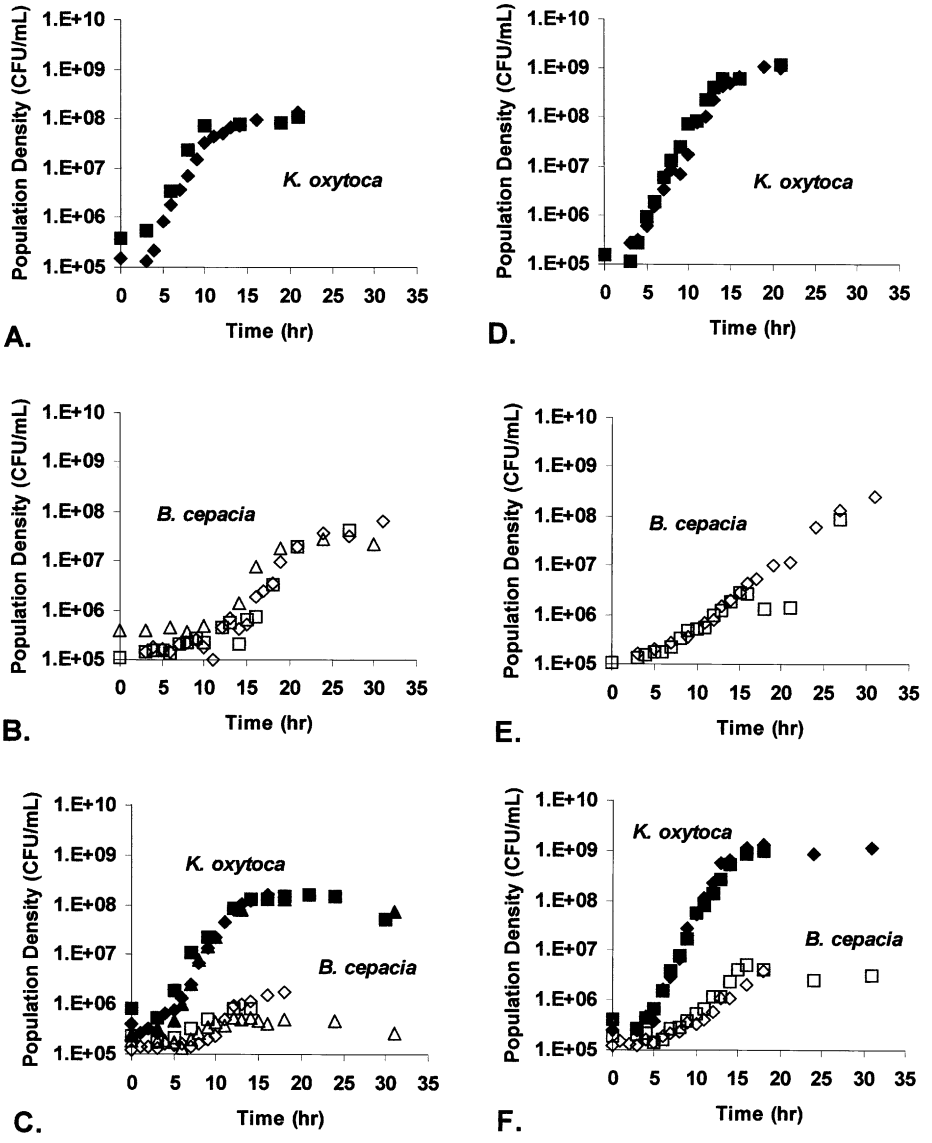

Figure 2. Single and dual-species batch growth curves. (A, B) 70 mg/L dissolved organic carbon single-species batch growth curve for *K. oxytoca* and *B. cepacia*, respectively. (D, E) 700 mg/L dissolved organic carbon single-species batch growth curve for *K. oxytoca* and *B. cepacia*, respectively. (C, F) Dual-species *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) batch growth curves for the 70 mg/L and 700 mg/L dissolved organic carbon batch experiments, respectively. Data from duplicate or triplicate experiments are shown (diamonds: exp. 1; squares: exp. 2; triangles: exp. 3).

measured for the 70 mg/L and 700 mg/L DOC substrate concentration experiments were 1.0×10^6 CFU/mL and 4.4×10^6 CFU/mL, respectively. The dual-species *B. cepacia* maximum population densities were lower than recorded in single-species experiments. The planktonic maximum population densities of *K. oxytoca* were 100 to 300 times greater than those of *B. cepacia* in dual-species cultures, though both organisms had comparable maximum population densities in single-species cultures (Fig. 2).

Planktonic specific growth rates (Table 1) were obtained for each organism using the growth curves presented in Fig. 2. For the single-species batch experiments, the growth rate of *K. oxytoca* at both substrate concentrations was statistically greater (p-value < 0.010) and more than twice the growth rate of *B. cepacia*. Also, varying substrate concentration in the single-species cultures did not significantly change the organism's growth rate, suggesting zero-order growth rate kinetics for these two initial substrate concentrations. This implies that the growth rate calculated for each organism for both substrate concentrations is the maximum growth rate ($\mu = \mu_{max}$). Similar to the single-species batch experiments, the growth rate of *K. oxytoca* at both substrate concentrations in dual species was statistically higher (p-value < 0.020) and more than twice the growth rate of *B. cepacia*. The specific growth rate of each organism was similar when grown in a pure culture or in combination with the other organism (Table 1). In a batch culture, the organism that had the higher growth rate (*K. oxytoca*) had significantly higher dual-species maximum population densities.

Dissolved organic carbon (DOC) concentrations were monitored to quantify DOC utilization over time

Table 1. Single- and dual-species planktonic growth rates (μ) for the 70 mg/L and 700 mg/L DOC substrate concentration batch experiments

	Single-species μ (h^{-1})		*Dual-species μ (h^{-1})*	
	70 mg/L DOC	*700 mg/L DOC*	*70 mg/L DOC*	*700 mg/L DOC*
K. oxytoca	0.76 ± 0.06 (n = 2)	0.76 ± 0.05 (n = 2)	0.75 ± 0.13 (n = 3)	0.81 ± 0.07 (n = 2)
B. cepacia	0.32 ± 0.05 (n = 3)	0.30 ± 0.01 (n = 2)	0.27 ± 0.07 (n = 3)	0.29 ± 0.05 (n = 2)

for *K. oxytoca* in single-species culture, *B. cepacia* in single-species culture; and both organisms grown together. After 30 h of growth, both single-species experiments and the dual-species experiment utilized comparable amounts of DOC for each substrate concentration with a significant amount of DOC (67.9% ± 6.6% and 55.6% ± 4.6% for the 70 and 700 mg/L DOC substrate concentration experiments, respectively) remaining in each batch reactors. Dual-species batch experiments were also performed to monitor dissolved oxygen concentrations during exponential growth (6 h), early stationary phase (12 h), and late stationary phase of growth (30 h). Dissolved oxygen (D.O.) levels remained constant over time (5.9 ± 0.2 mg/L) for the 70 mg/L DOC substrate concentration. For the 700 mg/L DOC substrate concentration experiment, the D.O. concentration was 5.8 mg/L during exponential growth (t = 6 h). Low oxygen concentrations (<0.2 mg/L) were recorded during early stationary growth (t = 12 h), but rebounded to nonlimiting concentrations (> 4 mg/L) during late stationary growth phase (t = 30 h).

Biofilm Experiments. Fifty-four separate rotating-disk reactor (RDR) experiments were performed to quantify detached and biofilm population densities, as well as biofilm growth rates, of *K. oxytoca* and *B. cepacia* in single- and dual-species cultures. Four single-species RDR experiments were performed for each organism at substrate concentrations of 70 mg/L and 700 mg/L DOC (two experiments were destructively sampled after 48 h and two after 5 days). The single-species suspended cell plate counts for both organisms at all substrate concentrations tested reached steady-state conditions at ~48 h (Fig. 3). An average of the steady-state data (defined from 48 h to 5 days) for suspended biomass concentrations (Fig. 3) and biofilm biomass concentrations (Fig. 4) are presented in Table 2. In a single-species culture, an order of magnitude increase in substrate concentration resulted in an increase in the steady-state suspended (detached) cell population densities of *K. oxytoca* (p-value < 0.001) and *B. cepacia* (p-value < 0.04) (Fig. 3A, B, C, D). In addition, *K. oxytoca* had a greater steady-state suspended cell density than *B. cepacia* at both the 70 mg/L and 700 mg/L DOC substrate concentrations (p-value < 0.01 and p-value < 0.001, respectively). The biofilm population densities for each organism and substrate concentration were relatively constant with time between 48 h and 5 days (Fig. 4A, B) with the steady-state biofilm population density of *K. oxytoca* comparable to that of *B. cepacia* (Table 2).

To understand how *B. cepacia* and *K. oxytoca* interact together in a dual-species biofilm, four (70 mg/L DOC) or six (700 mg/L) RDR experiments were performed (each experiment was for a duration of either 48 h or 5 days). In addition, shorter dual species RDR experiments were performed for a duration of 18 h for both the 70 mg/L DOC (n = 1) and 700 mg/L DOC (n = 2) substrate concentrations. Similar to the single-species experiments, the effluent population densities reached steady-state conditions after ~50 h (Fig. 3E, F). In a dual-species RDR, an increase in substrate concentration from 70 to 700 mg/L DOC resulted in a significant increase in the steady-state suspended (detached) cell population density of *K. oxytoca* (p-value < 0.002) and a significant decrease in the steady-state suspended cell population density of *B. cepacia* (p-value < 0.01). The steady-state suspended cell population density of *B. cepacia* was significantly greater (p-value < 0.02) than that for *K. oxytoca* at the 70 mg/L DOC substrate concentration experiments (Fig. 3E), which was contradictory to the results obtained in dual-species batch reactor studies at the same substrate concentration where *K. oxytoca* was the dominant organism (Fig. 2C). At the 700 mg/L DOC concentration, the steady-state suspended cell population density for *K. oxytoca* was significantly greater (p-value < 0.001) than that for *B. cepacia*. Therefore, when the substrate concentration was increased by an order of magnitude, there was a shift in the steady-state suspended population density with the suspended population density of *K. oxytoca* greater than that of *B. cepacia*.

The dual-species biofilm population densities remained relatively constant with biofilm age for an 18-h, 48-h, and 5-day biofilm at these two substrate concentrations (Fig. 4D, E). Similar to the dual-species suspended cell results, an increase in substrate concentration resulted in an increase in the dual-species steady-state biofilm population density of *K. oxytoca* (p-value < 0.005) and a decrease for *B. cepacia* (p-value < 0.05) (Table 2). This resulted in *B. cepacia* being the dominant organism in the dual-species biofilm at the 70 mg/L DOC substrate

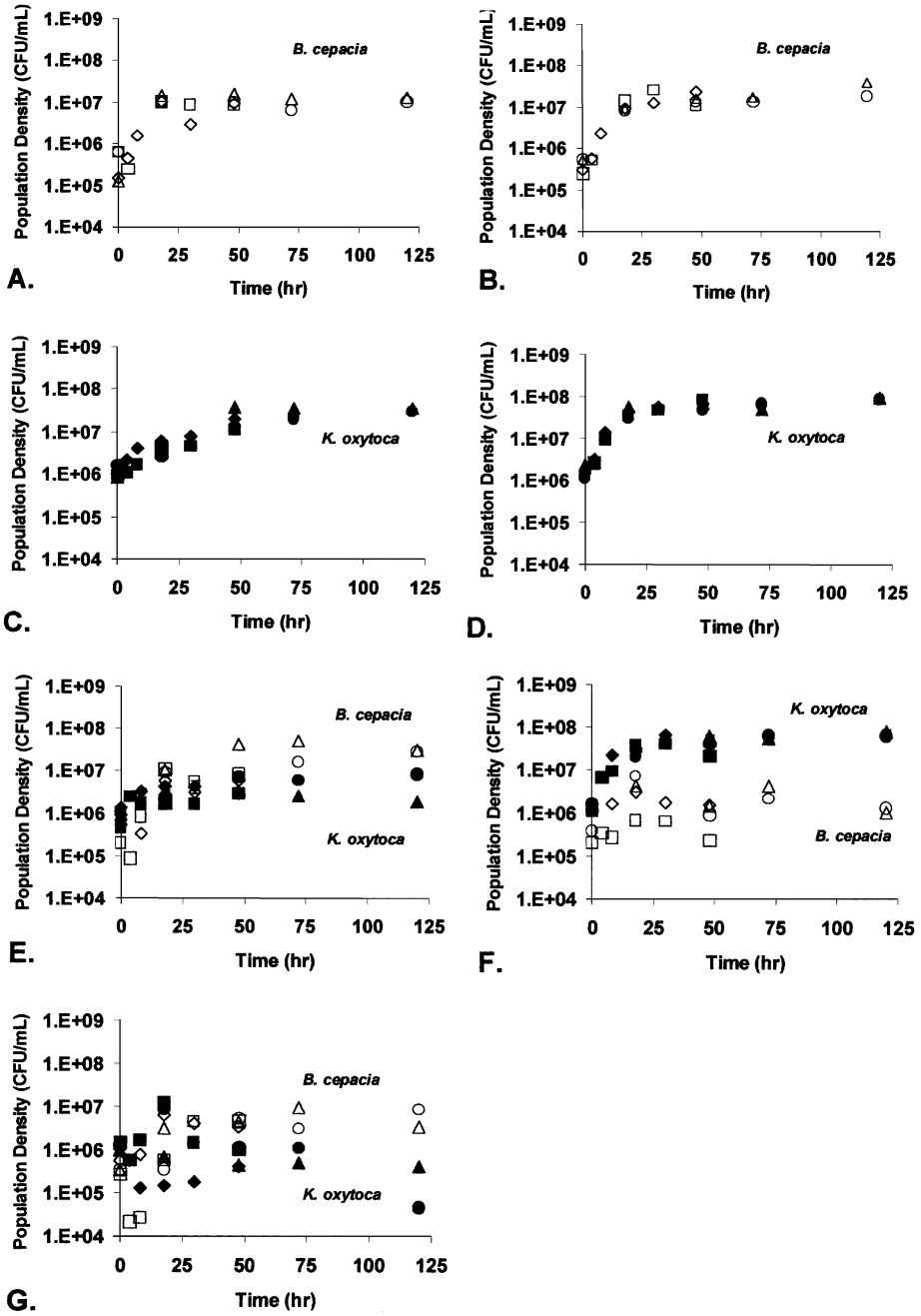

Figure 3. Effluent (suspended) population densities over time for *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) in single–species (A, B, C, D) and dual-species (E, F, G) rotating-disk reactors supplied substrate concentrations of 30 mg/L DOC (G), 70 mg/L DOC (A, C, E), and 700 mg/L DOC (B, D, F) during the 48-h and 5-day experiments.

concentration while *K. oxytoca* dominated at the 700 mg/L DOC concentration. The biofilm population density of the dominant organism in the dual-species culture (Fig. 4D or E) was comparable to what it achieved in the monoculture biofilm (Fig. 4A or B), whereas the biofilm population density of the nondominant organism was more than an order of magnitude lower than when grown alone. To further explore the link between substrate concentration and dual-species population density, another set of rotating-disk reactor (RDR) experiments was performed using a substrate concentration of 30 mg/L DOC (Fig. 3G and 4C). Separate RDRs supplied 30 mg/L DOC were destructively sampled after 18 h ($n = 2$), 48 hours ($n = 3$), and 5 days ($n = 2$). The decrease in substrate concentration from 70 mg/L to 30 mg/L DOC decreased both the *K. oxytoca* and *B. cepacia* suspended population densities (p-value < 0.005 and p-value < 0.02, respectively) but did not significantly change the biofilm population densities. The steady-state suspended and biofilm population density of *B. cepacia* was greater than that of *K. oxytoca* (p-value < 0.005 and p-value < 0.02, respectively) in the RDRs supplied 30 mg/L DOC. The steady-state biofilm population densities from the 30 mg/L DOC experiments were combined with the steady-state

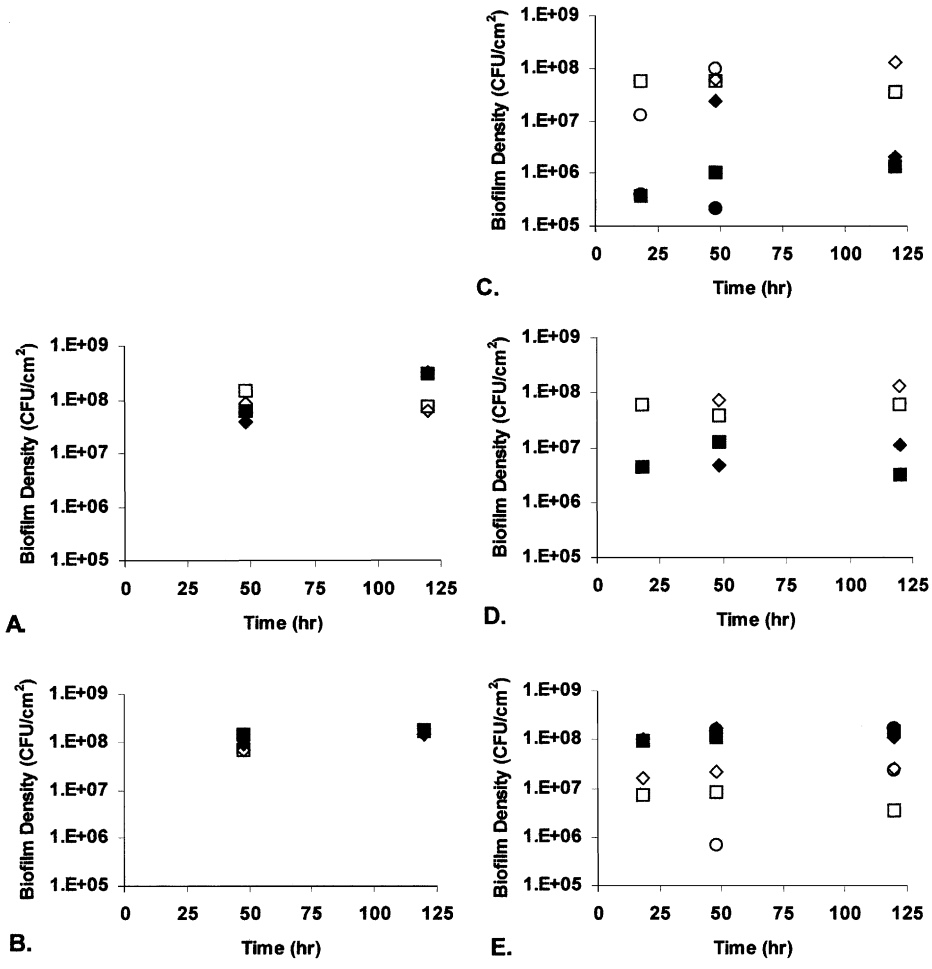

Figure 4. Biofilm population densities for *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) in single-species (A, B) and dual-species (C, D, E) rotating-disk reactors supplied substrate concentrations of 30 mg/L DOC (C), 70 mg/L DOC (A, D), and 700 mg/L DOC (B, E). Each data point represents a different rotating-disk reactor experiment.

biofilm population densities from the higher substrate concentrations (70 mg/L and 700 mg/L DOC) (Fig. 5). As the substrate concentration is increased from 30 mg/L DOC to 70 mg/L DOC, the dual-species biofilm population densities of both organisms remain constant with *B. cepacia* the dominant organism. An increase in substrate concentration from 70 mg/L DOC to 700 mg/L DOC decreased the *B. cepacia* biofilm population density and

Table 2. Steady-state dual-species suspended population densities (X_1), biofilm population densities (X_b), and specific biofilm growth rates (μ) for the 30 mg/L, 70 mg/L, and 700 mg/L DOC substrate concentration experiments[a]

	Single-species		*Dual-species*		
	Media concentration		*Media concentration*		
	70 mg/L DOC	*700 mg/L DOC*	*30 mg/L DOC*	*70 mg/L DOC*	*700 mg/L DOC*
X_1 (CFU/mL) × 10^7					
K. oxytoca	2.52 ± 1.08 (8)	7.08 ± 1.64 (8)	0.06 ± 0.04 (9)	0.44 ± 0.24 (8)	4.53 ± 2.09 (12)
B. cepacia	1.06 ± 0.29 (8)	1.92 ± 0.91 (8)	0.53 ± 0.22 (9)	2.34 ± 1.70 (8)	0.18 ± 0.16(12)
X_b (CFU/cm²) × 10^8					
K. oxytoca	1.77 ± 1.49 (4)	1.38 ± 0.35 (4)	0.06 ± 0.10 (5)	0.08 ± 0.05 (4)	1.45 ± 0.30 (6)
B. cepacia	0.92 ± 0.37 (4)	1.10 ± 0.51 (4)	0.77 ± 0.38 (5)	0.76 ± 0.42 (4)	0.14 ± 0.11 (6)
μ (h^{-1})					
K. oxytoca	0.16 ± 0.09 (4)	0.49 ± 0.15 (4)	0.24 ± 0.22 (5)	0.46 ± 0.19 (4)	0.24 ± 0.13 (6)
B. cepacia	0.14 ± 0.06 (4)	0.25 ± 0.12 (4)	0.08 ± 0.02 (5)	0.27 ± 0.19 (4)	0.18 ± 0.15 (6)

[a] X_1 and X_b values are an average (± SD) of the effluent and biofilm densities, respectively, measured between 48 h and 5 days reactor operation from Fig. 3 and 4, respectively. μ is an average (± SD) of the steady-state biofilm growth rate measured from Equation (1) using the suspended population density just prior to destructive sampling and the biofilm population density from destructive sampling for each rotating-disk reactor. Sample number given in parenthesis.

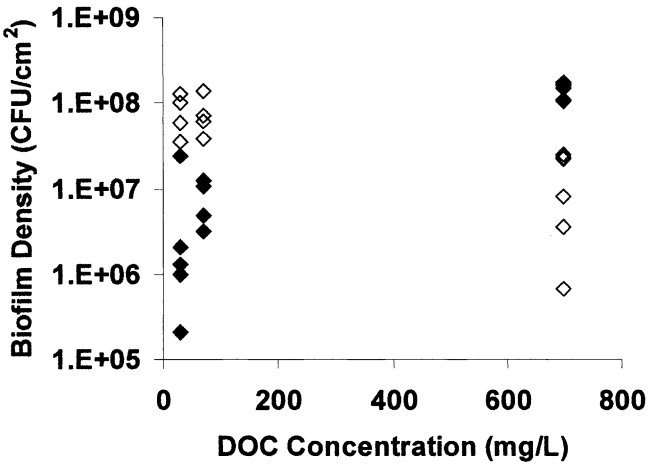

Figure 5. Steady-state dual-species biofilm population densities of *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) for 30 mg/L, 70 mg/L, and 700 mg/L substrate concentrations. Each data point represents a different rotating-disk reactor experiment.

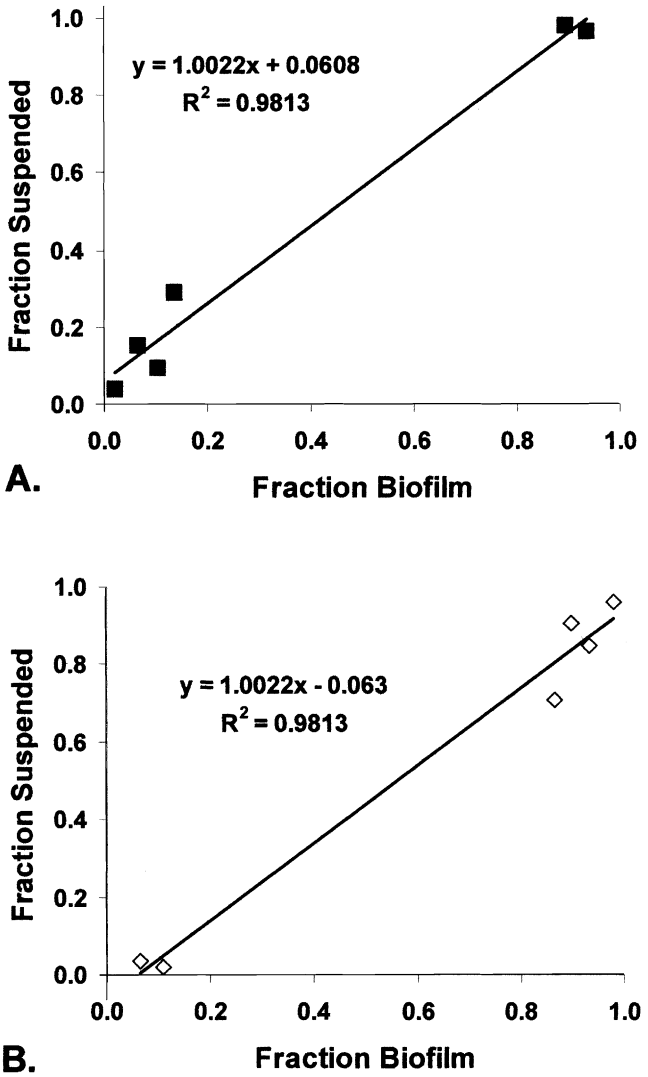

Figure 6. Comparison of the suspended biomass fraction to the biofilm biomass fraction for (A) *K. oxytoca* and (B) *B. cepacia* in the rotating-disk reactors supplied 30 mg/L, 70 mg/L, and 700 mg/L substrate concentrations. Fractions were calculated using average suspended (Fig. 3) and biofilm (Fig. 4) population densities from 48-h and 5-day sampling.

increased the *K. oxytoca* biofilm population density, resulting in *K. oxytoca* becoming the dominant population in the biofilm. To examine how the fraction of each organism in a biofilm related to the fraction of detached cells, the suspended biomass fraction of *K. oxytoca* (Fig. 6A) and *B. cepacia* (Fig. 6B) was plotted against the biofilm biomass fraction of each organism using average data from 48-h and 5-day sampling for all three substrate concentrations. The slope calculated using linear regression analysis was close to one (1.0022), which indicates that the fractions of biomass measured in the effluent are representative of the fractions measured in the biofilm. This suggests the detention time of the RDR was fast enough to prevent significant suspended growth and that the biofilm fraction on the removable coupons of the RDR is representative of the biofilm fraction throughout the reactor. A separate analysis comparing the fraction of total suspended biomass (suspended cell density × volume) to the total biofilm biomass (biofilm cell density × volume) for each organism in dual species indicates that the biofilm biomass makes up the majority (between 70% and 95%) of the total biomass in the reactor at steady state.

Specific biofilm growth rates were calculated using the suspended and biofilm population densities from each rotating-disk reactor experiment that was destructively sampled during steady-state (48-h and 5-day) conditions. Average steady-state specific growth rates were calculated for *K. oxytoca* and *B. cepacia* for each substrate concentration and inoculation scenario (single- or dual-species) (Table 2). The single-species specific biofilm growth rates of *K. oxytoca* increased from 0.16/h to 0.49/h when the substrate concentration was increased from 70 mg/L DOC to 700 mg/L DOC (p-value < 0.01), whereas that for *B. cepacia* remained constant between 0.14/h and 0.25/h (Table 2). The single-species growth rate of *K. oxytoca* and *B. cepacia* were comparable at the 70 mg/L DOC substrate concentration, but the single-species growth rate of *K. oxytoca* was greater than that of *B. cepacia* at the 700 mg/L DOC substrate concentration (p-value < 0.05). The dual-species growth rates of *K. oxytoca* and *B. cepacia* varied between 0.08/h and 0.46/h for all three substrate concentrations examined (Table 2). No statistical difference in dual-species growth rate was measured between each organism at each substrate concentration. Effluent dissolved organic carbon (DOC) concentrations were monitored over time to quantify

substrate utilization in the single- and dual-species rotating-disk reactors. The amount of DOC consumed during the 35-min detention time of the rotating-disk reactor for the 70 mg/L and 700 mg/L DOC substrate concentration experiments was 22.8% (±3.8%) and 23.1% (±9.7%) of the initial DOC concentration, respectively.

Discussion

According to existing descriptions of multispecies biofilm population dynamics [33], the faster-growing organisms should outcompete those that grow more slowly. In the batch reactors, single- and dual-species planktonic growth rates successfully predicted that the faster-growing organism (*K. oxytoca*) would have a higher population density than the slower-growing organism (*B. cepacia*) in dual-species planktonic cultures. Therefore, single-species or dual-species planktonic growth rates could be used to describe dual-species planktonic interactions. Research has shown that the faster-growing organism also dominates a dual-species biofilm [3, 31]. However, in our case, planktonic growth rates did not predict which organism would have a greater dual-species biofilm population density in the RDR experiments. The single- and dual-species specific biofilm growth rates for *K. oxytoca* were lower than in planktonic cultures, though the growth rates for *B. cepacia* were comparable between the two systems. This decrease in growth rate for *K. oxytoca* in a biofilm resulted in values not statistically different from the biofilm growth rates calculated for *B. cepacia*. Therefore, biofilm growth rates, in addition to plantonic growth rates, could not be used to describe the dual-species population distributions observed in the biofilm.

Substrate concentration played an important role in manipulating the relative abundance of *B. cepacia* and *K. oxytoca* in a dual-species biofilm culture. *B. cepacia* was the dominant organism in the dual-species biofilm at the lower (30 and 70 mg/L DOC) substrate concentrations. When this was increased to 700 mg/L DOC, there was a shift in the steady-state biofilm and suspended (detached) population densities with *K. oxytoca* now being greater than that for *B. cepacia*. Therefore, varying the substrate concentration provided a mechanism to control the fraction of each organism in the dual-species biofilm reactors. Previous research suggests a relationship between population dynamics and substrate concentration [6, 14, 18]. In a chemostat experiment using oral bacterial populations [18], certain organisms were isolated at greater frequencies at high substrate concentrations, whereas other organisms were isolated at higher frequencies at lower substrate concentrations. Camper et al. [6] observed that slower-growing organisms survived in higher numbers at lower substrate concentrations, a trend that was observed in the research presented herein. Increasing the substrate concentration has been shown to negatively affect the aerobic population in a biofilm reactor due to competition for dissolved oxygen and space in the biofilm [22]. Oxygen limitation at higher substrate concentrations may have affected the dual-species population distribution of the aerobic organism, *B. cepacia*, and the facultative organism, *K. oxytoca*, in the RDR experiments. Results from the dual-species batch experiments indicate that oxygen transfer into solution from constant mixing at room temperature appeared to have compensated for the oxygen demand from the bacterial populations at the 70 mg/L DOC substrate concentration. However, oxygen-limiting values in the 700 mg/L DOC batch reactors were recorded at 12 h, which is just before the batch culture entered stationary phase (Fig. 2F). Oxygen demand decreased in stationary phase and the oxygen concentration was able to rebound to non-oxygen-limiting values. A period of low oxygen at the high (700 mg/L DOC) substrate concentration could have limited the growth of the aerobic (*B. cepacia*) population resulting in a population density two orders of magnitude less than that of *K. oxytoca*. However, the same trend in species dominance occurred at the low (70 mg/L DOC) substrate concentration where oxygen was not limiting. Therefore, oxygen limitation may not be the mechanism defining the relative abundance of these two organisms in batch systems. In a biofilm, biomass growth equals detachment during steady-state conditions, indicating that oxygen utilization from biofilm growth is a function of the suspended (detached) biomass population density in the RDR. Though the total suspended biomass population in the dual-species 700 mg/L DOC rotating-disk reactor experiments ($4.71 \pm 2.13 \times 10^7$ CFU/mL, $n = 12$) was greater (p-value < 0.05) than the total suspended biomass population in the dual-species 70 mg/L DOC rotating-disk reactor experiments ($2.78 \pm 1.66 \times 10^7$ CFU/mL, $n = 8$), it is unclear whether this difference in biomass concentration is sufficient to increase oxygen demand to cause the shift in population dominance with change in substrate concentration. Further analysis is needed to fully understand the effects of oxygen limitation on both organisms.

K. oxytoca was the dominant organism in the batch experiments, but was not always the dominant organism in the rotating-disk reactors. This inconsistency in species dominance may be due to the inherent operational dynamics of the two reactor systems. For example, the leveling off of the populations over time in the batch reactors was likely caused by either the utilization of a limiting nutrient or production of by-products (which could be described simply as ''overcrowding''). Both of these phenomena would not occur to the same degree in the rotating-disk reactor because nutrients are constantly being resupplied and waste is constantly being removed.

This provides further explanation why kinetics from batch experiments did not correlate with those from continuous-flow systems.

In conclusion, *K. oxytoca* and *B. cepacia* behaved differently in biofilm mode than in planktonic mode with regard to dual-species specific growth rate and population density. Kinetics obtained from either single- or dual-species batch planktonic systems did not describe dual-species biofilm population densities. Single- and dual-species biofilm growth rates also did not correlate with dual-species biofilm population densities. The dual-species population densities could be described by changes in substrate concentration. At high substrate concentrations (700 mg/L DOC), *K. oxytoca* had a higher fraction of the total population than *B. cepacia*. At low substrate concentrations (30 and 70 mg/L DOC), there was a shift in the population distribution with *B. cepacia* becoming the dominant organism. This information enabled the use of substrate concentration to control the population densities of *K. oxytoca* and *B. cepacia* in a dual-species porous media biofilm reactor such that the population density of the TCE-degrading organism (*B. cepacia*), as well TCE degradation, was optimized [15]. Further research is needed to understand the exact reason this population shift occurs and if similar trends exist when other organisms are combined.

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