Effect of co-substrate concentration on dual-species population distribution, permeability reduction and trichloroethylene (TCE) biodegradation in porous media
by John Komlos, Jr

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy In Engineering
Montana State University

Abstract:
Dual-species microbial interactions have been extensively reported for batch and continuous culture environments but very little research has been performed on dualspecies interaction in a biofilm on the surface of a well-mixed reactor and even less research has been performed on porous media biofihns. The fundamental motive for this research was to gain a better understanding of the interaction between two microbial species when grown together in an engineered biofilm. Two organisms were combined together in planktonic, rotating disk, and porous media reactors to determine which variables controlled population distribution. The feasibility of using planktonic and biofilm growth kinetic data to predict dual-species porous media interactions was addressed. In addition, the ability to control the activities of each organism in a dualspecies porous media environment was examined. The two bacterial species used in this research were Burkholderia cepacia PR1-pTOM31c, an aerobic organism capable of constitutively mineralizing trichloroethylene (TCE), and Klebsiella oxytoca, a highly mucoid, facultative organism capable of reducing porous media permeability.

This research demonstrated the importance of growth rate and substrate concentration to predict dual-species interactions in batch, rotating disk and porous media reactors. The substrate concentrations used were different dilutions of LBG media resulting in dissolved organic carbon (DOC) concentrations of 30, 70 and 700 mg/L. In batch reactors, planktonic growth rates predicted the dual-species planktonic population distribution, with the faster growing organism (K. oxytoca) "outcompeting" the slower growing organism (B. cepacia). In the rotating disk and porous media reactors, however, biofilm growth rates did not correlate with the dual-species biofilm population distribution. The biofilm population distribution did correlate with substrate concentration, with B. cepacia having a greater dual-species population density than K. oxytoca at a low (30 mg/L DOC) substrate concentration and K. oxytoca having a greater dual-species population density at a high (700 mg/L DOC) substrate concentration. In addition, an increase in substrate concentration resulted in a decrease in TCE degradation and an increase in permeability reduction. This research demonstrated the effectiveness of using substrate concentration to control population density, TCE degradation and permeability reduction in a dual-species porous media bioreactor.

EFFECT OF CO-SUBSTRATE CONCENTRATION ON DUAL-SPECIES POPULATION DISTRIBUTION, PERMEABILITY REDUCTION AND TRICHLOROETHYLENE (TCE) BIODEGRADATION IN POROUS MEDIA

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APPROVAL

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John Komlos Jr.

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliography style, and consistency, and is ready for submission to the College of Graduate Studies.

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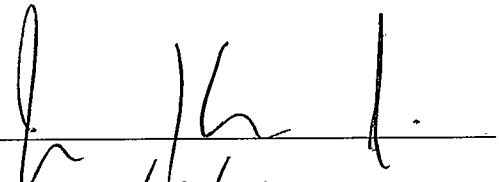
Signature 

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ABSTRACT

Dual-species microbial interactions have been extensively reported for batch and continuous culture environments but very little research has been performed on dual-species interaction in a biofilm on the surface of a well-mixed reactor and even less research has been performed on porous media biofilms. The fundamental motive for this research was to gain a better understanding of the interaction between two microbial species when grown together in an engineered biofilm. Two organisms were combined together in planktonic, rotating disk, and porous media reactors to determine which variables controlled population distribution. The feasibility of using planktonic and biofilm growth kinetic data to predict dual-species porous media interactions was addressed. In addition, the ability to control the activities of each organism in a dual-species porous media environment was examined. The two bacterial species used in this research were *Burkholderia cepacia* PR1-pTOM$_{31c}$, an aerobic organism capable of constitutively mineralizing trichloroethylene (TCE), and *Klebsiella oxytoca*, a highly mucoid, facultative organism capable of reducing porous media permeability.

This research demonstrated the importance of growth rate and substrate concentration to predict dual-species interactions in batch, rotating disk and porous media reactors. The substrate concentrations used were different dilutions of LBG media resulting in dissolved organic carbon (DOC) concentrations of 30, 70 and 700 mg/L. In batch reactors, planktonic growth rates predicted the dual-species planktonic population distribution, with the faster growing organism (*K. oxytoca*) "outcompeting" the slower growing organism (*B. cepacia*). In the rotating disk and porous media reactors, however, biofilm growth rates did not correlate with the dual-species biofilm population distribution. The biofilm population distribution did correlate with substrate concentration, with *B. cepacia* having a greater dual-species population density than *K. oxytoca* at a low (30 mg/L DOC) substrate concentration and *K. oxytoca* having a greater dual-species population density at a high (700 mg/L DOC) substrate concentration. In addition, an increase in substrate concentration resulted in a decrease in TCE degradation and an increase in permeability reduction. This research demonstrated the effectiveness of using substrate concentration to control population density, TCE degradation and permeability reduction in a dual-species porous media bioreactor.

CHAPTER 1

INTRODUCTION

The fundamental motive for this research is to gain a better understanding of the interaction between two microbial species when grown together in an engineered biofilm. An understanding of how organisms co-exist in biofilms can be applied to engineered systems such as trickling filters, vapor phase reactors for volatile organic compounds and subsurface bioremediation systems for the removal of xenobiotic compounds. Predicting which variables control the population distribution of a dual-species culture could allow for the creation of an engineered system in which population density is manipulated to provide the appropriate reactions of each organism present. General questions that need to be addressed for the successful creation and implementation of an engineered multi-species biofilm include:

1) Will the two microbial species co-exist?
2) Can their populations be controlled/predicted?
3) Do the desired activities of each organism persist?
4) Will either species be outcompeted by indigenous organisms?
5) Can (single-species planktonic) growth parameters from the literature be used to predict dual-species biofilm parameters?
6) Are biofilm growth/activity characteristics in rotating disk reactor biofilms similar to or significantly different than biofilms in porous media?

The example engineered biofilm system that has been chosen for this research involves designing a reactive/reduced permeability dual-species biofilm barrier in porous media for the control and treatment of trichloroethylene (TCE) contaminated groundwater.

Background

Bacteria in the environment are rarely found in mono-cultures, but instead as part of a community. Examples of processes that involve a community of bacteria in the environment include biodegradation, tooth decay, denitrification, methanogenisis, biofouling, wastewater treatment, composting, microbially induced corrosion, sulfate reduction as well as other processes (Caldwell and Costerton, 1996). Many of the processes mentioned above take place in porous media, yet very little research has been performed examining the interactions between two or more organisms in this environment. Further understanding of how mixed species populations interact in porous media could lead to enhanced in-situ bioremediation or bioaugmentation technologies as well as improved engineered systems (eg. bioreactors).

Planktonic Microbial Interactions

The quantitative study of factors that affect microbial growth can be traced back over 100 years (Ward, 1895). Since then, microbial growth has been extensively studied in both batch and chemostat cultures. In addition to the study of organisms in mono-

cultures, extensive work has also been performed to describe interactions in mixed microbial consortia.

Positive microbial interactions include commensalism, where one organism benefits from the presence or activity of another organism while the benefactor is unaffected (James et al. 1995). An example of commensalism is the interaction between *Nitrosomonas* and *Nitrobacter*, two organisms responsible for the microbial conversion of ammonia to nitrate (nitrification). *Nitrosomonas* converts ammonia to nitrite. *Nitrobacter* utilize the nitrite and convert it to nitrate while *Nitrosomonas* are not benefited directly by *Nitrobacter*.

Mutualism is another positive microbial interaction in which both organisms positively affect each other. Algae produce oxygen that is essential to aerobic organisms and aerobic organisms produce CO_2 that is essential to algae. Synergism is a type of mutualism in which the formation of specific products is greater in mixed than in pure cultures (Meers, 1973). Wolfaardt et al. (1994) demonstrated that a mixed culture could degrade a particular herbicide as the sole carbon and energy source, though none of the individual organisms were able to degrade the herbicide in pure culture.

Negative microbial interactions include one organism producing a product that is toxic to another organism (ammensalism). Predation is an interaction where one organism is consumed by another (amoebae attacking a bacterium) and parisitism is an interaction where one organism is invaded intracellularly by another. *Bdellovibrio bacteriovorus* attaches to another bacteria, penetrates the cell, grows and replicates causing the prey cell to lyse.

Competition is considered a negative interaction and is defined as situations in which the populations of two species are mutually limiting because of their joint dependence on a common factor or factors external to them (Meers, 1979). Two bacterial populations competing for a single limiting nutrient in a spatially homogeneous environment leads to exclusion of one of the competitors if the system is at steady-state (time-invariant) conditions (Fredrickson and Stephanopoulos, 1981) with the organism with the higher growth rate dominating (Powell, 1958). However, both populations can co-exist at steady-state when each is limited by a different resource (Drzyzga et al. 2001; Tilman, 1977).

The type of reactor used in the planktonic study of the dual-species interactions of the same two organisms can yield different results. Tilman (1977) found that both batch and continuous flow (chemostat) reactors provided similar kinetic data and dominant steady-state population when compared using the same organisms while Cao and Alaerts (1995) observed different population distributions using the same organisms (coexistence between populations in batch cultures but one organism dominating in a continuous flow system).

Bacteria Attached to Surfaces

The majority of dual-species studies have been performed using suspended cells in batch or chemostat experiments yet the majority of microorganisms in the environment are attached to surfaces (Costerton et al. 1995) and these attached cells are responsible for the majority of the metabolic activity in the system (Fletcher, 1986; Geesey et al. 1978). These attached cells are called biofilms.

Suspended (planktonic) vs. Biofilm Cells

Suspended and biofilm cells are exposed to different environment conditions. In well-mixed planktonic cultures, each individual cell has equal access to oxygen and nutrients. In a biofilm, however, significant biofilm thickness can cause concentration gradients through diffusion limitations (Revsbech and Jorgensen, 1996). Diffusion limitation could result in cells near the bottom of the biofilm receiving little or none of the nutrients required for growth. In addition, diffusion limitation could provide biofilm cells with enhanced resistance to disinfection by antimicrobial agents (Stewart and Raquepas, 1995). These concentration gradients also allow for ecological diversity in a biofilm (Bradshaw et al. 1997; Revsbech and Jorgensen, 1986; Wanner and Gujer, 1986). In nutrient-rich environments, organization of bacteria in biofilms will reduce the overall rate of growth of the population through nutrient and gaseous gradients (Wimpenny, 1995). Wentland et al. (1996) reported gradients in biofilm growth rates from the air/agar interface of a colony biofilm (higher growth rate) to the center of the colony biofilm (lower growth rate). Sharp et al. (1998b) measured a lower activity and expression of a degradative pathway in biofilm cultures compared to suspended cultures at comparable growth rates. Different genes are upregulated when a previously planktonic cell attaches to a surface, creating a different phenotype (Costerton et al. 2000). This could also provide another explanation for differences in disinfection rates and growth rates between planktonic and biofilm cells.

Dual-Species Biofilms

Multi-species interactions have been extensively examined in both batch and chemostat (continuous culture) environments, but very little research has been performed on multi-species interactions in a biofilm. The definition given by Fredrickson and Stephanopoulos (1981) for two organisms competing for the same limiting nutrient does not incorporate biofilm formation. Biofilms account for the majority of situations where microbial activity is of interest, yet very little research has been performed on biofilm population dynamics. Therefore, the mechanisms needed to establish a functional dual- or multi-species biofilm are not clear.

Microbial Interaction in a Biofilm. Similar to planktonic interactions, multiple-species in a biofilm may have a positive or negative affect on each species' growth. A multi-species biofilms containing aerobic and anaerobic bacteria can provide an environment for anaerobic organisms to survive, even when the bulk water is oxygenated, because the aerobes in the upper layers of the biofilm utilize the oxygen (Bradshaw et al. 1997; Revsbech and Jorgensen, 1986). In addition, a dual-species biofilm can produce a thicker biofilm compared to either one of the two species in pure culture (Siebel and Characklis (1991). However, multi-species in a biofilm can cause negative interactions. For example, Møller et al. (1997) reported a stable biofilm can be reduced to isolated colonies by predation.

Initial Colonization. It is known that a biofilm can trap particles (Drury et al. 1993), suggesting that an established biofilm may retain other organisms present in the

bulk fluid. Furthermore, research has demonstrated that many different dual-species scenarios can occur. An established biofilm can be beneficial to an inoculated organism (Banks and Bryers, 1992; Ciardi et al. 1987; Schwarz et al. 1987). Also an inoculated organism can be beneficial to organisms already present in a biofilm (Cowan et al. 1991). However, an established biofilm of another species can hinder the colonization of an inoculated species (Bibel et al. 1983; Sturman et al. 1994) or an inoculated species can have a negative affect on the established biofilm (Møller et al. 1997). McEldowney and Fletcher (1987) observed that the attachment of one species could be influenced by the simultaneous or previous attachment of another species but in many cases there was no effect. They concluded there were no particular species combinations or conditions, i.e. surface composition or sequence of attachment, which consistently influenced attachment or detachment of an organism in a dual-species culture.

Effect of Growth Rate on Population Distribution. 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 the organism with the higher growth rate was not present in the highest population density, suggesting that factors other than growth rate may influence spatial distribution and relative cell numbers in biofilms. Stewart et al. (1997) also observed that two organisms with different growth rates could coexist in a biofilm.

Effect of Substrate Concentration on Microbial Cultures. Research has shown that substrate concentration plays an important role in rate of growth (Pinar et al. 1998), population size (Bühler et al. 1998), and population distribution (Camper et al. 1996; Cleland et al. 1997; Houtmeyers et al. 1980; Marsh et al. 1983) of single- and dual-species cultures. Houtmeyers et al. (1980) and Marsh et al. (1983) 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. Pinar et al. (1998) reported that the type of carbon source (sucrose or glycerol) affected *Klebsiella oxytoca's* maximum specific growth rate. In addition, when both carbon sources were combined, *K. oxytoca* completely utilized sucrose before utilizing glycerol in batch cultures but utilized both simultaneously in a chemostat. Substrate concentration played an important role in colonization of a mixed-species biofilm, with slower growing organisms surviving in higher numbers at lower substrate concentrations (Camper et al. 1996). The amount of nitrogen and phosphorous, as well as the ratio to each other, affects the size and distribution of microbial populations (Cleland et al. 1997). Substrate concentration (Beyenal and Lewandowski, 2000; Stoodley et al. 1999) and substrate composition (Møller et al. 1997) also affect mixed-species biofilm structure and population density.

Conclusion. Skillman et al. (1998) observed that organisms can coexist together in a biofilm, but the organisms could compete with one another as well, depending on the organisms present. It was concluded by James et al. (1995) that multi-species

interactions in a biofilm can be a function of the microorganisms present, the substratum the microorganisms attach to, as well as each microorganism's physiological parameters.

Biofilms in Porous Media.

A well-mixed biofilm is defined as a biofilm created when the bulk fluid experiences completely mixed conditions, constant nutrient supply and constant waste removal. In literature this is described as a chemostat or a completely-mixed stirred tank reactor (CSTR). Porous media biofilms are defined as bacteria that grow on the surface of porous media (soil, glass beads, etc.). Both types of biofilms could experience mass transfer limitation deep in the biofilm, but in a porous media system, mass transfer limitation could occur in the bulk fluid. This could have a negative effect on aerobic organisms throughout a porous media system if oxygen is utilized at the beginning of the porous media. In addition, the hydrodynamics are different between a biofilm on the surface of a well-mixed reactor and a porous media biofilm. 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 porous media environment. These hydrodynamic differences between the two systems could influence the characteristics of the biofilm formed in the two reactors.

Biofilm Accumulation. When microbial cells come in contact with a surface, they may attach and grow. If the rate at which cells attach and grow is greater than the rate at which they detach from the surface, there will be accumulation of biomass on the surface (i.e. a biofilm). If substrate supply to the biofilm is sufficient, thick continuous biofilms

may be formed. This condition is referred to as "high substrate loading" (Cunningham et al. 1997). The relationship between substrate loading rate and biofilm morphology is discussed in detail by Rittmann (1993). Under high substrate loading conditions, the average biofilm thickness on individual media particles will increase, resulting in a corresponding decrease in effective pore space. In systems where the flow rate through the porous media remains constant, the average pore velocity will likely increase with the increasing biofilm thickness, while in systems where the piezometric gradient remains constant, pore velocity will decrease. Increased thickness may result in depletion of nutrients within the biofilm structure.

Effects of Biofilm Accumulation on Porosity and Permeability. The accumulation and activity of biofilms varies from point to point along individual porous media pore channels, and thus are considered to be microscale phenomena. Observations of biofilm accumulation and corresponding reduction in media porosity, permeability and other mass transport properties have been widely reported (Cunningham et al. 1997; Rittmann, 1993; Sharp et al. 1999; Taylor and Jaffe, 1990). Cunningham et al. (1991) observed that a permeability of 3 to 7 x 10^{-8}cm^2 persisted after biofilm thickness had reached a maximum value, suggesting that the biofilm accumulation process stabilizes so as to preserve a minimum permeability within the media-biofilm matrix. Thick biofilm accumulation and subsequent porous media permeability reduction has been modeled using the computer simulation program, AQUASIM (Wanner et al. 1995).

Biofilm Barrier Technology. The ability of thick biofilms to reduce porous media permeability has lead to an innovative technology to prevent contaminant migration in groundwater called subsurface biofilm barriers. The examples below demonstrate the effectiveness of biofilm barriers under laboratory conditions.

Bench scale experiments have shown that bacteria, uniformly distributed throughout sand columns and supplied adequate nutrients, were capable of significantly reducing the hydraulic conductivity throughout the columns by greater than 85% (Warwood et al. 1995). Although the initial hydraulic conductivities of columns packed with two types of sand were different (0.26-2.97 cm/min), thick biofilm formation resulted in similar hydraulic conductivities for both types of sand (0.04-0.16 cm/min) at steady state. The same column setup was used to determine the effects of nutrient starvation on permeability reduction in porous media (Warwood et al. 1995). Challenge of established mesoscale biofilm barriers with starvation conditions (no nutrients supplied) resulted in an increase in hydraulic conductivity after five to ten days. Rapid reduction in hydraulic conductivity followed the alleviation of the starvation challenge and suggests that the biofilm barrier population remained in the column under starvation conditions but was ineffective in maintaining reduced hydraulic conductivity. This provided a mechanism to control the extent of permeability reduction by varying nutrient concentration.

Biofilm barrier stability when exposed to common groundwater contaminants was also examined in porous media column studies (Warwood et al. 1995). Stability of an established biofilm barrier was unaffected by heavy metal concentrations in feed media

(1ppm strontium and 1ppm cesium). In addition, the stability of an established biofilm barrier was unaffected by 200 mg/L carbon tetrachloride in the feed media.

The effects of nutrient concentration and duration of addition were examined using two pilot-scale lysimeters that were designed and manufactured to facilitate evaluation of biobarrier performance in a two-dimensional configuration (Warwood et al. 1995). A consistent flow reduction of >99% was achieved over a 60 day period. Subsequent nutrient addition was not required to maintain the stated flow reduction over a 3-month period.

Effect of Hydraulic Gradient on Biofilm Barrier Stability. Hydraulic conductivity reduction was observed in the two experimental systems described in the above mesoscale biobarrier experiments. In the column experiments, hydraulic conductivity was reduced from an initial hydraulic conductivity between 0.26-2.97 cm /min to a final hydraulic conductivity of approximately 0.1 cm/min. When nutrient supply was turned off, biobarrier stability was lost and the hydraulic conductivity increased. In the second set of experiments using the 2-dimesional lysimeters, the initial hydraulic conductivity was reduced from between 4.0 and 4.8 cm/min to 3 x 10^{-5} cm/min (5 order of magnitude reduction). Furthermore, no increase in hydraulic conductivity was recorded for 60 days after nutrient supply was shut off. Both of these experiments were performed using the same organism, (*K. oxytoca*), the same nutrient and nutrient concentration, and similar porous media but had final hydraulic conductivities that varied by orders of magnitude. In addition, biofilm stability in the lysimeter experiment was maintained for a significantly longer period than the column experiment. One major difference between

these two systems is the hydraulic gradient. The hydraulic gradient of the column reactor was 1.33 ft/ft (Δh=4ft, Δl=3ft) and the hydraulic gradient of the lysimeter was 0.03 ft/ft (Δh=1.5in, Δl=4ft). In the lower hydraulic gradient system, the biofilm matrix could be exposed to lower pressure (shear) and therefore less likely to lose stability. This could result in reduced permeability conditions maintained even in the absence of nutrients. In the higher hydraulic gradient system, perhaps the biofilm matrix could not retain permeability reduction due to increased pressure and lack of nutrients needed for biofilm stability. It should be noted that the low hydraulic gradient found in the rectangular lysimeter is more typical of what would be found in the field.

Radial Flow Biofilm Barriers. The previous mesoscale biofilm barrier experiments were performed to determine how well biofilms could reduce porous media permeability. Another set of experiments was performed to determine if a biofilm barrier could be created under radial flow (field-relevant) conditions. The results indicate that a meso-scale biofilm barrier can be formed in the radial flow direction that reduced the hydraulic conductivity of the porous media by 75% and decreased the hydraulic flow rate through the soil from 1100 ml/min to 25 ml/min (Komlos et al. 1998). In addition, the injection of bacteria and nutrients in the vadose zone created a biofilm barrier above the existing water table that was capable of reducing hydraulic flow on top of the saturated zone barrier (Komlos et al. 1998). The creation of an elevated biofilm barrier in the field offers the potential for hydraulic containment of a dissolved contaminant plume.

Conclusion. Many important parameters need to be taken into account when studying biofilm accumulation in porous media. They include the porous media characteristics (hydraulic conductivity, hydraulic gradient, porosity), the microorganism characteristics, and the type and concentration of nutrient supplied to the system. Thick biofilms can be used to reduce porous media permeability. These biofilm barriers can manipulate soil hydraulic conductivity to allow for varying degrees of groundwater penetration, thus enabling the potential containment or diversion of a contaminant plume. For field application of this technology, other factors such as transport of bacteria through porous media must be taken into account.

Field-Scale Biofilm Barrier Technology

Selective plugging of permeable strata has the potential to prevent the migration of groundwater contaminants from hazardous waste sites (Cunningham et al. 1997; Komlos et al 1998; Warwood et al 1995). The penetration of starved microorganisms into porous media and subsequent resuscitation by nutrient addition is a conceivable method to achieve this subsurface plugging.

Bacterial Starvation to Increase Bacterial Transport. The uniform distribution of bacteria in porous media necessary create a biofilm barrier that will prevent contaminant migration is dependent on the successful transport of bacteria and nutrients through the subsurface. The injection of bacteria and/or nutrients into the subsurface may cause plugging near the injection well, limiting the distance bacteria can be transported through porous media (Shaw et al. 1985). Starving the bacteria results in a reduction in cell size

and biofilm production that prevents plugging near the injection well and allows the bacteria to transport further through porous media (MacLeod et al. 1988, Lappin-Scott et al. 1988, Lappin-Scott and Costerton 1992 and Cusack et al. 1992).

Biofilm Barrier Implementation. Center for Biofilm Engineering (CBE) bench-scale investigations using thick biofilms as a subsurface barrier have been shown to be a promising alternative for the containment of hazardous waste plumes (Cunningham et al. 1991, Warwood et al. 1995, James et al. 1995 and Cunningham et al. 1997). These laboratory experiments have demonstrated the effectiveness of injecting both bacteria and then nutrients into porous media, producing a thick, uniform biomass matrix capable of reducing aquifer permeability/hydraulic conductivity. The use of biofilm barriers in the field would involve 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 pore space is virtually sealed by bacterial growth and EPS (extracellular polysaccharide) production (Costerton, 1994).

Biofilm Barrier Capabilities. Biofilm barriers are an attractive alternative to traditional subsurface barrier technologies (slurry walls, grout curtains, sheet pilings) because biofilm barriers do not require excavation, may utilize indigenous organisms, require minimal maintenance, have no depth limitations, and are cost effective to install and maintain (Hiebert et al. 2001). Biofilm barrier technology can be combined with bioremediation technologies to simultaneously degrade a contaminant while hindering its migration. Examples include funnel and gate technology, where strings of biofilm

barriers are used to funnel a contaminant into a gate area where remediation of the contaminant can be performed on a localized scale. Also, a bacterial strain could be isolated from a hazardous waste site for its ability to not only form a stable biofilm barrier, but also for its ability to degrade the contaminant in question. This reactive biofilm barrier could degrade a contaminant while slowing its migration. Another type of reactive biofilm barrier creates anaerobic conditions in and downstream of the biobarrier through the activity of the bacterial species. The anaerobic environment could be used to prevent the bacterial oxidation of sulfide minerals, such as acid-generating mine tailings.

Field-Scale Demonstration of Biofilm Containment Barrier. A field demonstration of biofilm barrier technology was performed to show the feasibility of using bacteria to control groundwater flow (Cunningham, 2000, Hiebert et al. 2001). The test site was 40m wide, 56m long, 6.1m deep and lined with impermeable plastic. A highly mucoid *Pseudomonas* strain was inoculated into a series of injection wells along the width of the site followed by nutrients consisting of molasses as the primary growth substrate and nitrate as the primary electron acceptor once oxygen was depleted. As the biofilm barrier developed, a steady decrease in hydraulic conductivity occurred over time, reaching steady-state conditions after a three-week period. Hydraulic conductivity reductions of over 99% were measured across the barrier (Hiebert et al. 2001).

Reactive/Reduced Permeability Biofilm Barrier

A developing, advanced application of biofilm barrier technology is the use of reactive bacterial populations to produce reactive biofilm barriers. Reactive biofilm

barrier technology offers a means of degrading a contaminant while simultaneously reducing its migration. Since recalcitrant compounds, such as trichloroethylene (TCE), show little natural attenuation, the development of a TCE reactive biofilm barrier could lead to a significant advancement in bioaugmentation technology. Reactive biofilm barriers can be developed in two ways: 1) using a single bacterial population to reduce the soil hydraulic conductivity and simultaneously degrade the contaminant; 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 produce copious amounts of biofilm while carrying out the desired reaction. A single-species reactive/reduced permeability biofilm barrier has successfully reduced porous media permeability and degraded nitrate on the bench scale (Komlos et al. 1998). A dual-species reactive biofilm barrier 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.

Biodegradation of Trichloroethylene

Trichloroethylene (TCE) is a halogenated aliphatic organic compound that is primarily used to remove grease from fabricated metal parts and some textiles (U.S. Environmental Protection Agency, 1985). It has also been used in the past as a general anesthetic (Agency for Toxic Substances and Disease Registry, 1993). There is no known natural source of TCE (U.S. Environmental Protection Agency, 1985). The Environmental Protection Agency (EPA) lists TCE as one of approximately 90

contaminants in its National Primary Drinking Water Regulations (www.epa.gov as of 7/01). Acute short-term exposure has caused death in humans (Agency for Toxic Substances and Disease Registry, 1993). It has been documented to cause tumors in mice (U.S. Environmental Protection Agency, 1985) but confirming its link to cancer in humans has been inconclusive due to small sample sizes and presence of other chemicals. The EPA considers TCE as an intermediate between a probable and possible human carcinogen (Agency for Toxic Substances and Disease Registry, 1993). Because it is a very effective degreaser, its production has increased from approximately 260,000 lb. in 1981 to 320 million lb. in 1991 (www.epa.gov/OGWDW/dwh/t-voc/trichlor.html as of 7/01). From 1987 to 1993, according to the Toxics Release Inventory, TCE releases to water totaled over 100,000 lbs (www.epa.gov/OGWDW/dwh/t-voc/trichlor.html as of 7/01). TCE's increased production and presence in groundwater, combined with it being a suspected carcinogen, make research to remove TCE from the environment of extreme importance.

Trichloroethylene Remediation. Methods to remove TCE from groundwater are usually expensive (chemical degradation), require further disposal after removal from groundwater (charcoal adsorption), or release TCE into the environment (air stripping) (Fliermans et al. 1988). Therefore the removal of TCE from groundwater through biological degradation is an attractive option.

Anaerobic TCE Biodegradation. TCE can be biodegraded anaerobically under methanogenic conditions (Bouwer and McCarty, 1983; Parsons et al. 1984; Vogel and

McCarty, 1985). The anaerobic degradation process is reductive dechlorination (replacement of a chlorine atom with a hydrogen atom) (Kleopfer et al. 1985) with 1,2-dichloroethylene (Kleopfer et al. 1985; Parsons et al. 1984) and vinyl chloride (Parsons et al. 1984; Vogel and McCarty, 1985) as end products. Incomplete dechlorination of TCE with vinyl chloride as an end product is of concern because it also is a threat to human health. Vogel and McCarty (1985) also reported partial TCE mineralization to CO_2. Freedman and Gossett (1989) reported reductive dechlorination of TCE to the environmentally friendly compound of ethylene.

Aerobic TCE Biodegradation. Anaerobic degradation of TCE is is a slow process (Bouwer and McCarty, 1983) often resulting in the recalcitrant and mutagenic vinyl chloride as an end product. Aerobic TCE degradation is significantly faster than anaerobic degradation (Shields and Reagin, 1992) but until recently was only observed as a co-metabolic process with another chemical, such as methane (Wilson and Wilson, 1985; Fogel et al. 1986; Fliermans et al. 1988), toluene (Nelson et al. 1987; Nelson et al. 1988), propane (Fliermans et al. 1988), phenol (Nelson et al. 1987; Nelson et al. 1988) or ammonia (Arciero et al. 1989).

Constitutive Aerobic TCE Biodegradation. *Burkholderia cepacia* G4 is one such environmental isolate capable of co-metabolically degrading TCE (Folsom et al. 1990; Nelson et al. 1986; Nelson et al. 1987) via the toluene ortho-monooxygenase (TOM) pathway (Shields et al. 1991). Sheilds and Reagin (1992) were able to develop a constitutive, aerobic, TCE-degrading organism (*Burkholderia cepacia* G4 5223 PR1)

from the wild-type co-metabolic *B. cepacia* G4. This strain was able to degrade TCE in microcosm experiments (Krumme et al. 1993) and gas-phase bioreactors (Shields et al. 1994). Another strain (*B. cepacia* $PR1_{301}$) was developed from the environmental isolate, *B cepacia* G4, that is able to carry out constitutive TCE degradation without the insertion of additional genetic material (Munakata-Marr et al. 1996). This strain has proved successful in lab experiments (Munakata-Marr et al. 1996; Munakata-Marr et al. 1997) and an adhesion deficient variant (*B. cepacia* ENV435) was successfully bioaugmented to remediate a TCE-contaminated site (Steffan et al. 1999).

TCE-Degrading Biofilm Barrier. The TCE-degrading organism used in the experiments presented in this dissertation was *Burkholderia cepacia* PR1-$pTOM_{31c}$. This strain was also developed from *B. cepacia* G4 and has the ability to constitutively express the TOM pathway with kanamycin resistance as a selective marker (Shields et al. 1995). Unfortunately, *B. cepacia* PR1-$pTOM_{31c}$ is unable to form a stable biofilm capable of reducing porous media permeability (Sharp, 1995). As mentioned previously, research has shown that a biofilm, even of a different species, may enhance attachment of an organism compared to a clean surface (Banks and Bryers, 1992). Therefore, it would be desirable to combine *B. cepacia* with *Klebsiella oxytoca,* a thick biofilm-forming organism, in an attempt to create a stable TCE-degrading, permeability reducing biofilm. Karthikeyan et al. (1999) reported that a contaminant-degrading organism enabled the survival of another organism by reducing the contaminant to non-toxic levels. Therefore, the possibility exists for the TCE-degrading organism to lower TCE concentrations to levels non-toxic to the thick biofilm-former.

Goals and Objectives

In order to implement a multi-species biofilm in porous media, important questions must be addressed. Of foremost concern are whether the two (or more) species will coexist and what variables affect the interactions between the species. In addition, methods must be developed to control the population distribution of each organism to ensure that an appropriate microbial population exists to perform the desired reactions(s). And finally, the desired activity of the multi-species system must persist. Therefore the goals of this research are:

1) Examine the feasibility of combining an aerobic TCE-degrading organism with a facultative thick biofilm forming organism.

Before full-scale experiments could begin, it was essential to have an understanding of how these two organisms interact. A series of preliminary experiments were performed to understand what factors influence the interaction of *B. cepacia* and *K. oxytoca* in a dual-species biofilm. The results conclude that these two organisms can co-exist and that the method of inoculation did not significantly affect the population distribution in a porous media reactor (Komlos et al. 1999). Additional experiments suggest that substrate concentration might be an important factor in manipulating the population distribution in a porous media reactor (Komlos et al. 2001). This provided the basis for the research presented herein.

2) Determine which variables (growth rate, substrate concentration) control the population distribution in dual-species biofilm reactors.

The successful establishment and implementation of an engineered dual-species biofilm relies on the ability to understand which variables are important in providing an adequate population of each organism to maximize the desired activities.

3) Determine if planktonic and biofilm single- and dual-species growth rates can predict dual-species interactions in biofilm systems.

Growth rates obtained from planktonic cultures may be significantly different than growth rates in a biofilm. In addition, the rate of growth of an organism in pure culture may be different than if the organism was grown in mixed culture. Therefore, the use of planktonic growth kinetics may not provide an adequate representation of how that organism behaves in a biofilm. Similar discrepancies between single- and dual-species cultures may also occur. This research will examine if growth rate data from more simple experiments can be used to describe more complicated systems.

4) Determine if biofilm characteristics (growth rate, population density) are similar or significantly different in rotating disk reactor biofilms and porous media biofilms.

Growth rates obtained from biofilm growing on the surface of a well mixed rotating disk reactor may vary significantly from growth rates of porous media biofilms due to increased mass transfer limitations and hydrodynamic differences in a porous media system compared to a rotating disk reactor. Use of biofilm data from a rotating

disk reactor to describe porous media growth could affect the ability to accurately predict porous media behavior.

5) Determine the effects of substrate concentration on TCE biodegradation and permeability reduction in a porous media reactor inoculated with dual-species cultures.

Substrate concentration was identified as an important variable to control the population distribution of both organisms in dual-species biofilms. Experiments were performed to determine if varying substrate concentration could also be used to maximize the activities of *B. cepacia* (TCE degradation) and *K. oxytoca* (permeability reduction).

Thesis Contents

Chapter 1 - Introduction

Summary of research applicability and background literature support.

Chapter 2 - Materials and Methods

Description of all materials used and experimental methods developed and implemented for the completion of this research.

Chapter 3 - Results

Experimental results of batch, rotating disk and porous media column experiments used to examine bacterial interactions of *Burkholderia cepacia* and *Klebsiella oxytoca* in different systems. The results of these interaction experiments were used to design and optimize an engineered biofilm reactor capable of TCE degradation and permeability reduction in porous media.

Chapter 4 - Discussion

Discussion of interactions between *K. oxytoca* and *B. cepacia* in planktonic, rotating disk and porous media reactors and how these interactions affect TCE degradation and permeability reduction in porous media.

Chapter 5 - Conclusion

Overview of key conclusions and implications to bioremediation technology.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains

The bacterial isolates used for these experiments were *Burkholderia cepacia* PR1-pTOM$_{31c}$ and *Klebsiella oxytoca*. *B. cepacia* is an aerobic bacterium that can constituatively degrade trichloroethylene (TCE) via a cometabolic process using the toluene ortho-monooxygenase (TOM) pathway (Shields and Reagin, 1992). The genetic information for the TCE degradative pathway is located on the plasmid, TOM$_{31c}$. The plasmid also encodes for the resistance to the antibiotic kanamycin (Shields et al. 1995). This resistance to kanamycin (and *K. oxytoca's* lack of resistance) was used for selection of *B. cepacia* in dual-species cultures. Sharp (1995) 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 its inability to form a thick biofilm, *B. cepacia* was chosen as the reactive, non-mucoid bacterial strain for this experiment.

K. oxytoca is a highly mucoid, facultative anaerobic bacterium. The strain used in this work 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). Its ability to form thick biofilms, and its resistance to the antibiotic streptomycin, made it an ideal candidate for use as the mucoid organism in the dual-species experiments. *K. oxytoca* was identified and quantified in the dual-species cultures through its resistance (and *B. cepacia's* lack of resistance) to streptomycin.

Bacterial Isolation and Characterization

Selective and Non-Selective Plating Techniques

Selective and non-selective nutrient agar plates were used to characterize the dual-species populations. *B. cepacia* was selected on either modified Luria-Bertani (LBG) agar plates (10g 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) amended with 0.05g/L kanamycin (Sigma Chemical Co., St. Louis, MO) added after autoclaving and incubation for 45 minutes in a 55° C water bath or phenol agar plates (15g Bacto agar per liter of hydrocarbon minimal medium (HCMM2) with 94.1 mg/L phenol (J.T. Baker Chemical Co., Phillipsburg, NJ) amended with 0.05g/L kanamycin. HCMM2 media contains 2.84 g of sodium sulfate (Na_2SO_4), 1.37 g ammonium chloride (NH_4Cl), 1.515 g potassium phosphate monobasic (KH_2PO_4), 1.58 g sodium phosphate dibasic (Na_2HPO_4), sodium hydroxide (NaOH) ~ pH 7.2, 0.01125 g calcium chloride ($CaCl_2$) and 0.0967 g magnesium chloride ($MgCl_2$) per liter of nano-pure water. All chemicals for HCMM2 media were purchased from Fisher Scientific (Pittsburgh, PA). *K. oxytoca* was selected

on Brain Heart Infusion (BHI) agar plates (4 g BHI media (Becton Dickinson, Sparks, MD), and 15 g Bacto agar per liter of distilled water) amended with 0.1 g/L filter sterilized streptomycin sulfate (Fisher Scientific, Pittsburgh, PA) added 45 minutes after autoclaving. R2A (Becton Dickinson, Sparks, MD) was used as the non-selective nutrient agar to determine total cell numbers and provide a total cell balance.

Protein Assay

Measuring the weight of biomass protein was another method used to quantify biomass. Total protein was analyzed using the BCA Protein Assay Kit (Pierce, Rockland, IL). The process involves the reduction of Cu^{+2} to Cu^{+1} by protein in the sample, which was then quantified by colorimetric detection of the Cu^{+1} at an absorbance of 562 nm using a reagent containing bicinchoninic acid (BCA). Colorimetric detection was performed using a Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY). The LBG media used in all experiments contained dissolved proteins, which would also be detected by the BCA protein assay. Therefore, the samples were rinsed of any remaining dissolved protein through centrifugation at 7660 x g for 20 minutes, supernatant poured off, and the biomass pellet was resuspended in phosphate buffered saline solution (8.7 g NaCl, 0.4 g KH_2PO_4, 1.23 g K_2HPO_4 per liter distilled water).

Inoculum Preparation

To prepare a viable, TCE-degrading culture of *B. cepacia* for inoculation into the reactors, a loop-full of *B. cepacia* was transferred from a frozen culture (-70°C in 2% peptone (Becton Dickinson, Sparks, MD), 20% glycerin (Fisher Scientific, Pittsburgh,

PA)) to a phenol/kanamycin agar plate and incubated at 30°C for 48 hrs. A colony from the phenol/kanamycin plate was transferred to a LBG/kanamycin agar plate and incubated at 30°C for 24 hours. A colony from the LBG/kanamycin plate was transferred to 100 ml LBG broth (without kanamycin), and incubated for 18 hours 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 hours at 36°C on a horizontal shaker (150 rpm).

K. oxytoca was transferred from a frozen culture (-70°C in 2% peptone, 20% glycerin) and incubated at 30°C for 24 hours on a BHI agar plate amended with streptomycin. A colony was transferred to a 100 ml LBG broth and incubated at 36°C on a horizontal shaker (150 rpm). After 18 hours, 1 ml was transferred to 100 ml fresh LBG broth and incubated for 18 hours at 36°C on a horizontal shaker (150 rpm).

TCE Degradation Ability

To verify *B. cepacia's* ability to degrade TCE, 24-hour TCE disappearance assays were performed. Trichloroethylene (Aldrich Chemical Company, Milwaukee, WI) was diluted in methanol (Fisher Scientific, Pittsburgh, PA) to 75 mg/L. Two mL cell suspensions and 50 μL of 75 mg/L TCE were placed in 20-mm crimp-seal bottles (17 mL total capacity) yielding an aqueous phase TCE concentration of 0.64 mg/L. Controls with 2 mL sterile LBG media were used to measure abiotic TCE loss in the bottles. The bottles were capped with Teflon™-lined rubber stoppers (cat#10144802, West Pharmaceutical Services, Lionville, PA) and crimped before incubation at 36°C (inverted

and shaken at 150 rpm). Headspace samples (200 μL) from each vial were analyzed using a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 2.44 m by 2 mm I.D. glass column packed with 1% SP1000 on 60/80 Carbopak B (Supelco, St. Louis, MO) and a Tracor 700A Hall Electrolytic Conductivity Detector (Finnigan MATT, Austin, TX). The injector and detector temperatures on the gas chromatograph were 200°C and 250°C, respectively. The Hall reactor temperature was 900°C and Hall electrolyte was 1-propanol (Fisher Scientific, Pittsburgh, PA). The oven temperature was initially 150°C for one minute and increased at a rate of 10°C/min to a final temperature of 200°C for two minutes. The carrier gas was helium (29 mL/min) and the reactor gas was H_2 (20 mL/min). Samples were analyzed initially and then again at 24 hours to determine the disappearance of TCE over a 24-hour period. This loss was compared to controls to quantify the extent of TCE degradation for each sample.

Dissoved Organic Carbon

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 um filter. Inorganic carbon was removed by adding two drops of 20% phosphoric acid into two mL of sample to reduce the pH, thus converting any inorganic carbon to CO_2. Subsequent air sparging for six minutes removed the CO_2 from the sample. The system was calibrated using a 10 mg/L carbon standard. For the solutions containing 30, 70, or 700 mg/L DOC initial substrate concentrations, 500, 200

or 20 µl of sample was injected into the carbon analyzer, respectively, to obtain carbon concentrations of approximately 10 mg/L.

Dissolved Oxygen

Dissolved oxygen (D.O.) was measured using an Accumet AP64 Series handheld dissolved oxygen meter (Fisher Scientific, Pittsburgh, PA). The probe was calibrated in water-saturated air at room temperature prior to each use. The D.O. probe measures temperature internally and a barometric pressure typical of the elevation of Bozeman, MT (637 mmHg at 4800 ft) was used for calibration. Zero oxygen concentration calibration was performed by immersing the probe in either a sodium sulfite solution (3 g Na_2SO_3/300 mL water) or water purged with nitrogen gas. With both methods, the oxygen concentration measurement decreased to approximately 0.08 mg/L and then rose to approximately 1.5 mg/L. Therefore, when the dissolved oxygen measurements from a particular reading showed this same trend, its D.O. reading was recorded to be 0.1 mg/L. For the column experiments, the dissolved oxygen meter was fitted to a flow through system made from a 40 ml centrifuge tube with an O-ring. Inlet and outlet ports were tapped in the side. The flow through system was designed to attach to the influent and effluent tubing of the column.

Single and Dual Species Planktonic (Batch) Experiments

For the single-species experiments, 50 µl of growth culture containing either *B. cepacia* or *K. oxytoca* was transferred to 500 ml of a 1:10 or 1:100 diluted LBG broth (70

mg/l and 700 mg/l dissolved organic carbon, respectively). For the dual-species experiments, 50 µl of *B. cepacia* growth culture and 50 µl of *K. oxytoca* growth culture were transferred simultaneously. The number of *B. cepacia* and *K. oxytoca* cells transferred to each flask was 7.3×10^7 +/- 2.1×10^7 colony forming units (CFU) and 1.3×10^8 +/- 4.9×10^7 CFU, respectively. Flasks were incubated at room temperature on a horizontal shaker (150 rpm) and 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. Each experiment was performed in duplicate to ensure repeatability.

Single and Dual Species Biofilm Experiments

Reactor Design

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 for varying substrate concentrations. A rotating disk reactor is a one liter glass beaker containing a magnetically driven rotor with six 1.27 cm diameter biofilm test-surface coupons (Figure 1). A drain spout on the side of the RDR provided a constant volume of 180 ml and a wetted surface area (including stir plate) of 253 cm^2. A complete description of an RDR can be found in Zelver, et al. (1999).

Continuous nutrient addition (different dilutions of LBG media resulting either 30, 70, 700 mg/l DOC) was supplied to the reactors using a peristaltic pump at a rate between 5.4 and 5.7 ml/min resulting in a detention time of 32-34 minutes. Flow rates were established using a graduated cylinder and stopwatch. This particular detention time is approximately half the doubling time of the quickest growing organism (*K. oxytoca*), thus minimizing suspended cell doubling in the reactor. A cell balance on the biofilm and suspended region allows for the biofilm growth rate to be determined. Flow breaks were situated upstream of the reactor to prevent bacterial contamination of the nutrient reservoir.

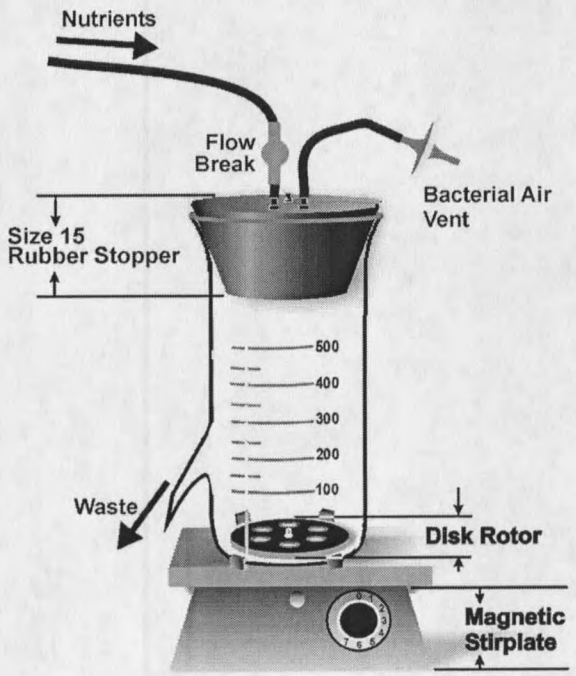

Figure 1 Rotating Disk Reactor.

Reactor Operation

Two ml of concentrated growth culture (1.6×10^{10} +/- 6.4×10^{9} CFU/ml and 9.5×10^{9} +/- 2.4×10^{8} CFU/ml for *K. oxytoca* and *B. cepacia*, respectively) was added to each rotating disk reactor containing 180 ml of media through syringe injection and left in batch mode for one hour to allow the microorganisms to attach initially to the surface. After one hour, nutrient media was pumped through the reactor at the desired flow rate. The system ran for three hours to wash out the high concentration of inoculated cells in suspension before sampling began (therefore, t=0 was four hours after inoculation). Effluent samples from each reactor were taken periodically for five days and were used to determine when steady-state conditions were obtained. After five days of operation, the reactors were taken off-line and destructively sampled to remove the bacteria from the disks using a procedure described by Zelver, et al. (1999). The cells were then homogenized at 13,500 rpm for 30 seconds and then grown on selective agar plates (plated in triplicate). The series of RDR experiments performed included single-species experiments at 70 and 700 mg/L influent DOC substrate concentrations and dual-species experiments at 30, 70, and 700 mg/L influent DOC substrate concentration. Each experiment was repeated to ensure reproducibility.

Porous Media Column Experiments

Reactor Design

The porous media column reactors used in this experiment were custom-made from glass chromaflex columns (Kimble Kontes, Vineland, NJ). The columns were 30

cm long and had an inner diameter of 4.8 cm, yielding an empty volume of 543 ml. The columns were filled with 1mm glass beads (BioSpec Products Inc., Bartlesville, OK). One of the columns had three miniert sampling ports (Alltech, Deerfield, IL) along the length of the column (Figure 2). TCE was fed to the reactors via a 1/4 inch Swaglok tee (Idaho Valve and Fitting, Idaho Falls, ID) from a syringe pump. Another Swaglok tee is located between the TCE injection point and the influent of the column, which serves as an influent sampling port and bacterial injection point. Between the TCE injection point and influent sampling point is an in-line mixing chamber constructed from a glass flow break with a stir bar inside. Teflon tubing (0.64 cm) (Cole-Parmer Instrument Company, Niles, IL) was attached to both ends of the flow break and connected to the Swagelok tees.

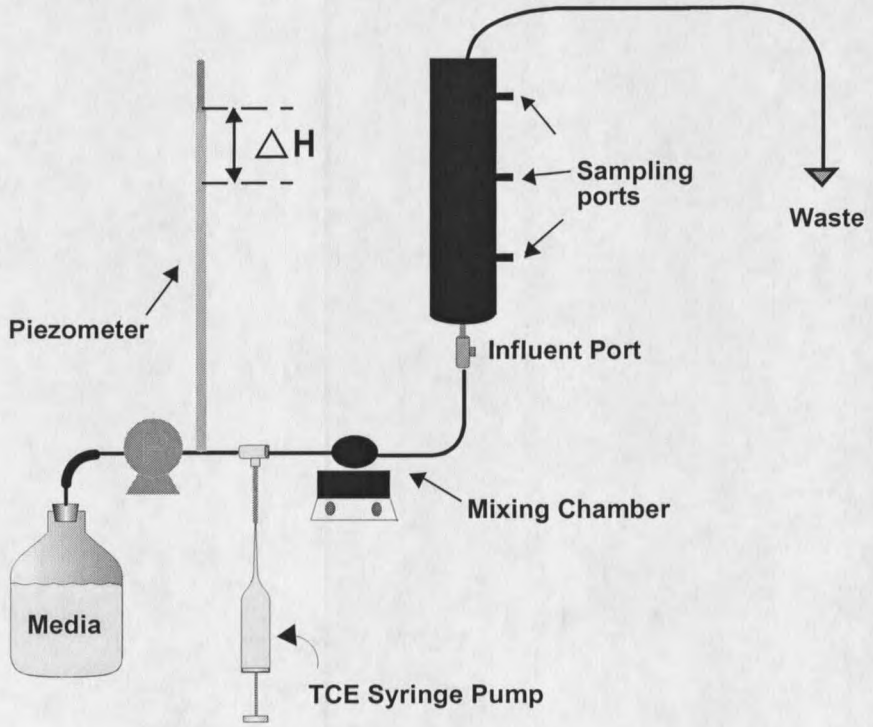

Figure 2 Schematic of Porous Media Column with Sampling Ports.

Reactor Preparation

Prior to each experiment, the column was rinsed with ethanol. Beads were autoclaved, rinsed with deionized (DI) water to remove any remaining nutrients and then re-autoclaved. Beads were then poured into the column along with autoclaved D.I. water so that the column was completely saturated. All tubing was rinsed and autoclaved. Tubing was attached to the column and 1 liter of a 10% bleach solution was pumped through the column for ~ 3 hrs followed by 1 liter of sodium thiosulfate (Fisher Scientific, Pittsburgh, PA) for ~ 3 hrs. Eight to ten liters of autoclaved D.I. water was then rinsed through the column for ~24 hrs. R2A counts from the effluent of the column before the experiment began were $<10^3$ CFU/mL.

Fluorescein Breakthrough Curves

Fluorescein breakthrough curves were obtained before bacteria addition to quantify the hydrodynamics of the reactor and measure any losses of a conservative tracer. A Hamilton gas-tight 10ml syringe (cat # 1010, Fisher Scientific, Fair Lawn, NJ) filled with 1290 mg/L (0.3mM) fluorescein (Sigma Chemical Company, St. Louis, MO) was continuously pumped (0.1 ml/hr) from a Kd Scientific syringe pump (Fisher Scientific) and allowed to mix with the main flow line (Q=2.4 ml/min). Column influent and effluent samples were analyzed by measuring the absorbance using a Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY) at a wavelength of 490nm.

Abiotic TCE Breakthrough Curves

A nine-hour TCE breakthrough curve was performed before bacteria were added to the column to measure any abiotic losses in the system. Water saturated with TCE (TCE solubility = 1100 mg/L @ 25°C) was continuously pumped (0.16 ml/hr) from the syringe pump and allowed to mix with fluid in the nutrient flow line (Q = 2.4ml/min) resulting in a TCE concentration to the column of approximately 1.2 mg/L. The syringe pump was turned on for nine hours and TCE concentrations from the influent and effluent were measured every 10-20 minutes until steady-state condition were reached and then every 70-90 minutes during steady-state. TCE concentrations from the sampling ports were monitored periodically. A 1 ml gas-tight syringe (1MR-GT, SGE Chromatography Supplies, Austin, TX) with an 11.5 cm long .22 gage needle (NO961, SGE Chromatography Supplies, Austin, TX) was used to obtain aqueous samples from inside the column at the sampling ports.

Reactor Operation

Before the bacteria were inoculated into the column, effluent dissolved oxygen concentrations, influent and effluent dissolved organic carbon concentrations and hydraulic head measurements across the column were obtained. In addition, selective plating and absorbance techniques were used to verify the effectiveness of the reactor preparation procedure.

At the beginning of the experiment, 50 ml of each organism's growth culture was injected into the column. The mass of *B. cepacia* and *K. oxytoca* inoculated into each column was 5.7×10^{10} +/- 8.7×10^{9} CFU and 1.6×10^{11} +/- 3.4×10^{10} CFU, respectively.

Different dilutions of LBG media, resulting in 30, 70 or 700 mg/L initial DOC, were pumped through the system at a rate of 2.4 ml/min for 70 hours. Effluent samples were periodically taken to monitor dissolved oxygen concentrations, dissolved organic carbon concentrations and population densities over time.

Destructive Sampling (before TCE)

After 70 hours, the column was taken off-line and approximately five grams of beads were removed from the influent and effluent ends of the column and put in separate test tubes. Fifteen mL of phosphate buffered saline solution was added incrementally to each tube (5 mL, 5 mL, 3 mL, 2 mL). After each addition, the test tube was vortexed for one minute to detach the bacteria from the beads and the supernatant was poured off. Microscopic analysis revealed that this "bead bashing" procedure removed virtually all of the biofilm from the beads. The resulting supernatants were homogenized and plated in triplicate to determine population dynamics throughout the column.

Biotic TCE Breakthrough Curve

After 70 hours of nutrient flow to the column, the column was taken off-line and a small amount of beads were removed from the influent and effluent of the column as described above. The column was put back on-line and three pore volumes of nutrients were run through the column before the TCE experiment began. Water saturated with TCE (TCE solubility = 1100 mg/L @ 25°C) was continuously pumped (0.16 ml/hr) from the syringe pump into the column resulting in a TCE concentration to the column of approximately 1.2 mg/L. The syringe pump was turned on for nine hours and TCE

concentrations from the influent and effluent were measured every 10-20 minutes until steady-state conditions were reached and then every 70-90 minutes during steady-state. TCE concentrations from the sampling ports were monitored periodically.

Destructive Sampling (after TCE)

At the end of the TCE experiment, the column was taken off-line, drained and approximately five grams of beads were removed from the influent, middle and effluent of the column and put in separate test tubes. Fifteen mL of phosphate buffered saline solution was added incrementally to each tube (5 mL, 5 mL, 3 mL, 2 mL). After each addition, the test tubes were vortexed for one minute to detach the bacteria from the beads and the supernatant was poured off. The resulting supernatants were homogenized and plated in triplicate to determine population dynamics throughout the column.

TCE Analysis

A half ml of liquid was extracted from a sampling port and combined with a half mL of hexane (Fisher Scientific, Pittsburgh, PA) in a 2 ml glass vial (cat # 337511AA, Fisher Scientific, Pittsburgh, PA) and closed with a Teflon/silicone/Teflon screw cap (cat #0337529A, Fisher Scientific, Pittsburgh, PA). The vial was shaken at 150 rpm for 10 minutes and 2 µl of the hexane phase was injected into the Hewlett Packard 5890 Series II gas chromatograph with a Tracor 700A Hall Electrolytic Conductivity Detector that was previously described in this chapter.

Specific TCE Degradation Rate

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

Eq. 1 $$k = \frac{(TCE_{in} - TCE_{out}) * Q}{X_b * W_b}$$

where k is the specific TCE degradation rate (mass TCE/time/biomass) and Q is flow rate (L^3/t) through the porous media column. The total weight (W_b) of 1mm beads in a packed column was 771 grams. X_b is the bacterial population of the TCE-degrading organism (*B. cepacia*) desorbed from the glass beads collected at the end of the TCE breakthrough curve. This compares with literature values of specific TCE degradation rates that are in terms of mass of TCE degrading bacteria and not population of TCE degrading bacteria. The literature approach was not feasible because methods to quantify biomass in a dual-species culture, such as measuring particulate protein or carbon concentrations, cannot distinguish between each organism. Therefore, selective plating techniques were used to differentiate the two organisms. To compare single- and dual-species TCE degradation rates to literature values, *B. cepacia's* densities were converted from a population basis (CFU) to a weight basis (gram biomass) using a standard curve that compared plate counts to protein concentrations for *B. cepacia* grown in a batch culture with LBG media at different population densities (Figure 3).

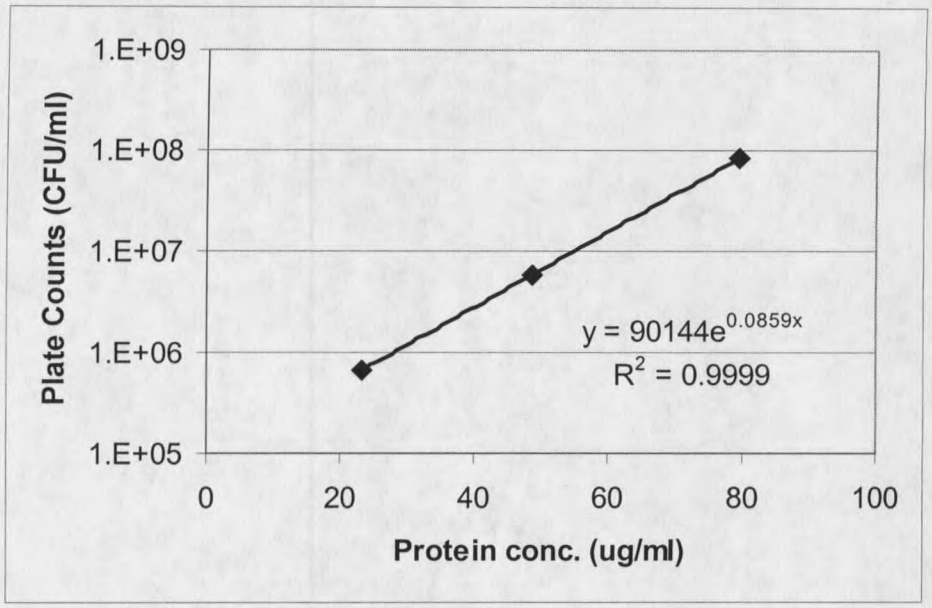

Figure 3 Standard curve of plate counts vs protein concentration for *B. cepacia* at different population densities grown on LBG media.

Growth Rate Determination

Batch Growth

In a batch reactor, bacterial growth for a population of cells can be divided into lag, exponential, stationary, and death phases (Madigan et al. 1997). Lag phase can be described as an acclimation period when a bacterial culture is introduced to fresh media and it is dependent on the history of the bacterial culture and growth conditions. Exponential phase of growth is the period where the cell number doubles at a constant rate. Stationary phase of growth occurs when an essential nutrient for growth is used up or a waste product of the organism builds up to an inhibitory level causing exponential growth to cease. In stationary growth there is no net increase or decrease in growth. Death phase occurs when population decay becomes greater than population growth.

Planktonic (Batch) Growth Rate

Bacterial cell growth can be defined by equation 2 (Madigan et al. 1997).

Eq. 2 $$\frac{dX}{dt} = \mu X$$

Where: X = cell density (biomass/volume)

μ = instantaneous growth rate constant (1/time)

t = time

Integrating equation 2 yields a description of bacterial cell growth in a batch reactor (Equation 3).

Eq. 3 $$X = X_0 e^{\mu t}$$

Where: X_0 = cell density at the beginning of exponential growth

Planktonic growth rates were calculated by solving equation 3 for μ 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.

Doubling Time

The interval for one cell to form into two is called a generation and the time required for this to occur is called the generation time or doubling time. Cell doubling time can be obtained by rearranging equation 3 to solve for time resulting in equation 4.

Eq 4. $$t_{doubling} = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$$

Where: $t_{doubling}$ = time for cell to double (time)

Biofilm Growth Rate. Biofilm growth rates were obtained by performing a planktonic cell mass balance on the suspended region of the rotating disk reactor (eq. 5a) and a biofilm mass balance (eq. 5b) on the total surface area of the rotating disk reactor and porous media column. This method had previously been used to measure specific biofilm growth rates in a RotoTourque reactor (Van Der Wende et al. 1989).

Eq. 5a: $$V\frac{dX_1}{dt} = Q(X_0 - X_1) + \mu_p X_1 V + r_d X_b A$$

Eq. 5b: $$A\frac{dX_b}{dt} = \mu_b X_b A - r_d X_b A$$

Where:

V = volume of fluid (L^3)

X_1 = Effluent (planktonic) cell density (CFU/L^3)

Q = flow rate (L^3/t)

X_0 = influent cell concentration (CFU/L^3)

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)

μ_b = biofilm growth rate (1/t)

Running the reactor to steady-state allows the biomass cell balance (eq. 5b) to be simplified to biofilm growth rate equaling biofilm detachment rate (eq. 6).

Eq. 6 $$\mu_b = r_d$$

Steady-state conditions combined with no cells added, allows equation 5a to be combined with equation 6 to yield a solution for the biofilm growth rate (Eq. 7).

Eq. 7 $$\mu_b = \frac{1}{A} \times \frac{X_1}{X_b}(Q - \mu_p V)$$

Hydrualic Conductivity

The hydraulic conductivity (K) of the column was monitored over time to quantify the effects of substrate concentration on permeability reduction using Darcy's Law (eq. 8):

Eq. 8 $$K = -\frac{Q}{A}\left(\frac{\Delta L}{\Delta H}\right)$$

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

Statistics

Comparison between data sets was done using a two sample t-test to provide p-values to accept or reject the null hypothesis that the true means between two sample groups are equal. The level of significance was defined as 0.05. Data sets are considered significantly different if the p-value was lower than 0.05. All p-value calculations were performed using statistical software, Minitab 13.20 (Minitab Inc., State College, PA).

CHAPTER 3

RESULTS

Single- and dual-species batch, rotating disk and porous media reactor experiments were performed to better understand the interactions between two microbial species when grown together in an engineered biofilm. The two organisms used throughout these experiments were *Burkholderia cepacia* PR1-pTOM$_{31c}$ and *Klebsiella oxytoca*. The main goal of these experiments was to determine which variables influenced the creation and control of a dual-species biofilm. The feasibility of using batch single- and dual-species growth kinetics to describe dual-species interactions in biofilms grown in a well-mixed reactor and in porous media was also examined.

In addition, the effects of this dual-species interaction on cell activity (trichloroethylene (TCE) degradation or permeability reduction) for different substrate concentrations were examined.

Planktonic (Batch) Experiments

A series of batch reactor experiments were performed to examine the behavior of *K. oxytoca* and *B. cepacia* growth in single- and dual-species planktonic cultures. These experiments determined which organism had the higher growth rate and if growth rate determined population dominance in batch cultures. In addition, the effect of substrate concentration on growth rate and population density of these two organisms in single-

and dual-cultures was determined. The batch reactors consisted of one liter flasks filled with 500mL of different dilutions of LBG media resulting in an initial concentration of either 70 mg/L or 700 mg/L dissolved organic carbon (DOC). The 70 mg/L DOC substrate concentration media contained 1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl and 0.1 g dextrose (glucose) per liter of distilled water. The 700 mg/L DOC media used the same ingredients, but at an order of magnitude higher concentration. The single-species batch reactors were inoculated with 50 μL of either *K. oxytoca* or *B. cepacia* growth culture. Dual-species batch reactors were inoculated simultaneously with 50 μL of *K. oxytoca* and 50 μL of *B. cepacia* growth culture. The mass of *K. oxytoca* and *B. cepacia* cells inoculated into each reactor was $1.3 \times 10^8 \pm 4.9 \times 10^7$ CFU and $7.3 \times 10^7 \pm 2.1 \times 10^7$ CFU, respectively. Selective plating techniques were used to monitor bacterial population densities over time and DOC concentrations were monitored to measure substrate utilization over time. Single and dual-species growth curves were compared for each substrate concentration. These results, along with DOC analysis, were used to describe the single- and dual-species population distributions.

Single-Species Results

Population Densities. Samples were removed from the batch reactors and analyzed using selective plating techniques to monitor single-species *K. oxytoca* and *B. cepacia* planktonic populations over time for the 70 mg/L dissolved organic carbon concentration (Figure 4). A solid line defines the exponential growth phase for each organism. The initial population density for the *K. oxytoca* batch experiment was 2.7×10^5 CFU/mL. *K. oxytoca* experienced a three hour lag period before entering exponential

(log) growth phase. The *K. oxytoca* population density started to level off and appeared to enter stationary phase after approximately eight hours of exponential growth. The stationary phase population density of *K. oxytoca* was 1.2 x 10^8 CFU/mL.

The initial population density for the *B. cepacia* batch experiments was 1.1 x 10^5 CFU/mL. *B. cepacia* remained in lag phase for six hours, which was twice as long as *K. oxytoca's* lag phase. Exponential growth lasted for approximately 17 hours before stationary phase began. The stationary phase population density of *B. cepacia* was 6.7 x 10^7 CFU/mL, which was slightly lower than *K. oxytoca* at the same substrate concentration.

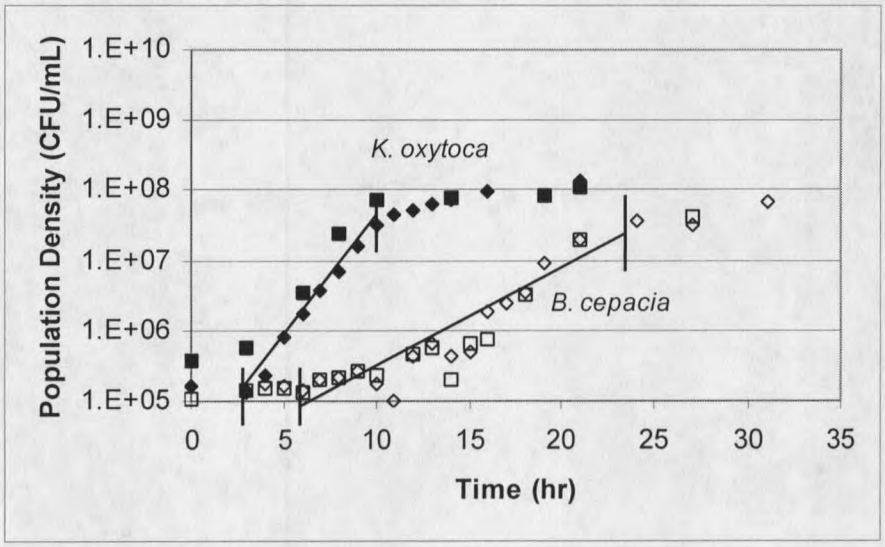

Figure 4. Single-species *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) population density over time for the 70 mg/L dissolved organic carbon batch experiments. Solid line indicates exponential growth phase.

Another set of single-species batch experiments used a diluted LBG media containing 700 mg/L dissolved organic carbon (Figure 5). A solid line defines the exponential growth phase for each organism. The initial population density for the *K.*

oxytoca batch experiments was 1.6 x 10^5 CFU/mL. *K. oxytoca* experienced a three hour lag period before entering exponential (log) growth phase. The *K. oxytoca* population density started to level off and appeared to enter stationary phase after approximately ten hours of exponential growth. The stationary phase population density of *K. oxytoca* was 1.1 x 10^9 CFU/mL. The *K. oxytoca* stationary phase population densities for the 700 mg/L carbon substrate concentration were an order of magnitude higher than that at the 70 mg/L DOC substrate concentration.

The initial population density for the *B. cepacia* batch experiments was 1.1 x 10^5 CFU/mL. *B. cepacia* remained in lag phase for six hours, which was once again twice as long as *K. oxytoca's* lag phase. Exponential growth lasted for approximately 22 hours before stationary phase began. The final time point for *B. cepacia* in Figure 5 is at 30 hours, which is just when *B. cepacia* started to enter stationary phase. A separate batch experiment analyzed the *B. cepacia* population density over a longer period of time. After 48 hours of growth, *B. cepacia's* stationary phase population density was 6.8 x 10^8 CFU/mL. The stationary phase *B. cepacia* population densities were slightly lower than *K. oxytoca* for the 700 mg/L DOC substrate concentration. The *B. cepacia* stationary phase population densities were an order of magnitude higher in the 700 mg/L DOC experiments compared to the 70 mg/L DOC experiments.

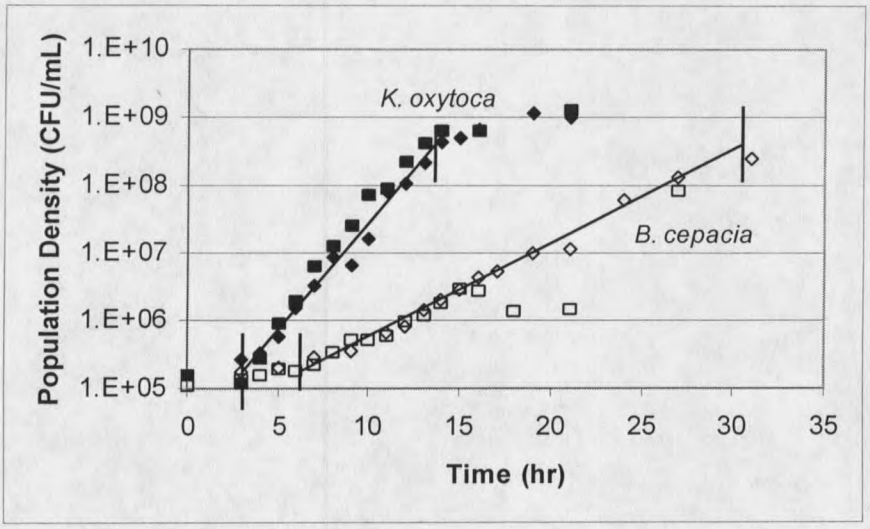

Figure 5. Single-species *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) population density over time for the 700 mg/L dissolved organic carbon batch experiments. Solid line indicates exponential growth phase.

Specific Growth Rate. A single-species growth rate was obtained for each organism from the graphs of population density over time for both initial substrate concentrations (70 mg/L and 700 mg/L dissolved organic carbon) (Table 1). As described in Chapter 2, a growth rate was calculated using linear regression of the log phase of growth (eq. 3).

K. oxytoca's growth rate at the 70 mg/L and 700 mg/L DOC initial substrate concentrations was 0.76/hr and 0.76/hr, respectively. These growth rates were similar to the batch growth rate reported for *Klebsiella pneumonia* KP1 (0.84/hr +/- 0.23/hr) grown in a media containing 100 mg/L glucose (Stewart et al. 1997). *K. oxytoca's* growth rate at the 70 and 700 mg/L DOC substrate concentration was statistically greater (*p-value* < *0.010*) and more than twice that of *B. cepacia's* (0.30/hr and 0.30/hr, respectively). 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 (70 and 700 mg/L DOC) (Table 1). This implies that the growth rate calculated for each organism for both substrate concentrations is the maximum growth rate ($\mu=\mu_{max}$).

Table 1. Single-species planktonic growth rates (μ) for *K. oxytoca* and *B. cepacia* at 70 mg/L and 700 mg/L dissolved organic carbon substrate concentrations. Values are an average of duplicate experiments and errors represent +/- the standard deviation.

	μ (hr^{-1})	
mg/L (carbon)	70	700
K. oxytoca	0.76 ± 0.06	0.76 ± 0.05
B. cepacia	0.30 ± 0.03	0.30 ± 0.01

Dissolved Organic Carbon. Dissolved organic carbon (DOC) concentrations were monitored over time to quantify substrate utilization in the single-species batch reactors.

A graph of DOC utilization over time for a "low" initial substrate concentration (described above as 70 mg/L DOC) batch experiment shows that the rate of DOC utilization was greater by the faster growing *K. oxytoca* at the beginning of the experiment compared to *B. cepacia*, but both organisms had comparable amounts of DOC remaining in the batch reactor after 30 hours (Figure 6). After 30 hours of growth, a significant amount of DOC (70% and 60% for *K. oxytoca* and *B. cepacia*, respectively) remained in the batch reactors even though both systems entered stationary phase of growth.

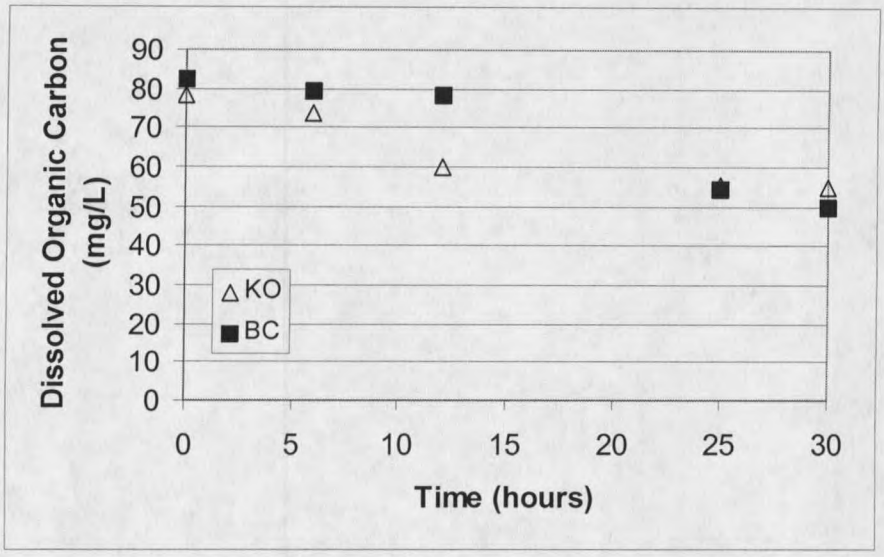

Figure 6. Dissolved organic carbon (DOC) concentrations over time for single-species *K. oxytoca* and *B. cepacia* at the 70 mg/L DOC initial substrate concentration batch experiments.

A graph of DOC concentration over time for the "high" initial substrate concentration (described above as 700 mg/L DOC) is presented in Figure 7. Similar to the 70 mg/L DOC experiment, the rate of DOC utilization by the faster growing *K. oxytoca* was greater at the beginning of the experiment compared to *B. cepacia*. The amount of DOC remaining in the batch reactor after 30 hours, however, was similar for each organism. A significant amount of DOC (60% and 51% for *K. oxytoca* and *B. cepacia*, respectively) remained in the batch reactors even though both systems entered stationary phase of growth.

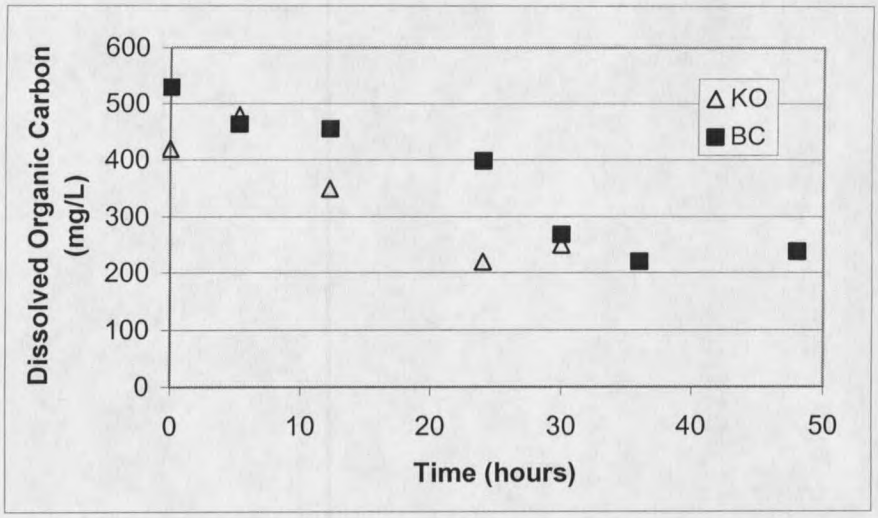

Figure 7. Dissolved organic carbon (DOC) concentrations over time for single-species *K. oxytoca* and *B. cepacia* at the 700 mg/L DOC initial substrate concentration batch experiments.

Dual-Species Results

Dual-species batch experiments were performed to determine how *B. cepacia* and *K. oxytoca* would interact and also to observe the effect substrate concentration had on growth rate and population distribution.

Population Densities. Both organisms were inoculated simultaneously into a single batch reactor with a diluted LBG media containing either 70 mg/L or 700 mg/L DOC and population density was monitored over time using selective plating techniques.

The results of the 70 mg/L DOC substrate concentration dual-species experiment with regard to the *K. oxytoca* population density (Figure 8) was similar to that observed in the single-species 70 mg/L DOC experiments. *K. oxytoca* entered exponential growth phase after approximately three hours and remained in exponential growth phase for approximately nine hours. The stationary phase population density for *K. oxytoca* in

dual-species culture (1.6 x 10^8 CFU/mL) was similar to that observed in the single-species 70 mg/L DOC experiment.

The *B. cepacia* growth curve for the dual-species 70mg/L DOC experiment (Figure 8) was different than the single-species observations. *B. cepacia* in a dual-species culture entered exponential growth at approximately the same time as in the single-species experiments, but remained in exponential growth for a much shorter period of time (approximately 10 hours) compared to single-species experiments. This resulted in a stationary phase population density (1.6 x 10^6 CFU/mL) almost two orders of magnitude lower than *B. cepacia's* population density when grown alone (single species).

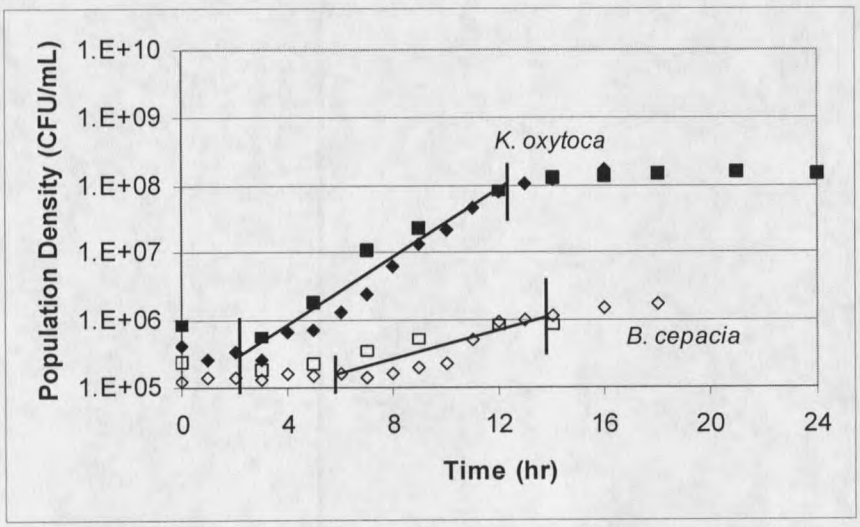

Figure 8. Dual-species *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) population density over time for the 70 mg/L dissolved organic carbon batch experiments. Solid line indicates exponential growth phase.

The growth curves obtained from the dual-species 700 mg/L batch experiments (Figure 9) were similar to those from the 70 mg/L batch experiments. *K. oxytoca* entered exponential growth after approximately four hours and remained there for approximately

10 hours before entering stationary phase. The stationary phase population density of *K. oxytoca* at the 700 mg/L DOC concentration (1.1 x 10^9 CFU/mL) was an order of magnitude higher than the dual-species *K. oxytoca* stationary phase concentration at 70 mg/L DOC.

For the 700 mg/L DOC dual-species batch experiment (Figure 9), *B. cepacia* entered exponential growth at approximately the same time as the 70 mg/L DOC batch experiment (six hours) and remained in exponential growth phase for approximately 10 hours before entering stationary phase. The stationary phase population density for *B. cepacia* at 700 mg/L (3.8 x 10^6 CFU/mL) was similar to the dual-species stationary *B. cepacia* population density at 70 mg/L (1.6 x 10^6 CFU/mL). The dual-species *B. cepacia* stationary phase population densities were two to three orders of magnitude less than *B. cepacia*'s single-species stationary phase population densities.

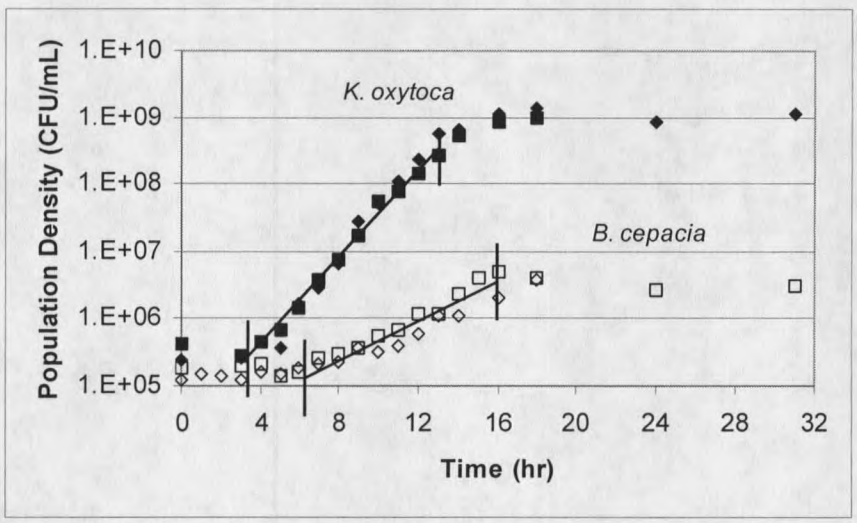

Figure 9. Dual-species *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) population density over time for the 700 mg/L dissolved organic carbon batch experiments. Solid line indicates exponential growth phase.

Specific Growth Rate. Dual-species growth rates were obtained for each organism from the graphs of population density over time for both substrate concentrations (70 mg/L and 700 mg/L dissolved organic carbon) (Table 2). Growth rates were calculated using linear regressions of data from the log phase of growth.

Similar to the single-species batch experiments, *K. oxytoca's* growth rate at the 70 mg/L and 700 mg/L DOC substrate concentrations (0.68/hr and 0.81/hr, respectively) was statistically (*p-value* < *0.040*) higher and more than twice that of *B. cepacia's* (0.28/hr and 0.29/hr, respectively). Also, varying substrate concentration in the dual-species cultures did not significantly change the organism's growth rate suggesting zero-order growth rate kinetics for these two substrate concentrations (70 and 700 mg/L) (Table 2).

Table 2. Dual-species planktonic growth rates (μ) for *K. oxytoca* and *B. cepacia* at 70 mg/L and 700 mg/L dissolved organic carbon substrate concentrations. Values are an average of duplicate experiments and errors are +/- the standard deviation.

	μ (hr^{-1})	
mg/L (carbon)	70	700
K. oxytoca	0.68 ± 0.03	0.81 ± 0.05
B. cepacia	0.28 ± 0.07	0.29 ± 0.04

Dissolved Organic Carbon. Dissolved organic carbon (DOC) was monitored over time to quantify substrate utilization in the dual-species batch reactor. DOC concentrations over time for the dual-species "low" substrate concentration (described above as 70 mg/L DOC) experiments are presented in Figure 10 along with the single species *K. oxytoca* and *B. cepacia* data presented earlier (Figure 6). All three batch experiments (single-species *K. oxytoca*, single *B. cepacia*, and dual-species) had a similar

initial DOC concentration (77.7 mg/L +/- 4.7 mg/L). The dual-species batch reactor utilized DOC at approximately the same rate at the beginning of the experiment as the single-species *K. oxytoca* batch experiment, which was significantly faster than the single-species *B. cepacia* experiments. All three systems had similar DOC concentrations after 30 hours (52.5 mg/L +/- 2.6 mg/L).

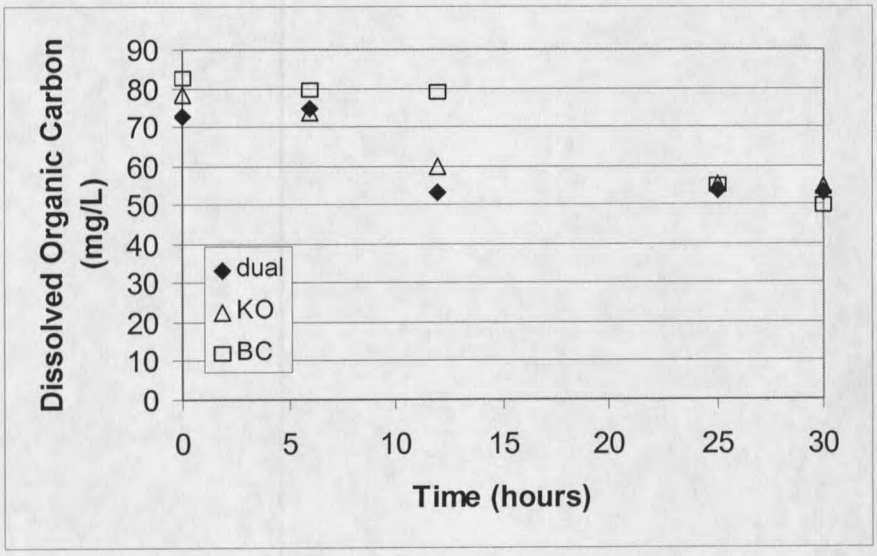

Figure 10. Dissolved organic carbon concentrations over time for the single species *K. oxytoca*, single-species *B. cepacia*, and dual-species inoculation scenarios at the 70 mg/L DOC substrate concentration batch experiments.

DOC concentrations over time for the dual-species "high" substrate concentration (described above as 700 mg/L DOC) experiments are presented in Figure 11 along with the single species *K. oxytoca* and *B. cepacia* data presented earlier (Figure 7). All three batch experiments (single-species *K. oxytoca*, single *B. cepacia*, and dual-species) had a similar initial DOC concentration (489 mg/L +/- 60.3 mg/L). The dual-species batch reactor utilized DOC at the beginning of the experiment at approximately the same rate as the single-species *K. oxytoca* batch experiment, which was faster than the single-species

B. cepacia experiments. All three systems had similar DOC concentrations after 30 hours (271 mg/L +/- 31.8 mg/L). Similar to the single-species batch experiments, the dual-species batch experiments did not completely utilize all the DOC available, even though each organism entered stationary phase of growth.

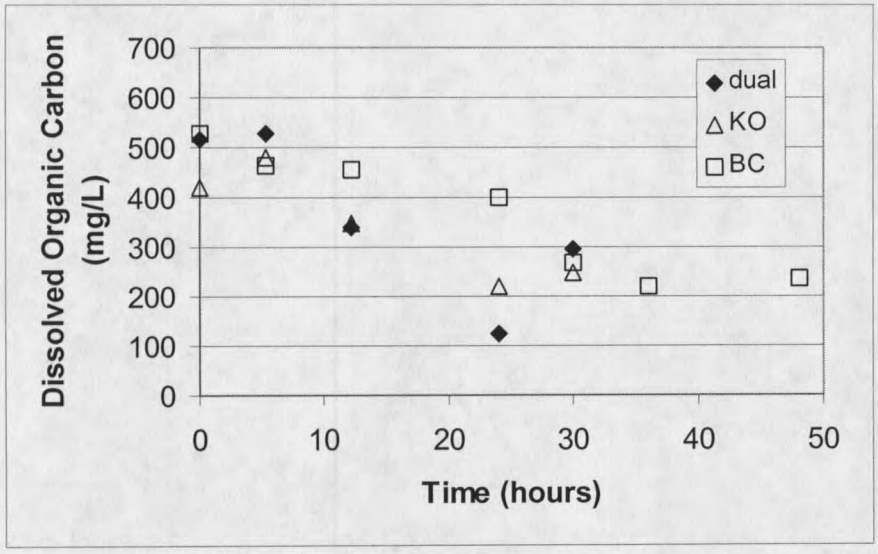

Figure 11. Dissolved organic carbon concentrations over time for the single species *K. oxytoca*, single-species *B. cepacia*, and dual-species inoculation scenarios at the 700 mg/L DOC substrate concentration batch experiments.

Rotating Disk Reactor (Biofilm) Experiments

Biofilm growth rate experiments were performed using rotating disk reactors (RDR) (Figure 1) to determine if *K. oxytoca* had a higher biofilm growth rate than *B. cepacia* and if the faster growing organism would dominate the dual-species biofilm. In addition, the effects of substrate concentration on single- and dual-species growth rates and population densities of *B. cepacia* and *K. oxytoca* biofilms were examined. As discussed in the Materials and Methods section, the RDR facilitates the development of

biofilm on removable coupons under well-mixed conditions. Substrate concentrations of 70 and 700 mg/L dissolved organic carbon (DOC) were used with the single-species inoculum and substrate concentrations of 30, 70 and 700 mg/L DOC were used with the dual-species inoculum. Single- and dual-species biofilm growth rates and population densities were compared for the different substrate concentrations and to the batch reactor results.

Single-Species Results

Effluent Population Density. After the bacteria were inoculated into the rotating disk reactor, the effluent population densities were monitored over time using selective plating techniques. The effluent plate count data are presented in Figures 12 and 13. In a chemostat, the effluent concentration is equal to the suspended cell concentration in the reactor. Therefore, the effluent plate counts are equivalent to detached suspended densities. Measures were taken to minimize suspended cell growth by setting the detention time of the fluid in the completely mixed reactor significantly less than the planktonic growth rate of the faster growing organism. Using the planktonic growth rate values obtained from the batch experiments, the cell doubling time of the faster growing organism was calculated using equation 4 to be 0.91 hours. The detention time of the rotating disk reactor was 0.55 hours.

The effluent plate counts for the 70 mg/L DOC substrate concentration indicate steady-state conditions occurred after approximately 50 hours for both organisms (Figure 12). After five days of operation, the single-species effluent population density of *K.*

oxytoca (3.2 x 10^7 CFU/mL) was higher and statistically different (*p-value* < *0.020*) than *B. cepacia's* effluent population density (1.2 x 10^7 CFU/mL).

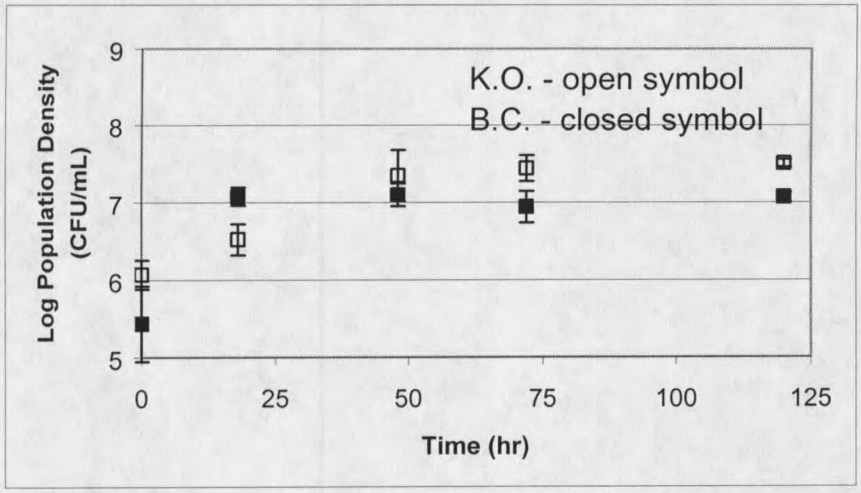

Figure 12. Single-species effluent population densities for *K. oxytoca* (open symbols) and *B. cepacia* (closed symbols) for the 70 mg/L DOC substrate concentration rotating disk reactor experiments. Values are average of duplicate experiments and error bars are +/- standard deviation.

The effluent plate counts for the 700 mg/L DOC substrate concentration also indicate that steady-state conditions occurred after approximately 50 hours for both organisms (Figure 13). After five days of operation, the steady-state effluent population density of *K. oxytoca* (9.1 x 10^7 CFU/mL) was slightly higher than *B. cepacia's* effluent population density (2.9 x 10^7 CFU/mL).

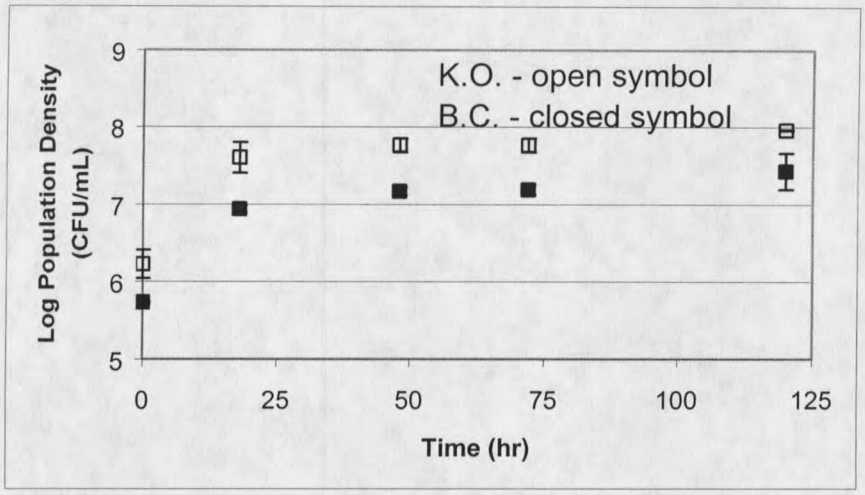

Figure 13. Single-species effluent population densities for *K. oxytoca* (open symbols) and *B. cepacia* (closed symbols) for the 700 mg/L DOC substrate concentration rotating disk reactor experiments. Values are average of duplicate experiments and error bars are +/- standard deviation.

Increasing the substrate concentration by an order of magnitude resulted in a three-fold increase in the *K. oxytoca* steady-state population density and a slight increase in the *B. cepacia* steady-state population density (Table 3).

Table 3. Single-species planktonic population densities (X_1) after five days of rotating disk reactor operation. Values are average of duplicate experiments and errors are +/- the standard deviation.

	X_1 (CFU/mL) x 10^7	
	70 mg/L DOC	700 mg/L DOC
K. oxytoca	3.21 +/- 0.37	9.13 +/-0.14
B. cepacia	1.17 +/- 0.17	2.88 +/- 1.48

Biofilm Population Density. After 5 days of constant nutrient addition, the rotating disk reactors (RDR) were taken apart and the biofilm scraped from the removable disks, homogenized and the biofilm density was quantified using selective plating techniques.

The steady-state biofilm population density of *K. oxytoca* (3.05 x 10^8 CFU/cm^2) was statistically greater (*p-value* < *0.010*) than *B. cepacia's* biofilm population density (6.9 x 10^7 CFU/cm^2) at the 70 mg/L DOC substrate concentration (Table 4). Both organisms had comparable biofilm population densities at the 700 mg/L DOC substrate concentration.

An order of magnitude increase in the substrate concentration (70 mg/L to 700 mg/L DOC) resulted in a single-species *K. oxytoca* steady-state biofilm population (1.6 x 10^8 CFU/cm^2) that was half the *K. oxytoca* single-species, steady-state population at the 70 mg/L DOC substrate concentration (3.1 x 10^8 CFU/cm^2) (Table 4). Increasing the substrate concentration by an order of magnitude resulted in the *B. cepacia* single species steady-state population to double from 6.9 x 10^7 CFU/cm^2 to 1.5 x 10^8 CFU/cm^2. All single-species steady-state biofilm population densities were within a half order of magnitude of each other, regardless of substrate concentration.

Table 4. Single-species biofilm population densities (X_1) after five days of rotating disk reactor operation. Values are average of duplicate experiments and errors are +/- the standard deviation.

	X_b (CFU/cm^2) x 10^8	
	70 mg/L DOC	700 mg/L DOC
K. oxytoca	3.05 +/- 0.19	1.60 +/- 0.22
B. cepacia	0.69 +/- 0.09	1.53 +/- 0.18

Suspended vs Biofilm Populations. The total suspended population in the rotating disk reactor at steady-state (X_1 x V) was compared with the total biofilm population in the rotating disk reactor at steady-state (X_b x A) (Table 5). Biofilm biomass was approximately 90% of the total biomass for the 70 mg/L DOC substrate concentration

and between 70% and 90% of the total biomass for the 700 mg/L DOC substrate concentration.

Table 5. Comparison of total suspended and total biofilm biomass in the single-species rotating disk reactor experiments for two substrate concentrations (70 and 700 mg/L DOC). Values are average of duplicate experiments +/- the standard deviation.

	70 mg/L DOC		700 mg/L DOC	
	Suspended Biomass (CFU x 10^{10})	Biofilm Biomass (CFU x 10^{10})	Suspended Biomass (CFU x 10^{10})	Biofilm Biomass (CFU x 10^{10})
K. oxytoca	0.58 +/-0.07	7.73 +/- 0.49	1.64 +/- 0.03	4.05 +/- 0.56
B. cepacia	0.21 +/- 0.03	1.75 +/- 0.23	0.52 +/- 0.27	3.87 +/- 0.44

Growth Rate Analysis. Biofilm growth rates were determined by inputting the steady-state effluent (Table 3) and biofilm population densities (Table 4), as well as the planktonic growth rates (Table 1), into the biofilm growth rate equation (eq. 7). The single-species biofilm growth rates are presented in Table 6. Results show a significant increase in growth rate from 0.08/hr to 0.45/hr with an order of magnitude increase in substrate concentration (70 mg/L to 700 mg/L DOC) for *K. oxytoca* (*p-value* < *0.015*). *B. cepacia's* biofilm growth rate did not change with change in substrate concentration. *K. oxytoca's* single-species' growth rate did not change when the substrate concentration was increased in the planktonic (batch) analysis but increased significantly when the substrate concentration was increased in the single-species biofilm experiments. *K. oxytoca's* single-species growth rate is comparable to *B. cepacia's* at the 700 mg/L DOC substrate concentration. Unlike the batch experiments, *K. oxytoca's* growth rate is lower and statistically different than *B. cepacia's* at the 70 mg/L DOC substrate concentration (*p-value* < *0.015*).

Table 6. Single-species biofilm growth rates (μ). Values are average of duplicate experiments +/- the standard deviation.

	μ (hr^{-1})	
DOC (mg/L)	70	700
K. oxytoca	0.08 ± 0.01	0.45 ± 0.06
B. cepacia	0.18 ± 0.002	0.20 ± 0.09

Dissolved Organic Carbon. Effluent dissolved organic carbon (DOC) concentrations were monitored over time to quantify substrate utilization in the single-species rotating disk reactors (Table 7). The amount of DOC consumed (and percent removed) during the 35 minute detention time of the rotating disk reactor was calculated by assuming the effluent DOC concentration at time zero was equal to the steady-state influent DOC concentration. The initial effluent DOC concentrations for the "low" substrate experiment (defined throughout as 70 mg/L DOC) were 74.0 mg/L and 73.8 mg/L for *K. oxytoca* and *B. cepacia*, respectively. Both *K. oxytoca* and *B. cepacia* single-species experiments had similar steady-state effluent DOC concentrations (56.9 and 60.0 mg/L DOC, respectively).

The initial DOC concentrations for *K. oxytoca* and *B. cepacia* at the "high" substrate concentration experiments (defined throughout as 700 mg/L DOC) were 634 mg/L and 638 mg/L, respectively. The steady-state effluent DOC concentrations for *K. oxytoca* and *B. cepacia* were 407 and 555 mg/L, respectively. The DOC concentrations from the "high" substrate concentration experiment suggest that both organisms utilize approximately the same amount of DOC and that, like the previous batch and rotating disk reactors, DOC was not completely utilized in the dual-species rotating disk reactors.

Though the concentration utilized between the "high" and "low" substrate concentration experiments varies by an order of magnitude, the percent removals are similar.

Table 7. Effluent dissolved organic carbon (DOC) concentrations for the single-species biofilm experiments +/- the standard deviation.

	Beginning DOC Conc. (mg/L)	Steady-State DOC Conc. (mg/L)	Concentration Utilized (mg/L)	% Utilized
"70 mg/L DOC"				
K. oxytoca	74.0 ± 4.8	56.9 ± 1.9	17.1 +/- 2.9	23.0 +/- 2.4
B. cepacia	73.8 ± 2.5	60.0 ± 6.6	13.8 +/- 4.0	18.8 +/- 6.1
"700 mg/L DOC"				
K. oxytoca	634 ± 149	407 ± 6.5	227 ± 156	33.8 ± 16.6
B. cepacia	648 ± 315	555 ± 194	109 ± 99.4	14.8 ± 8.1

Dual-Species Results

A series of dual-species experiments were performed using the rotating disk reactors (RDR) to examine the dual-species population distribution of *K. oxytoca* and *B. cepacia* in a biofilm. These results were compared to the single-species biofilm experiments, as well as to the planktonic data, to understand how microbial interaction changes between planktonic and biofilm systems. This research determined if single- and dual-species planktonic data could be used to describe biofilm reactions. Also, the ability to predict dual-species population distributions using single-species data (i.e. growth rates) was examined.

In addition to the 70 and 700 mg/L DOC substrate concentrations used in the batch and single-species rotating disk reactors, an additional set of experiments were performed using a lower substrate concentration (30mg/L DOC) to provide a further range of substrate concentrations to interpret the dual-species interactions in a biofilm.

Effluent Population Density. After both organisms were simultaneously inoculated into the RDR, selective plating techniques were used to quantify the effluent population densities over time.

The dual-species effluent population densities for the 30 mg/L substrate concentration experiments are presented in Figure 14. The system entered steady-state conditions after approximately 50 hours. *B. cepacia's* effluent population density after five days of growth (5.8 x 10^6 CFU/mL) was measurably greater than *K. oxytoca's* effluent population density (2.3 x 10^5 CFU/mL).

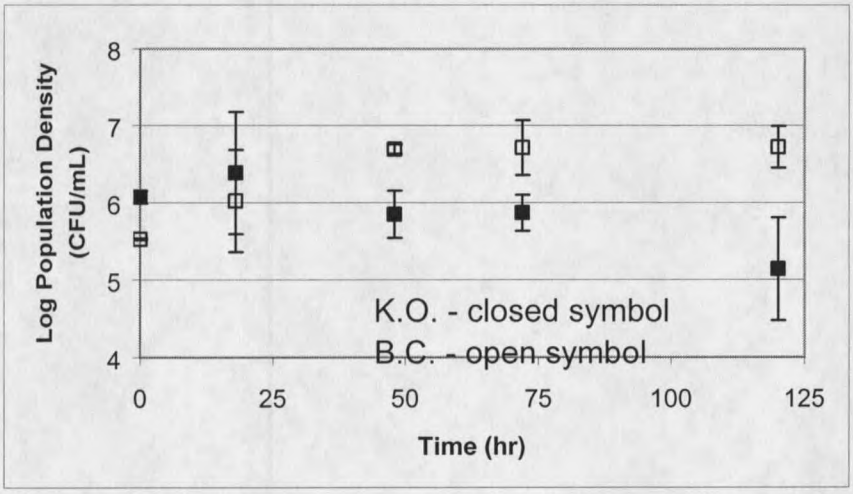

Figure 14. Dual-species effluent population densities for *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) for the 30 mg/L DOC substrate concentration rotating disk reactor experiments. Values are average of duplicate experiments and error bars are +/- standard deviation.

The dual-species effluent population densities over time for *K. oxytoca* and *B. cepacia* for the 70 mg/L DOC substrate concentration experiments can be found in Figure 15. The steady-state effluent population density for *B. cepacia* (2.9 x 10^7 CFU/mL) was

measurably greater than the *K. oxytoca* steady-state effluent population density (5.2 x 10^6 CFU/mL). In an ideal CSTR reactor, the effluent bacterial population density is assumed to be equal to the completely mixed suspended population. Therefore, *B. cepacia* had a planktonic population density greater than *K. oxytoca* at the 70 mg/L DOC, which is different than what was observed in the batch reactor at the same substrate concentration (*K. oxytoca* was the dominant organism).

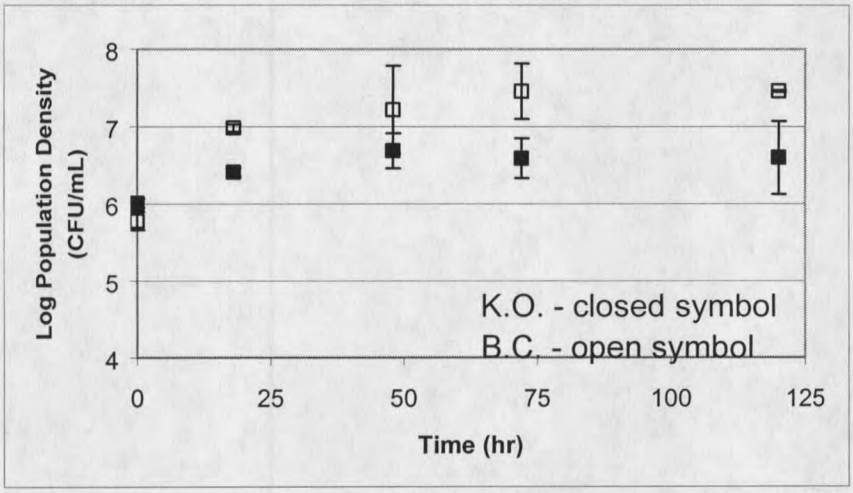

Figure 15. Dual-species effluent population densities for *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) for the 70 mg/L DOC substrate concentration rotating disk reactor experiments. Values are average of duplicate experiments and error bars are +/- standard deviation.

The effluent population densities over time for *K. oxytoca* and *B. cepacia* for the 700 mg/L DOC substrate concentration experiments can be found in Figure 16. The steady-state effluent population density for *K. oxytoca* (7.1 x 10^7 CFU/mL) was significantly higher (*p-value* < *0.002*) at steady-state than the *B. cepacia* effluent population density (1.2 x 10^6 CFU/mL). Recall that the RDR is a CSTR and the effluent population density is equal to the suspended population. Therefore, when the substrate

concentration was increased by an order of magnitude, there was a shift in the steady-state suspended population density with *K. oxytoca* now having a higher population density than *B. cepacia*.

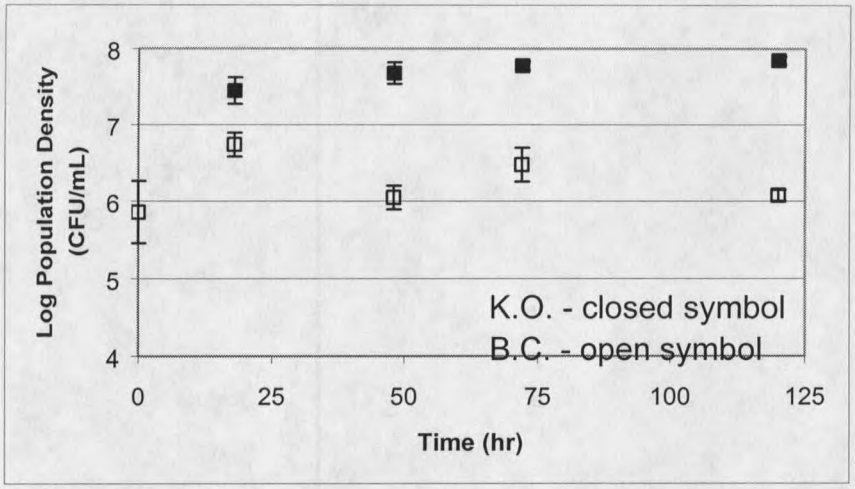

Figure 16. Dual-species effluent population densities for *K. oxytoca* (closed symbols) and *B. cepacia* (open symbols) for the 700 mg/L DOC substrate concentration rotating disk reactor experiments. Values are average of duplicate experiments and error bars are +/- standard deviation.

Table 8 provides a summary of the dual-species effluent population densities for all three substrate concentrations after five days of reactor operation. *K. oxytoca's* effluent population density increased significantly (*p-value* < *0.030*) when the substrate concentration was increased from 30 to 700 mg/L DOC. *B. cepacia's* dual-species effluent population distribution increased measurably between the 30 and 70 mg/L DOC substrate concentration and then decreased significantly between the 70 and 700 mg/L DOC substrate concentration (*p-value* < *0.002*). The sum of the steady-state effluent population density of both organisms in the dual-species experiment (Table 8) had values

similar to that organism's steady-state effluent population density by itself (single-species culture) (Table 3) for the 70 and 700 mg/L substrate concentrations.

Table 8. Dual-species planktonic population densities (X_1) after five days of rotating disk reactor operation. Values are average of duplicate experiments and errors are +/- the standard deviation.

	X_1 (CFU/mL) x 10^7		
	30 mg/L DOC	70 mg/L DOC	700 mg/L DOC
K. oxytoca	0.023 +/- 0.026	0.52 +/- 0.47	7.08 +/- 0.83
B. cepacia	0.58 +/- 0.34	2.89 +/- 0.08	0.12 +/- 0.02

Biofilm Population Density. After five days of operation, the RDRs were taken off-line, biofilm harvested from disks, and biofilm population density quantified using selective plating techniques. Table 9 contains the steady-state biofilm population densities for the dual-species biofilm experiments. In the 700 mg/L DOC substrate experiment, *K. oxytoca's* population density (1.6 x 10^8 CFU/cm^2) was an order-of-magnitude higher, but not statistically different, than *B. cepacia's* (1.3 x 10^7 CFU/cm^2). For the 30 mg/L DOC substrate concentration experiments, *B. cepacia's* population density (8.2 x 10^7 CFU/cm^2) was significantly higher ($p=0.033$) than *K. oxytoca's* biofilm population density (1.7 x 10^6 CFU/cm^2). For the 70 mg/L DOC substrate concentration experiments, *B. cepacia's* population density (9.8 x 10^7 CFU/cm^2) was measurably higher, but not statistically different, than *K. oxytoca's* population density (6.9 x 10^6 CFU/cm^2). At each substrate concentration, the sum of the dual-species biofilm population densities was comparable to the steady-state population density when each organism was grown separately.

Table 9. Dual-species biofilm population densities (X_b) after five days of rotating disk reactor operation. Values are average of duplicate experiments and errors are +/- the standard deviation.

	X_b (CFU/cm^2) x 10^8		
	30 mg/L DOC	70 mg/L DOC	700 mg/L DOC
K. oxytoca	0.017 ± 0.005	0.07 ± 0.05	1.61 ± 0.17
B. cepacia	0.82 ± 0.67	0.98 ± 0.53	0.13 ± 0.14

Suspended vs Biofilm Populations. The total (*K. oxytoca* plus *B. cepacia*) suspended population in the rotating disk reactor at steady-state (X_1 x V) was compared with the total biofilm population in the rotating disk reactor at steady-state (X_b x A) (Figure 17). Total biofilm biomass remained constant with change in substrate concentration. Although suspended biomass increased with increasing substrate concentration, biofilm biomass was the majority of total biomass in the system.

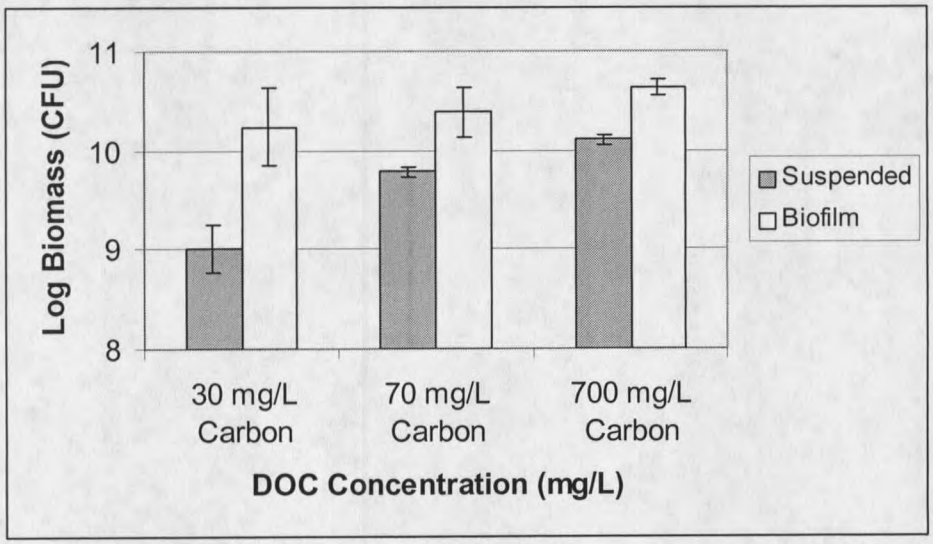

Figure 17. Total (*K. oxytoca* + *B. cepacia*) suspended and biofilm biomass from the dual-species rotating disk reactor experiments. Values are average of duplicate experiments +/- the standard deviation.

Growth Rate Analysis. The values of the rotating disk reactor's suspended and biofilm population densities were input, along with the dual-species planktonic growth rates (Table 2), into the biofilm growth rate equation (eq. 7) to calculate a specific biofilm growth rate for each organism (Table 10). This equation was derived from a mass balance around the suspended and biofilm sections of the rotating disk reactor. *K. oxytoca's* growth rate increased when the substrate concentration was increased from 30 to 70 mg/L DOC and remained constant between the 70 and 700 mg/L DOC substrate concentration experiments. *B. cepacia's* growth rate increased when the substrate concentration was increased from 30 to 70 mg/L DOC and remained constant between the 70 and 700 mg/L DOC substrate concentration experiments. *K. oxytoca's* growth rate was slightly higher than that for *B. cepacia* for all three dual-species experiments. Though changes in dual-species growth rates were recorded, all dual-species biofilm growth rates were not significantly different using a 0.05 level of significance.

When *K. oxytoca* was combined with *B. cepacia*, its biofilm growth rate increased significantly ($p\text{-value} < 0.035$) at the 70 mg/L DOC substrate concentration and remained constant at the 700 mg/L DOC substrate concentration compared to the single-species biofilm growth rates in Table 6. *B. cepacia's* dual-species growth rate did not change significantly at the 70 and 700 mg/L DOC substrate concentration compared to the single-species biofilm growth rates.

Table 10. Dual-species biofilm growth rates (μ) for the three dissolved organic carbon (DOC) substrate concentration experiments. Values are average of duplicate experiments and errors are +/- the standard deviation.

	μ (hr^{-1})		
	30 mg/L DOC	70 mg/L DOC	700 mg/L DOC
K. oxytoca	0.14 ± 0.17	0.57 ± 0.13	0.33 ± 0.07
B. cepacia	0.09 ± 0.03	0.39 ± 0.22	0.20 ± 0.19

Dissolved Organic Carbon. Dissolved organic carbon (DOC) was monitored over time to quantify substrate utilization in the dual-species rotating disk reactors (Table 11). An increase in substrate concentration resulted in an increase in the amount of DOC utilized and a decrease in the percent utilized. As in the previous batch and rotating disk reactors, DOC was not completely utilized in the dual-species rotating disk reactors.

Table 11. Effluent dissolved organic carbon (DOC) concentrations for the dual-species biofilm experiments.

Target DOC Conc. (mg/L)	Beginning DOC Conc. (mg/L)	Steady-State DOC Conc. (mg/L)	Concentration Utilized (mg/L)	% Utilized
30	29.9	15.4	14.5	48.5
70	72.7	53.5	19.3	26.5
700	556	497	59.0	10.6

Porous Media Experiments

Dual-Species Population Dynamics

The above experiments demonstrated that growth rates could be used to predict dual-species dominance in batch cultures but could not be used to predict dual-species dominance in a biofilm grown in well-mixed conditions (rotating disk reactor). Varying substrate concentration, however, enabled the manipulation of the dual-species population distribution in the rotating disk reactor biofilm. A series of column

experiments were performed to quantify the steady-state population densities of *Klebsiella oxytoca* and *Burkholderia cepacia* in porous media and determine if trends observed in a biofilm grown in a well-mixed system would be similar to those in a porous media system. Both types of biofilms could experience substrate limitation, but in a porous media system substrate limitation could occur in the bulk fluid. This could have a negative effect on aerobic organisms throughout a porous media reactor if oxygen is utilized at the beginning of the reactor. In addition, hydrodynamic differences between a rotating disk reactor and a porous media reactor could influence the density of the biofilm. The effects of these differences on the population distribution and growth rate will be addressed.

The porous media columns used in these experiments were 30 cm long, 4.8 cm diameter and filled with 1 mm glass beads (Figure 2). Both organisms were inoculated simultaneously into the porous media column and nutrient (different dilutions of LBG media resulting in either 30, 70 and 700 mg/L dissolved organic carbon) was pumped through the column at a rate of 2.4 ml/min to establish steady-state conditions. After 70 hours, the column was taken off-line and beads were removed from the entrance and exit of the column to quantify the biofilm biomass. The column was put back on-line and TCE was continuously pumped through the column for nine hours to examine TCE degradation in a dual-species porous media reactor. This section will address dual-species population distribution in porous media (the first 70 hours) and the following section will discuss TCE degradation and permeability reduction in a dual-species column.

Effluent Population Distribution. Selective plating techniques were used to analyze effluent samples over time at the three substrate concentrations for the dual-species experiments.

The results from the 30 mg/L dissolved organic carbon (DOC) experiment show that the *K. oxytoca* population density was comparable to that of *B. cepacia* at the beginning of the experiment (Figure 18). Over the duration of the experiment, the *B. cepacia* population density increased while the *K. oxytoca* population decreased. After 70 hours, *B. cepacia's* effluent population density (1.5×10^7 CFU/mL) was six times higher, and statistically greater (*p-value* < *0.035*), than *K. oxytoca's* effluent population density (2.4×10^6 CFU/mL).

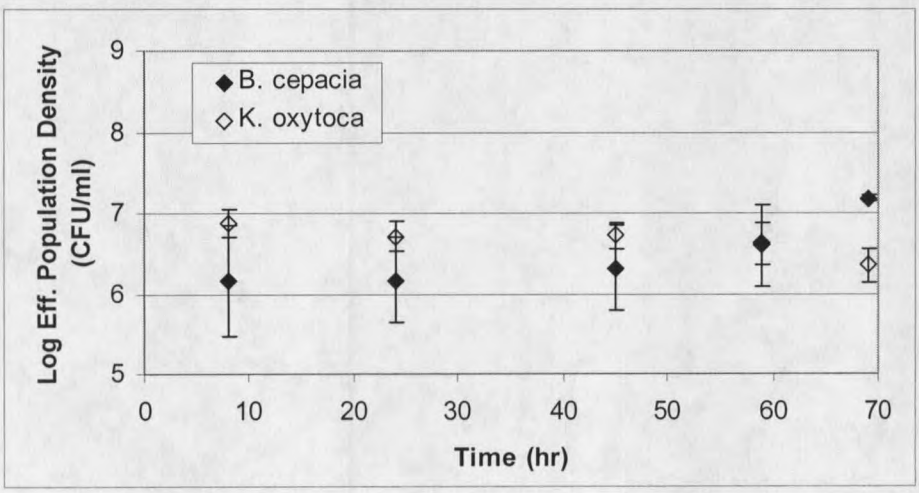

Figure 18. Dual-species porous media effluent plate counts of *K. oxytoca* (open symbols) and *B. cepacia* (closed symbols) for the 30 mg/L DOC substrate concentration experiments. Values are an average of duplicate experiments and error bars are +/- standard deviation.

Dual-species effluent population densities for the 70 mg/L dissolved organic carbon (DOC) substrate concentration experiment show that the *K. oxytoca* population

density was slightly greater than the *B. cepacia* population density at the beginning of the experiment (Figure 19). After 70 hours, *B. cepacia's* effluent population density (7.9 x 10^6 CFU/mL) was comparable to *K. oxytoca's* effluent population density (1.2 x 10^7 CFU/mL).

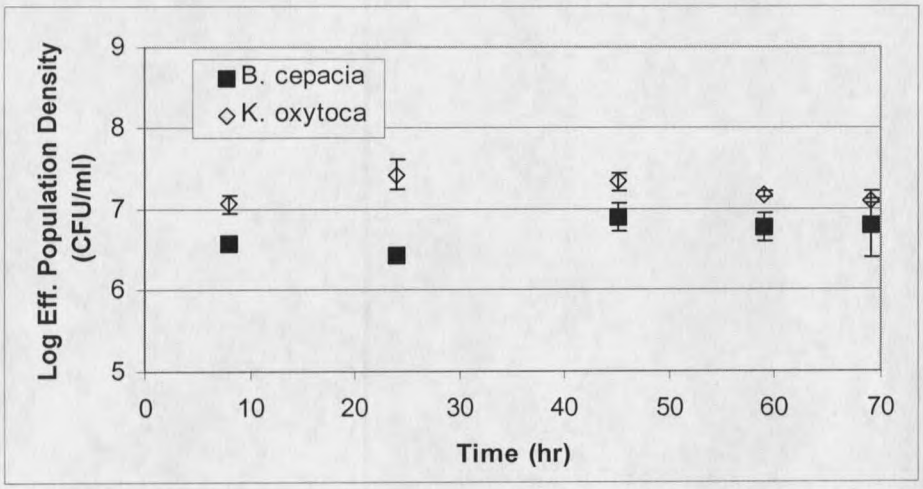

Figure 19. Dual-species porous media effluent plate counts of *K. oxytoca* (open symbols) and *B. cepacia* (closed symbols) for the 70 mg/L DOC substrate concentration experiments. Values are an average of duplicate experiments and error bars are +/- standard deviation.

The dual-species effluent population densities for the 700 mg/L dissolved organic carbon (DOC) substrate concentration experiments show that *K. oxytoca* was greater than *B. cepacia* at the beginning of the experiment (Figure 20). These initial conditions were similar to the other two substrate concentrations. The *K. oxytoca* population density remained higher than *B. cepacia's* for the duration of the experiment. After 70 hours, *K. oxytoca's* effluent population density (1.0 x 10^8 CFU/mL) was two orders of magnitude greater, and significantly higher (*p-value* < *0.010*), than *B. cepacia's* effluent population density (1.2 x 10^6 CFU/mL).

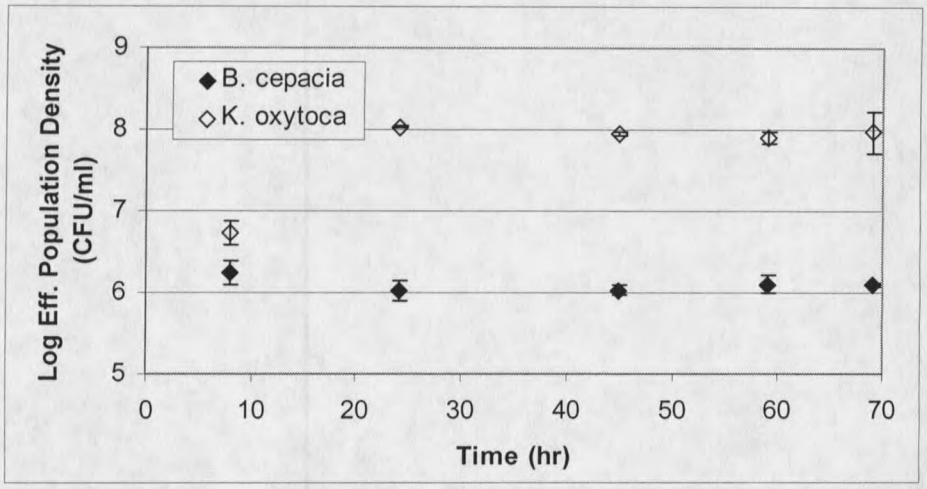

Figure 20. Dual-species porous media effluent plate counts of *K. oxytoca* (open symbols) and *B. cepacia* (closed symbols) for the 700 mg/L DOC substrate concentration experiments. Values are an average of duplicate experiments and error bars are +/- standard deviation.

Biofilm Population Distribution. After 70 hours of nutrient flow, each column was taken off-line and glass beads removed from the influent and effluent of the column. Bacteria were desorbed from the beads and selective plating techniques were used to quantify the dual-species biofilm population distribution for each organism at the entrance and exit of the column (Table 12).

For the 30 mg/L DOC substrate concentration, *B. cepacia's* porous media population density at the entrance and exit of the column (3.4×10^7 CFU/g bead and 1.2×10^8 CFU/g bead, respectively) was 5 and 22 times higher and significantly greater than (*p-values* < 0.040) *K. oxytoca's* porous media population density at the beginning and end of the column (6.0×10^6 CFU/g bead and 5.2×10^6 CFU/g bead, respectively). Both organisms' population densities remained constant from the beginning to the end of the column.

For the 70 mg/L DOC substrate concentration, *B. cepacia's* porous media population density at the entrance and exit was comparable to *K. oxytoca*. Both organisms' population density decreased from the entrance to the exit of the column.

For the 700 mg/L DOC substrate concentration, *K. oxytoca's* porous media population density at the entrance of the column (3.9×10^9 CFU/g bead) was three orders of magnitude greater and statistically different (*p-value* < *0.002*) than *B. cepacia's* density (2.3×10^6 CFU/g bead). *K. oxytoca's* density at the exit (3.2×10^7 CFU/g bead) was two orders of magnitude lower (*p-value* < *0.005*) than at the entrance of the column. This was only three times greater, but still statistically higher (*p-value* < *0.020*), than *B. cepacia's* density at the exit (1.1×10^7 CFU/g bead).

Table 12. Dual-species porous media population distribution (after 70hr) at the entrance and exit of the column. Values are an average of duplicate experiments +/- the standard deviation.

Substrate Concentration (mg/L DOC)	*K. oxytoca* (CFU/g bead) x 10^8		*B. cepacia* (CFU/g bead) x 10^8	
	Entrance	Exit	Entrance	Exit
30	0.060 ± 0.03	0.052 ± 0.019	0.34 ± 0.04	1.18 ± 0.8
70	2.0 ± 0.84	0.039 ± 0.012	2.4 ± 0.50	0.78 ± 0.93
700	39 ± 10	0.32 ± .025	0.023 ± 0.007	0.11 ± 0.021

The porous media biofilm data from the entrance and exit of the column was averaged for each experiment and summarized in Figure 21. *B. cepacia's* biofilm population in porous media was significantly higher (*p-value* < *0.020*) than *K. oxytoca* at the 30 mg/L substrate concentration experiment. Both organisms had similar biofilm population densities at the 70 mg/L DOC substrate concentration experiment. At the 700 mg/L DOC substrate concentration experiment there was a shift in population distribution

with *K. oxytoca's* population density higher than *B. cepacia's* (*p-value* < *0.005*). An increase in substrate concentration caused an increase in the *K. oxytoca* porous media population density. *B. cepacia's* porous media population remained constant between the 30 and 70 mg/L DOC substrate concentration and decreased significantly at the 700 mg/L DOC substrate concentration (*p-value* < *0.005*). Varying substrate concentration from 30 to 700 mg/L DOC resulted in a change in species dominance between these two organisms in porous media.

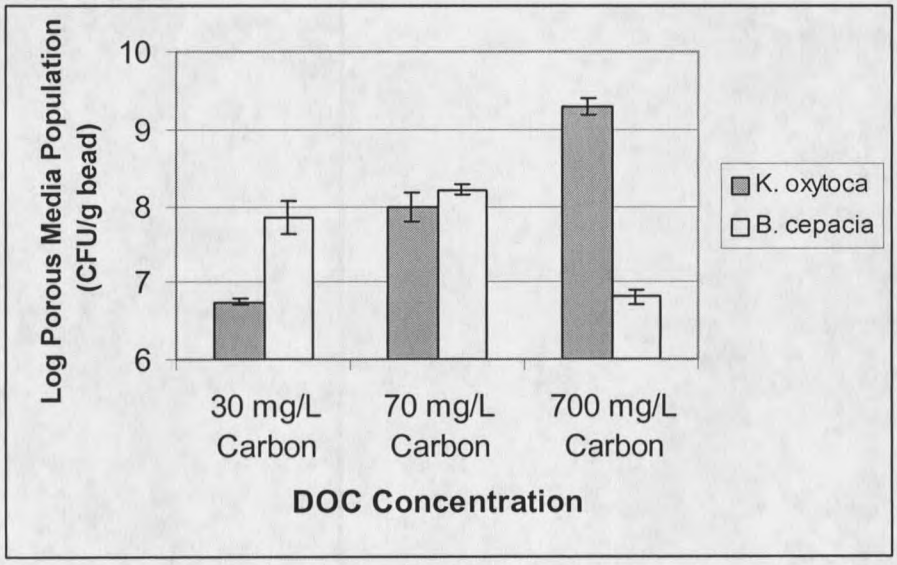

Figure 21. Dual-species porous media population densities of *K. oxytoca* and *B. cepacia* at steady-state for three different substrate concentrations. Values are the average of duplicate experiments and error bars are +/- standard deviation.

Porous Media Growth Rates. The biofilm growth rate equation used for the rotating disk reactor (eq. 7) assumes that the planktonic organisms grow at the exponential growth rate calculated from the batch experiments. In a porous media column, the planktonic organisms may grow exponentially at the very beginning of the

column. As the plug flow of nutrients and oxygen are utilized over the length of the column, however, the planktonic organisms are likely to have a lower growth rate further in the column. While the assumption of suspended organisms growing exponentially appears to be valid in the rotating disk reactor, it may not be the case in the porous media column. In addition, comparison of the total biofilm population after 70 hours to the total suspended population (Table 13) show that the suspended cells make up less than seven percent of the total cells in the system.

Table 13. Total biofilm and suspended populations (and fraction of total biomass) in the porous media column after 70 hours of growth. Values are average of duplicate experiments +/- standard deviation.

	Total Biofilm Biomass (CFU x 10^{10})	Total Suspended Biomass (CFU x 10^{10})	Total Biofilm Biomass (%)	Total Suspended Biomass (%)
30 mg/L DOC	6.27 ± 2.9	0.38 ± 0.06	93.8 ± 1.8	6.2 ± 1.8
70 mg/L DOC	20.4 ± 1.2	0.44 ± 0.19	97.9 ± 0.8	2.1 ± 0.8
700 mg/L DOC	153 ± 40	2.25 ± 1.3	98.6 ± 0.4	1.4 ± 0.4

Therefore, to calculate the growth rate of the organisms attached to the porous media it was assumed that planktonic growth was negligible and the biofilm growth rate equation (eq. 7) was rearranged as followed (eq. 9).

Eq. 9 $$\mu_b = \frac{Q}{A} \times \frac{X_1}{X_b}$$

Where: μ_b = biofilm growth rate (1/t)

Q = flow rate (L^3/t)

A = biofilm surface area (L^2)

X_1 = Effluent (planktonic) cell density (CFU/L^3)

X_b = biofilm cell density (CFU/L^2)

Porous media growth rates were calculated by inputting the steady-state effluent population densities (Figure 18, 19 and 20) and the average of the steady-state porous media population densities at the entrance and exit of the column (Figure 21) into equation 9 and summarized in Table 14. The porous media population densities are recorded on a per weight basis (CFU/g glass beads). By measuring the weight of the glass beads and calculating the surface area (19.2 cm^2 of surface area in one gram of glass beads), the porous media population densities were converted to a per surface area basis.

K. oxytoca's growth rate was comparable to *B. cepacia's* at the 30 mg/L and 70 mg/L DOC substrate concentration experiments and *B. cepacia's* growth rate was greater (*p-value* < *0.030*) than *K. oxytoca* at the high substrate concentration. All growth rates were between 0.01/hr and 0.08/hr. These rates were lower than those for the batch (planktonic) and rotating disk reactor experiments. If suspended growth was not ignored and equation 7 was used to calculate the specific biofilm growth rate in porous media, the resulting biofilm growth rate would be even smaller than that calculated using equation 9 due to the negative effect of the suspended growth term on the biofilm equation.

Table 14. Dual-species porous media growth rates (μ) for the three dissolved organic carbon (DOC) substrate concentration experiments. Values are average of duplicate experiments and errors are +/- the standard deviation.

	30 mg/L DOC	70 mg/L DOC	700 mg/L DOC
K. oxytoca	0.076 +/- 0.031	0.024 +/- 0.012	0.009 +/- 0.003
B. cepacia	0.040 +/- 0.016	0.009 +/- 0.009	0.035 +/- 0.005

Dissolved Organic Carbon (DOC) Concentrations. The substrate concentrations used in this experiment were the same as those used in the dual-species biofilm experiments (30, 70 and 700 mg/L DOC). Dissolved organic carbon was monitored from the influent and effluent of the column over time to quantify substrate utilization in the porous media columns (Table 15). Increasing the substrate concentration increased the amount of carbon utilized in the column. A significant amount of DOC remained after 70 hours of reactor operation.

Table 15. Steady-state influent and effluent dissolved organic carbon (DOC) concentrations from the dual-species porous media column experiments. Values are an average of steady state values over time +/- the standard deviation (n=3 for 30 and 70 mg/L DOC and n=2 for 700 mg/L DOC).

Substrate Concentration (mg/L DOC)	Influent DOC Conc. (mg/L)	Effluent DOC Conc. (mg/L)	Concentration Utilized (mg/L)	% Utilized
30	29.6 ± 1.8	20.2 ± 1.8	9.4 ± 1.1	31.8 ± 3.9
70	71.7 ± 5.4	50.3 ± 5.0	21.4 ± 8.0	29.5 ± 9.9
700	626 ± 16.6	527 ± 18.0	99.0 ± 1.4	15.8 ± 0.6

TCE Degradation in Porous Media

The previous section demonstrated the feasibility of combining *Burkholderia cepacia* and *Klebsiella oxytoca* in a porous media column and showed the importance of substrate concentration in controlling the fraction of each organism in dual-species biofilms. TCE breakthrough curves were run on the columns to determine if substrate concentration could also be used to optimize TCE degradation.

The two porous media columns used in this experiment were 30 cm long, 4.8 cm diameter glass columns filled with 1 mm glass beads. The columns were identical except

that one column did not have sampling ports and the other had three miniert sampling ports added along the length of the column (Figure 2). A complete description of the experimental set-up can be found in the materials and methods section (Chapter 2).

Both organisms were inoculated into each column simultaneously and nutrients (30, 70 or 700 mg/L DOC substrate concentration) were delivered to the column until steady-state conditions were established. TCE was then continuously added to the influent of the column for nine to twelve hours and TCE concentrations were monitored in the influent and effluent of the column. TCE degradation was quantified by calculating the difference between steady-state influent and effluent TCE concentrations.

Fluorescein Breakthrough Curve

Fluorescein breakthrough curves were performed on both columns (with and without sampling ports) before bacteria addition to quantify the hydrodynamics of the reactor and measure any losses of a conservative tracer (Figure 22). As described in Chapter 2, the fluorescein was injected into the main flow line via syringe pump. After the syringe pump was a chamber (with stir bar) to allow for complete mixing before the solution reached the influent sampling port. The influent and effluent fluorescein concentrations of each column behaved in a similar manner. The influent fluorescein concentrations reached steady-state conditions after approximately 50 minutes due, in part, to the mixing chamber (Figure 2). The fluorescein was detected in the effluent after 90 minutes and the effluent concentrations reached steady-state after approximately 150 minutes. A detention time of 100 minutes was calculated by measuring the time between the start of influent and effluent steady-state values. The breakthrough curves show

symmetrical effluent rising and falling segments, which are indicative of solute moving through homogeneous, isometric porous media. Influent fluorescein concentrations equaled effluent fluorescein concentration indicating no loss throughout the columns.

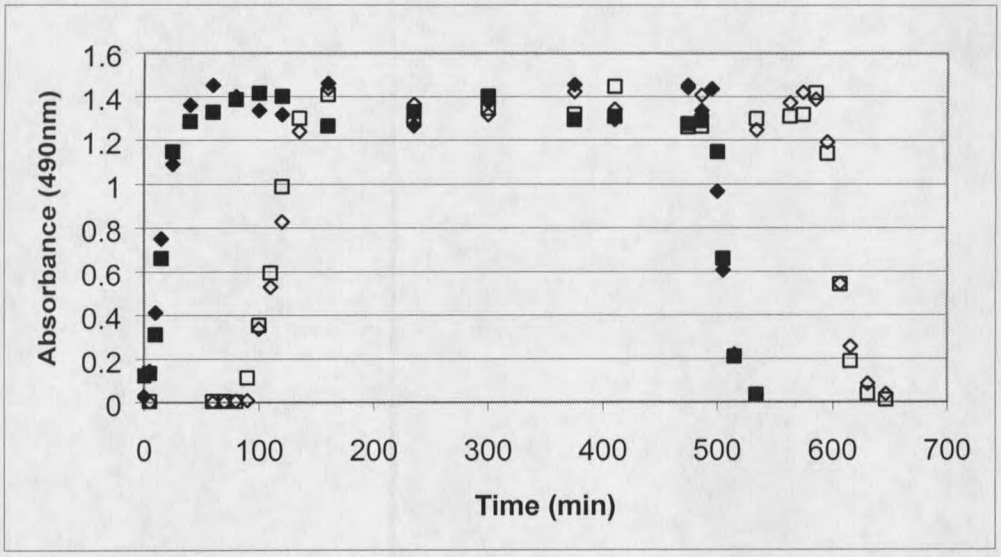

Figure 22. Fluorescein breakthrough curves. The column with ports is shown with diamonds and the column without ports are squares (closed = influent, open = effluent).

Abiotic TCE Breakthrough Curve

Abiotic TCE breakthrough curves were performed just before inoculation of bacteria into the column (Figure 23). The data shows influent and effluent TCE breakthrough curves for each substrate concentration (30, 70 and 700 mg/L DOC) experiment. Each experiment was duplicated. Steady-state influent TCE concentrations were equal to steady-state effluent TCE concentrations, indicating no abiotic loss of TCE in this system.

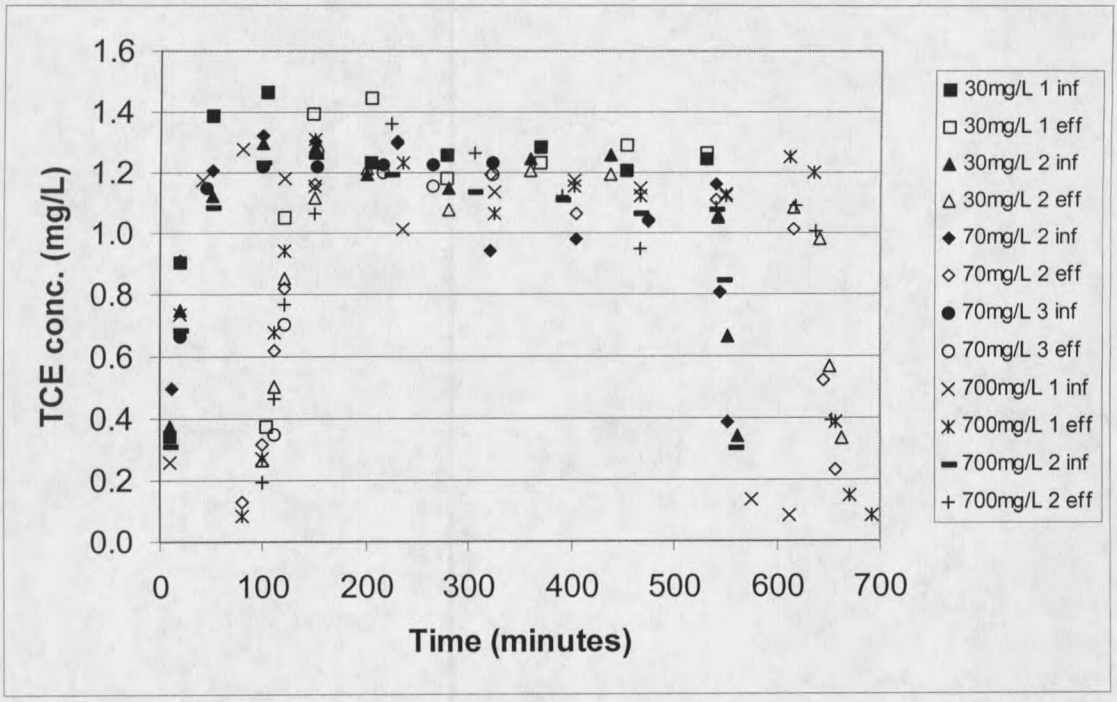

Figure 23. Abiotic TCE breakthrough curves ran on the porous media columns before bacterial addition.

Biotic TCE Analysis in Porous Media

Single- and dual-species TCE column experiments were performed. In the single-species column experiment, *B. cepacia* was inoculated into the porous media column and supplied diluted LBG media consisting of 30 mg/L DOC. After the column reached steady state, water saturated with TCE (TCE solubility = 1100 mg/L @ 25°C) was pumped (0.16 ml/hr) via syringe pump into the main nutrient line of the porous media column for twelve hours and TCE degradation and *B. cepacia* population density over time was monitored. In the dual-species TCE experiments, the column was inoculated with a dual-species culture containing *B. cepacia* and *K. oxytoca*. Different dilutions of LBG media (either 30, 70 or 700 mg/L DOC substrate concentration) were pumped

through the reactors for 70 hours at a flow rate of 2.4 ml/min. The dual-species population distribution up to 70 hours was presented in the previous section and will be summarized below. Water saturated with TCE (TCE solubility = 1100 mg/L @ 25°C) was then pumped (0.16 ml/hr) via syringe pump into the main nutrient line to the column for nine hours and influent and effluent TCE concentrations, as well as population densities and dissolved organic carbon concentrations, were monitored over time.

Single-Species TCE Degradation

Effluent Population Density. A pure culture of *B. cepacia* was inoculated into the porous media column fed the 30 mg/L DOC substrate concentration and effluent population density was monitored over time (Figure 24). Just before TCE was added to the column after 70 hours of growth, *B. cepacia's* population density was 7.0×10^6 CFU/mL. The TCE concentration pumped into the column was 1.07 ppm. The effluent concentration of *B. cepacia* remained constant (final effluent concentration was 8.1×10^6 CFU/mL) during the 12.5 hours of TCE addition, suggesting that this TCE concentration was not detrimental to the *B. cepacia* population over this period of time.

Porous Media Population Density. After 70 hours of growth without TCE and 12.5 hours of growth with TCE, the column was taken off-line and destructively sampled to quantify *B. cepacia's* population distribution along the length of the column (Table 16). The toluene ortho-monooxygenase (TOM) pathway is responsible for the degradation of TCE by *B. cepacia* and is located on the TOM plasmid. Also located on the TOM plasmid is a resistance marker to the antibiotic, kanamycin. Therefore, if *B.*

cepacia is capable of growth on kanamycin, its TCE degradation pathway is active. For this reason, and to monitor biological contamination in the column, LBG plates amended with kanamycin were used with the non-selective R2A plates to quantify the population with active TOM pathways as well as the total population.

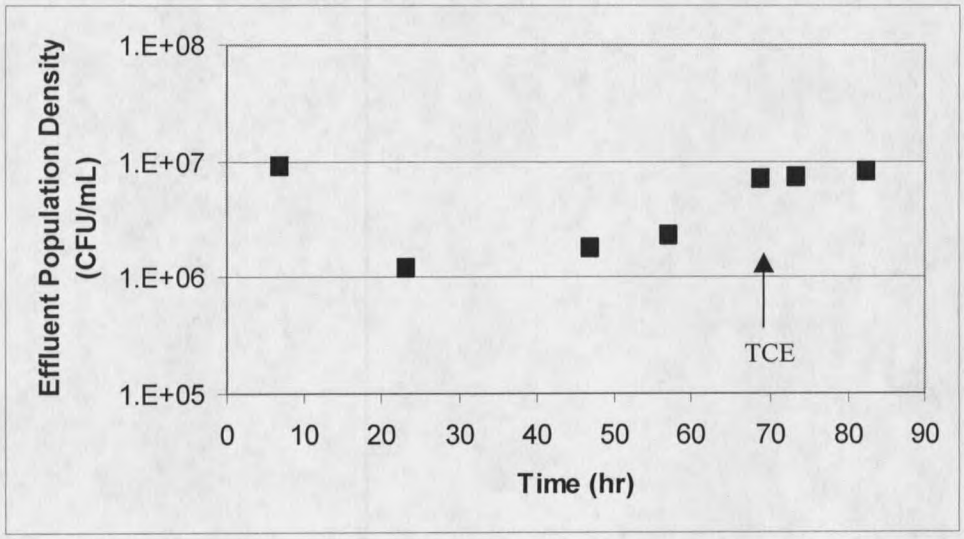

Figure 24. *B. cepacia's* effluent population density over time for the pure-culture porous media column experiment supplied 30 mg/L DOC substrate concentration.

The results from the destructive sampling (Table 16) show that *B. cepacia's* population density was lower at the influent of the column, but was relatively uniform throughout the column. Also, comparing the kanamycin plates with the R2A plates indicate that the majority of cells in the column had an active TOM pathway. There is a greater discrepancy between the LBG/kanamycin and R2A plates at the beginning of the column, where the highest load of TCE is present (recall that the TCE concentration used in this experiment was 1.2 mg/L). This loss of plasmid activity corresponds with Sharp

(1995), who observed that exposure to a TCE concentration of 3.5 mg/L could cause as much as 25% pTOM plasmid loss.

Table 16. Porous media population densities along the length of the column after the TCE breakthrough curve analysis for pure culture of *B. cepacia* fed the 30 mg/L substrate concentration. Values are an average of triplicate plate counts +/- the standard deviation.

	Porous Media Population Density (CFU/g glass bead) x 10^8			
	Entrance	Middle	Exit	Average
LBG/kanamycin plate counts	0.64 +/- 0.13	3.10 +/- 0.12	1.85 +/- 0.33	1.87 +/- 1.23
R2A Plate Counts	0.99 +/- 0.20	4.01 +/- 0.15	1.76 +/- 0.15	2.26 +/- 1.57

TCE Breakthrough Curve. The TCE breakthrough curve for the pure culture of *B. cepacia* in the porous media column fed 30 mg/L dissolved organic carbon can be found in Figure 25. The influent and effluent steady-state TCE concentrations were 1.07 mg/L and 0.23 mg/L, respectively. Results show that a column inoculated with a pure culture of *B. cepacia* and supplied 30 mg/L DOC substrate concentration removed 79.1% of the TCE.

A specific TCE degradation rate was calculated by inputting the steady-state influent and effluent TCE concentrations from the *B. cepacia* TCE breakthrough curve (Figure 25) and the single-species porous media population density into equation 1. The specific TCE degradation rate calculated for *B. cepacia* in single-species for the 30 mg/L DOC substrate concentration was 0.029 μg TCE/min/mg protein.

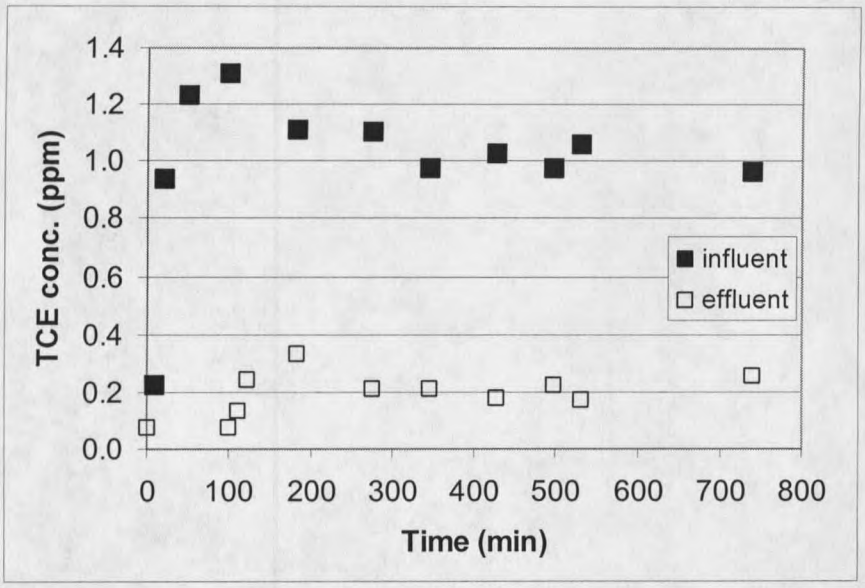

Figure 25. TCE breakthrough curve for *B. cepacia* at 30 mg/L DOC substrate concentration.

Dual-Species TCE Degradation

After a dual-species culture containing *B. cepacia* and *K. oxytoca* was inoculated into the porous media column, substrate as 30, 70 or 700 mg/L DOC was pumped through the reactors for 70 hours at a flow rate of 2.4 ml/min. After 70 hours, each column was taken off-line and glass beads removed from the entrance and exit of the column. Bacteria were desorbed from the beads and porous media population densities quantified using selective plating techniques. The column was put back on-line and flow was resumed for 4.5 hours (~3 retention times) to minimize any disturbance caused by removal of the beads. A constant supply of TCE was then pumped into the main nutrient line to the column via a syringe pump for nine hours. Influent and effluent TCE concentrations, as well as population densities and dissolved organic carbon concentrations, were monitored over time.

Effluent Population Densities. The dual-species effluent population densities of *K. oxytoca* and *B. cepacia* over time for the 30 mg/L substrate concentration are presented in Figure 26. *K. oxytoca* was the dominant organism for the first 48 hours, though not statistically different. *B. cepacia* became the dominant organism by t=69hr with populations statistically higher than *K. oxytoca* (*p-value* < *0.035*). The sum of both populations remained constant up to t=69 hr. The column was disturbed at t=69hr to remove beads (approximately 5 grams) from the beginning and end of the column for porous media population density analysis. The column was put back on-line and nutrient flow was pumped through for three detention times (~4.5hr) before TCE flow began. During TCE flow into the column, there was a slight rise in *B. cepacia* and a decrease in *K. oxytoca's* population density, though not statistically significant.

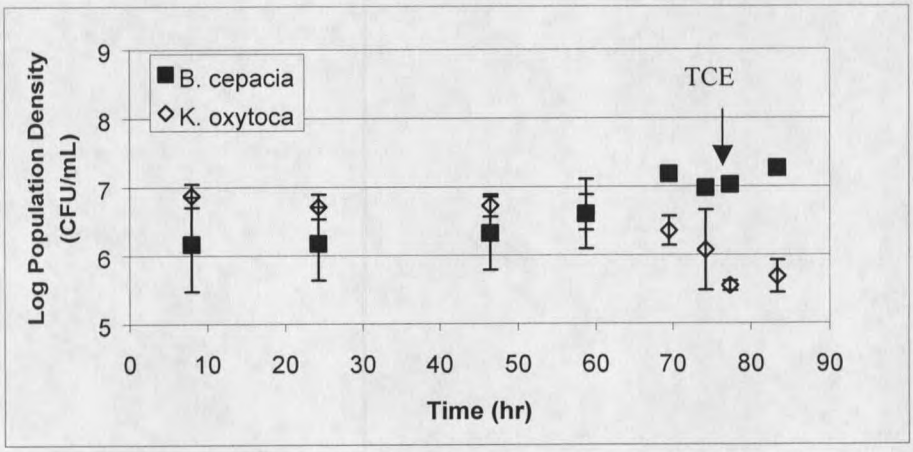

Figure 26. Dual-species porous media effluent plate counts of *K. oxytoca* and *B. cepacia* for the 30 mg/substrate concentration experiments. TCE was introduced to the column between t=74.25hr to t=83.4hr. Values are an average of duplicate experiments and error bars are +/- standard deviation.

The dual-species effluent population densities of *K. oxytoca* and *B. cepacia* over time for the 70 mg/L substrate concentration are presented in Figure 27. *K. oxytoca* was the dominant organism for the first 48 hours. During the next 20 hours, the difference in population density between the two organisms lessened and by t=69 hr the populations were comparable. The column was disturbed at t=69hr to remove beads from the beginning and end of the column for porous media population density analysis. The column was put back on-line and nutrient flow was pumped through for three detention times (~4.5hr) before TCE flow began. During TCE flow into the column, both organisms remained relatively constant.

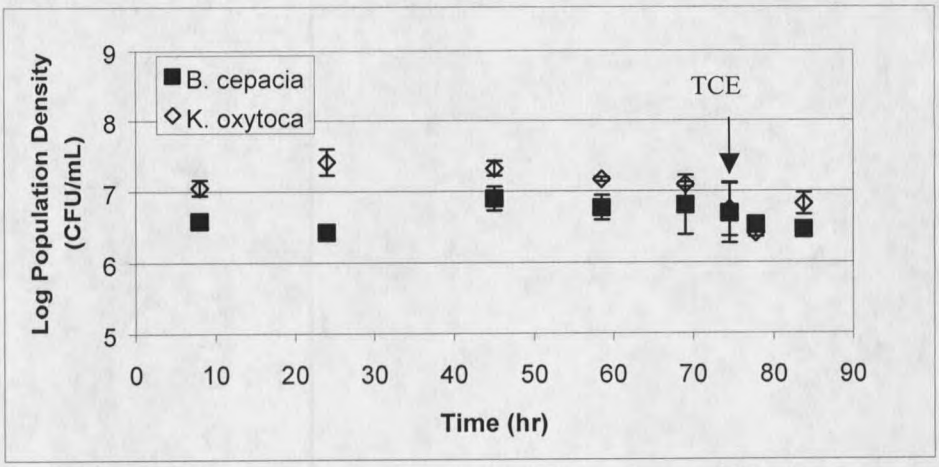

Figure 27. Dual-species porous media effluent plate counts of *K. oxytoca* and *B. cepacia* for the 70 mg/L DOC substrate concentration experiments. TCE was introduced to the column between t=74.5hr and t=83.75hr. Values are an average of duplicate experiments and error bars are +/- standard deviation.

The dual-species effluent population densities of *K. oxytoca* and *B. cepacia* over time for the 700 mg/L substrate concentration are presented in Figure 28. *K. oxytoca* was the dominant organism throughout the entire experiment and had an effluent population

density after 69 hours that was significantly greater than *B. cepacia* (*p-value* < *0.010*). *K. oxytoca's* effluent population density decreased slightly between t=69hr and t=74.75hr while *B. cepacia's* population density actually increased slightly. During TCE flow into the column, both organisms' population density remained stable.

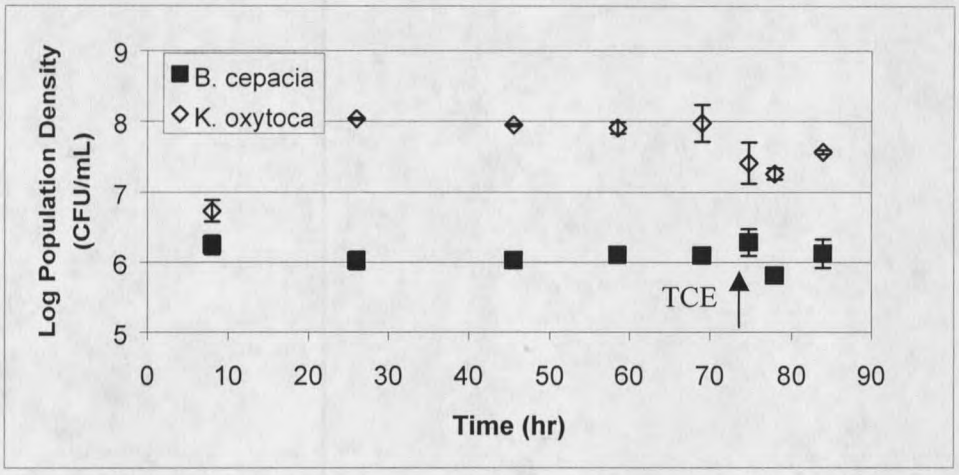

Figure 28. Dual-species porous media effluent plate counts of *K. oxytoca* and *B. cepacia* for the 700 mg/L DOC substrate concentration experiments. TCE was introduced to the column between t=74.8hr and t=83.9hr. Values are an average of duplicate experiments and error bars are +/- standard deviation.

Porous Media Population Density - Before TCE. After 70 hours of growth, each column was taken off-line and bacteria harvested from beads removed from the beginning and end of the column. This data was previous described in Table 12 and the paragraph preceding it.

For the 30 mg/L DOC substrate concentration, *B. cepacia's* porous media biofilm population density was an order of magnitude greater than *K. oxytoca*. *B. cepacia* and *K. oxytoca* had comparable porous media biofilm population densities at the 70 mg/L DOC substrate concentration. At the 700 mg/L DOC substrate concentration experiment there

was a shift in population distribution with *K. oxytoca's* population density higher than *B. cepacia's*.

Porous Media Population Density - After TCE. After TCE (initial concentration approximately 1.2 mg/L) was pumped through the columns for nine hours, the column was taken off-line and the biofilm harvested from the beginning, middle and end of the column (Table 17). *B. cepacia's* population density was higher than *K. oxytoca* throughout the length of the column for the 30 mg/L DOC substrate concentration. For the 70 mg/L DOC substrate concentration, *K. oxytoca* and *B. cepacia* had comparable population densities at the beginning of the column but *B. cepacia* had a higher population density in the middle and end of the column. For the 700 mg/L DOC substrate concentration experiment, *K. oxytoca's* population density at the beginning of the column (5.4×10^8 CFU/g bead) was two orders of magnitude higher (*p-value* < *0.001*) than *B. cepacia's* (6.3×10^6 CFU/g bead) but both organisms had comparable populations in the middle and end of the column.

Table 17. Dual-species porous media population densities after TCE addition was stopped. Values are an average of duplicate experiments +/- the standard deviation.

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

The biofilm populations from the entrance, middle, and exit of the column (Table 17) were averaged and are presented in Figure 29. *K. oxytoca's* population increased

between the 30 and 70 mg/L DOC substrate concentrations and between the 70 and 700 mg/L DOC substrate concentration. *B. cepacia's* population density remained constant between the 30 to 70 mg/L DOC substrate concentrations and between 70 and 700 mg/L DOC substrate concentration. *B. cepacia* had a higher population density at the 30 mg/L DOC substrate concentration but both organisms had comparable population densities at the 70 mg/L DOC substrate concentration experiments. *K. oxytoca* had the higher population density at the 700 mg/L substrate concentration experiment.

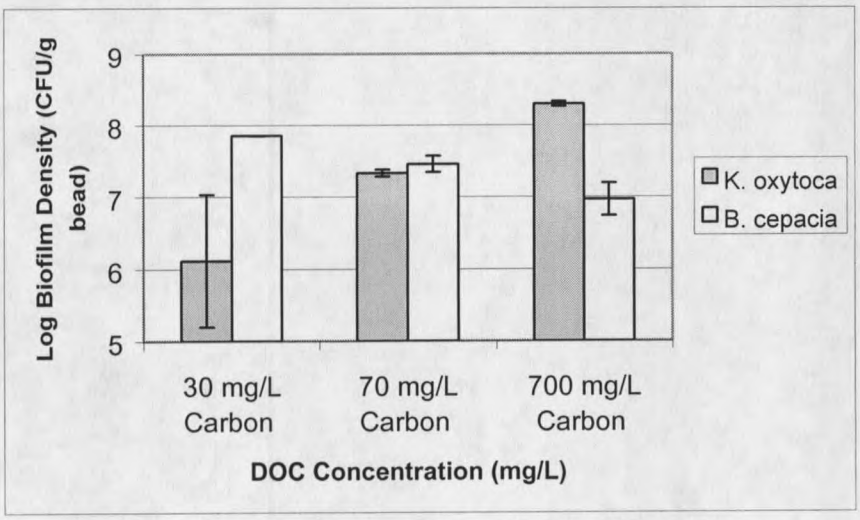

Figure 29. Dual-species porous media population densities of *K. oxytoca* and *B. cepacia* after TCE addition for three different substrate concentrations. Values are the average of duplicate experiments and error bars are +/- standard deviation.

TCE Degradation. TCE breakthrough curves were performed on the dual-species columns fed 30 mg/L DOC substrate concentration (Figure 30), 70 mg/L DOC substrate concentration (Figure 31), and 700 mg/L DOC substrate concentration (Figure 32). Probabilities were calculated by comparing the last four steady-state influent and effluent data points from duplicate experiments.

The dual-species TCE breakthrough curves for the 30 mg/L DOC substrate concentration experiments (Figure 30) show effluent TCE concentrations broke through the column at the same time as the abiotic TCE breakthrough curves (Figure 23) but had effluent TCE concentrations (0.59 mg/L) significantly lower (*p-value* < *0.001*) than the influent TCE concentrations (1.19 mg/L). The effluent TCE concentrations appeared to reach steady-state conditions after 150 minutes.

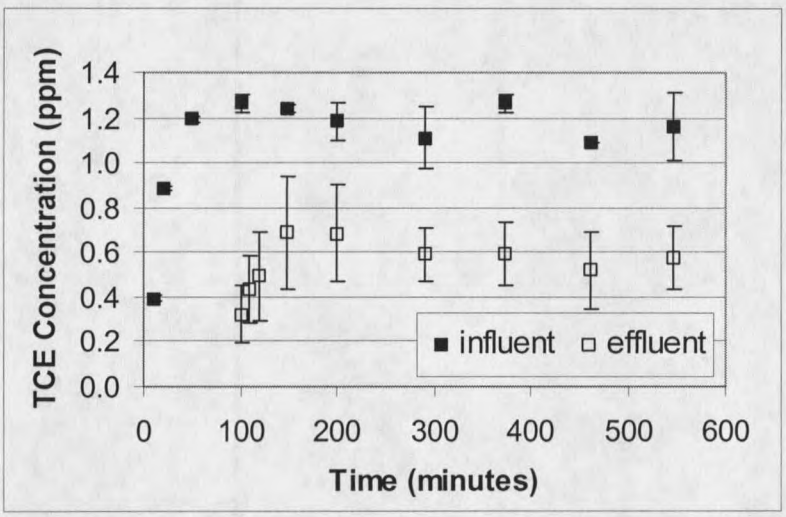

Figure 30. TCE Breakthrough curve for the dual-species column experiments fed 30 mg/L DOC substrate concentration. Values are average of duplicate experiments and error bars are +/- the standard deviation.

The dual-species TCE breakthrough curves for the 70 mg/L DOC substrate concentration experiments are presented in Figure 31. The steady-state influent TCE concentration (1.10 mg/L) is similar to the influent TCE concentration at the dual-species 30 mg/L substrate concentration experiment. The effluent steady-state TCE concentration (0.81 mg/L) was significantly lower (*p-value* < *0.001*) than the influent steady-state TCE concentration but not as low as the steady-state effluent concentration

in the 30 mg/L DOC substrate concentration TCE breakthrough curves; more TCE was removed at the lower substrate concentration experiment.

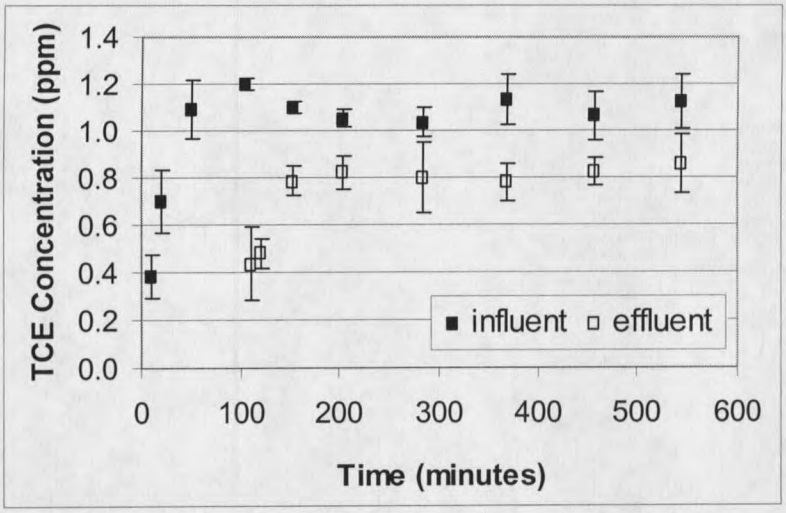

Figure 31. TCE breakthrough curve for the dual-species column experiments fed 70 mg/L DOC substrate concentration. Values are average of duplicate experiments and error bars are +/- the standard deviation.

The influent TCE steady-state concentration for the 700 mg/L DOC substrate concentration (1.15 mg/L) was similar to the other two experiments (30 and 70 mg/L DOC substrate concentrations). The effluent steady-state TCE concentration (1.16 mg/L) was comparable to the influent steady-state TCE concentration. Therefore, no removal of TCE was recorded between the influent and effluent of the 700 mg/L DOC substrate concentration columns (Figure 32).

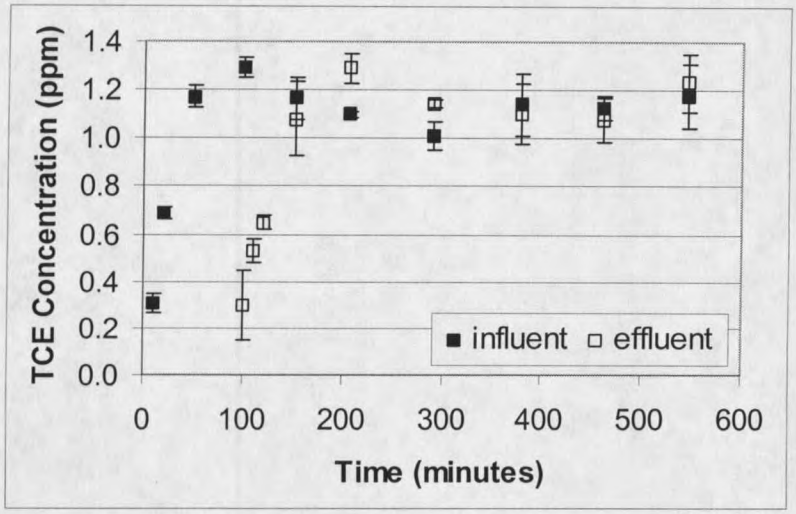

Figure 32. TCE Breakthrough curve for the dual-species column experiments fed 700 mg/L DOC substrate concentration. Values are average of duplicate experiments and error bars are +/- the standard deviation.

A percent removal was obtained by taking the difference between the influent and effluent steady-state TCE concentrations (Table 18) for the three substrate concentrations. The greatest amount of TCE degradation occurred in the low substrate concentration and increasing the substrate concentration caused a decrease in both TCE degradation and the specific TCE degradation rate calculated from equation 1. Sorption of TCE to biomass in the column was assumed to be negligible because no removal of TCE was observed at the high substrate concentration (where the highest population densities, mostly *K. oxytoca*, were observed).

Table 18. Dual-species TCE breakthrough curve data. Values are the average of replicate experiments +/- the standard deviation.

Substrate Concentration (mg/L DOC)	Steady-State Influent (mg/L)	Steady-State Effluent (mg/L)	% removal	Specific TCE Degradation Rate[1]
30	1.19 ± 0.04	0.59 ± 0.19	48.8 ± 16.5	0.024 ± 0.009
70	1.10 ± 0.05	0.81 ± 0.11	26.9 ± 6.9	0.014 ± 0.002
700	1.15 ± 0.02	1.16 ± 0.09	-0.5 ± 5.4	-0.0002 ± 0.004

[1] μg TCE/min/mg protein

Dissolved Oxygen Concentration. The constitutive degradation of TCE by *B. cepacia's* TOM pathway requires oxygen; therefore dissolved oxygen concentration throughout the column is an important variable. Munakata-Marr et al. (1997) reported the negative affect of oxygen utilization in a porous media column with respect to *B. cepacia's* ability to degrade TCE. Effluent oxygen concentrations were monitored over time for each experiment (Figure 33) using a Accumet AP64 series dissolved oxygen meter fitted to a flow through system made from a 40ml centrifuge tube with an O-ring and inlet and outlet ports tapped in the side. Only the low substrate concentration experiments (30 mg/L dissolved organic carbon) had measurable effluent oxygen concentrations.

TCE Degradation over Length of Column. The column with sampling ports was used to measure the fate of TCE over the length of the column for the three substrate concentrations. The 700 mg/L DOC substrate concentration experiments show a constant TCE concentration over distance (Table 19), indicating no loss of TCE in the column. The 30 mg/L DOC substrate concentrations show a decreasing TCE concentration over the length of the column. The 70 mg/L DOC substrate concentration experiments show a

decrease in TCE concentration from the influent sampling port to port 1 (6 cm into column) but then remains constant. The 30 mg/L DOC substrate concentration experiment had non-limiting effluent oxygen concentrations while the two higher substrate concentrations (70 and 700 mg/L DOC) were oxygen limited at the effluent (Figure 33). Oxygen present throughout the column allowed for a continual decrease of TCE throughout the column at the 30 mg/L DOC substrate concentration while the oxygen could have become limited before the first sampling port at the 70 mg/L DOC substrate concentration experiment, resulting in no more TCE degradation.

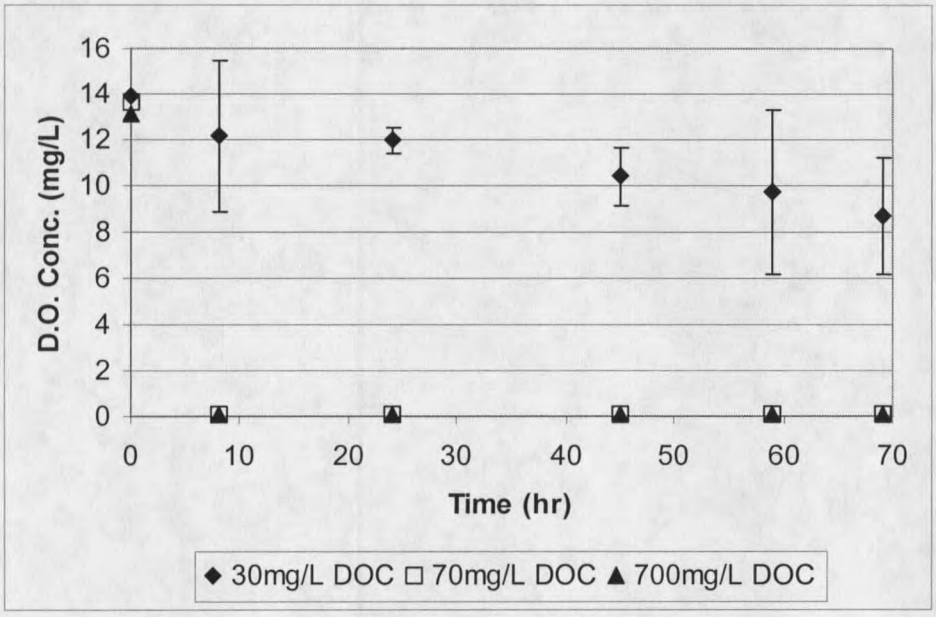

Figure 33. Effluent dissolved oxygen concentration over time. Data is average of replicate experiments and error bars are +/- standard deviation.

Table 19. TCE concentration (mg/L) over length of 30 cm long column for dual-species experiments.

Substrate Conc. (mg/L DOC)	Influent	Port 1 (6 cm)	Port 2 (14.5 cm)	Port 3 (22 cm)	Effluent
30	1.27	0.45	0.28	0.23	0.48
70	1.14	0.73	0.71	0.71	0.78
700	1.09	1.00	1.12	1.20	1.15

Note that the TCE concentrations inside the column for the 30 mg/L DOC substrate concentration were lower than the effluent concentration (Table 19). The values for each sampling port were taken from the center of the column. Figure 34 shows TCE concentrations from the center and along the wall of the column for the 30 mg/L DOC substrate concentration. TCE concentrations were higher along the wall of the column compared to the middle at each sampling port. This could be due to channeling of TCE along the side of the column or a lower TCE degrading population on the side of the column compared to the middle (thus higher TCE concentrations).

Permeability Reduction in Porous Media

Substrate concentration could be used to control population distribution and TCE degradation in the porous media column. The effect of substrate concentration on hydraulic conductivity was also examined. The hydraulic conductivity of the porous media column was monitored over time for each substrate concentration (Figure 35) by measuring the hydraulic head across the porous media column and inputting it, along with the flow rate, surface area and length of column, into Darcy's Law (eq. 8). The initial hydraulic conductivities for all three experiments ranged from 0.85 to 0.95

cm/min. The hydraulic conductivity for each substrate concentration reached steady-state conditions after approximately 20 hours. There was minimal hydraulic conductivity reduction for the 30 mg/L DOC substrate concentration experiment and the highest hydraulic conductivity reduction was recorded in the 700 mg/L DOC substrate concentration.

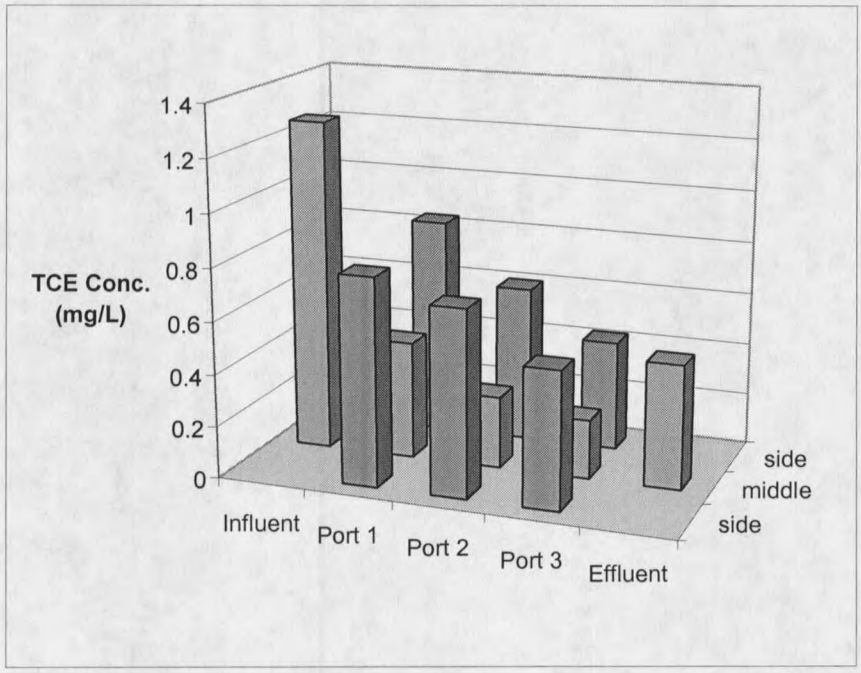

Figure 34. Steady-state TCE concentrations in porous media column inoculated with both organisms for the 30 mg/L DOC substrate concentration.

A summary of this data, as well as percent hydraulic conductivity reduction, can be found in Table 20. At the 30 mg/L DOC substrate concentration, a steady-state hydraulic conductivity of 0.79 cm/min was recorded. This resulted in a hydraulic conductivity reduction of less than 10%. The steady-state hydraulic conductivity was 0.52 cm/min during the 70 mg/L DOC substrate concentration experiment which resulted in a hydraulic conductivity reduction of 39%. An order of magnitude increase in

substrate concentration (70 to 700 mg/L DOC) resulted in a steady-state hydraulic conductivity of 0.19 cm/min, which resulted in a hydraulic conductivity reduction of 79%. An increase in substrate concentration resulted in an increased reduction in hydraulic conductivity.

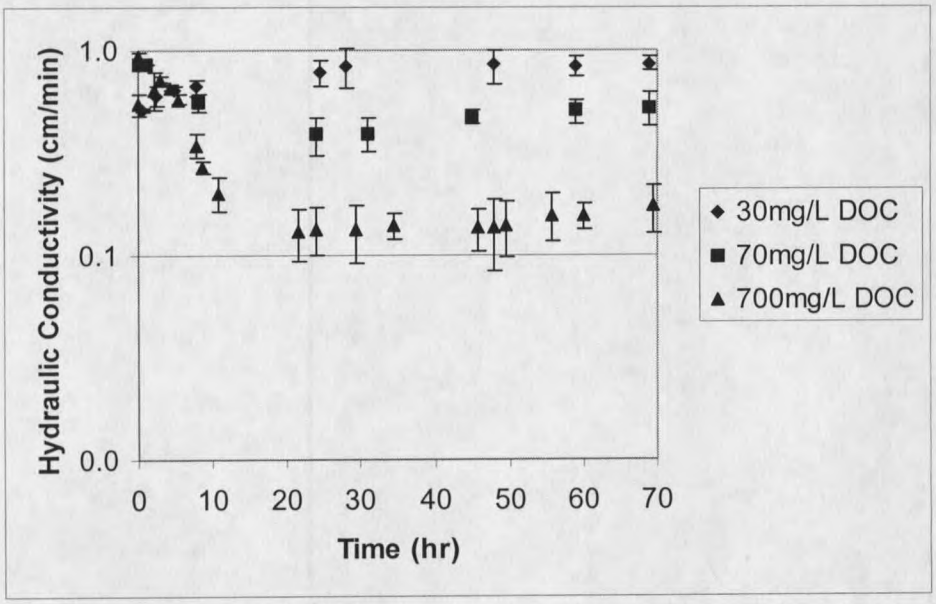

Figure 35. Hydraulic conductivity over time. Values are the average of duplicate experiments and error bars are +/- standard deviation.

Table 20. Summary of hydraulic conductivity (K) data for the three substrate concentrations. Data is average of duplicate experiments +/- standard deviation.

Substrate Conc. (mg/L DOC)	$K_{initial}$ (cm/min)	$K_{steady\ state}$ (cm/min)	Hydraulic Conductivity Reduction (%)
30	0.85 ± 0.02	0.79 ± 0.21	7.3
70	0.86 ± 0.0	0.52 ± 0.06	38.9
700	0.94 ± 0.04	0.19 ± 0.06	79.2

CHAPTER 4

DISCUSSION

Single- and dual-species batch, rotating disk and porous media reactor experiments were performed to better understand the interactions between two microbial species when grown together in an engineered biofilm. The main goal of these experiments was to determine what variables influenced the creation and control of a dual-species biofilm. The feasibility of using batch single- and dual-species growth kinetics to describe dual-species interactions in biofilms on the surface of a well mixed reactor and in porous media was examined. The two organisms used throughout these experiments were *Burkholderia cepacia* and *Klebsiella oxytoca.*

B. cepacia can constitutively mineralize trichloroethylene (TCE) under aerobic conditions and *K. oxytoca* can form thick biofilms capable of plugging porous media. In addition to studying the population dynamics of these two organisms, the effects of dual-species interaction on cell activity (TCE degradation or permeability reduction) in porous media for different substrate concentrations were examined.

Planktonic (Batch) Experiments

A series of batch reactor experiments were performed using single- and dual-species cultures of *K. oxytoca* and *B. cepacia*. The experiments used two different substrate concentrations (different dilutions of LBG media) resulting in dissolved organic

carbon (DOC) concentrations of approximately 70 and 700 mg/L. These experiments examined the effects of specific growth rate on each organism's population density in pure or dual-species cultures. The effect of substrate concentration on specific growth rate and population density was observed in single- and dual-species cultures.

Effect of Substrate Concentration
on Single-Species Growth

The stationary phase population densities of *K. oxytoca* for the 70 mg/L and 700 mg/L DOC substrate concentrations were 1.2×10^8 CFU/mL and 1.1×10^9 CFU/mL, respectively. The stationary phase population densities of *B. cepacia* for the 70 mg/L and 700 mg/L DOC substrate concentrations were 6.7×10^7 CFU/mL and 6.8×10^8 CFU/mL, respectively. An order of magnitude increase in substrate concentration resulted in an order of magnitude increase in stationary phase population density for both organisms in single-species. Bühler et al. (1998) also reported stationary phase population density to be proportional to substrate concentration.

Effect of Substrate Concentration on
Single-Species Specific Growth Rate

K. oxytoca's growth rate was 0.76/hr for both the 70 and 700 mg/L DOC substrate concentration batch experiments and *B. cepacia's* growth rate was 0.30/hr for both the 70 and 700 mg/L DOC substrate concentration batch experiments. 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 substrate concentrations.

Effect of Growth Rate on
Single-Species Population Density

K. oxytoca's growth rate (0.76/hr) was 2.5 times greater than *B. cepacia's* (0.30/hr) for each substrate concentration. *K. oxytoca's* stationary phase population density at the 70 and 700 mg/L DOC substrate concentration was 1.2 x 10^8 CFU/mL and 1.1 x 10^9 CFU/mL, respectively. *B. cepacia's* stationary phase population density was 6.7 x 10^7 CFU/mL and 6.8 x 10^8 CFU/mL, respectively. *K. oxytoca's* stationary phase population density was 1.8 and 1.6 times *B. cepacia* stationary phase population density. The organism that had the higher growth rate also had a slightly higher single-species stationary phase population density. A growth rate 2.5 times higher in the faster growing organism did not result in a stationary phase population density that was 2.5 times higher than the slower growing organism's population density. Even though growth rate predicted which organism would have the higher population density in single-species experiments, it did not fully predict the stationary phase difference in population density. Therefore, growth rate is not the only variable responsible for an organism's stationary phase single-species population density.

Growth Curve Analysis

The single-species batch growth curves for the 70 and 700 mg/L DOC substrate concentration experiments (Figures 4 and 5, respectively) were used to determine the lag, exponential (log) and stationary phases of growth for both organisms (Table 21). Lag phase was defined as the period of time between inoculation of bacteria and start of exponential growth. *K. oxytoca* had a lag phase of three hours for both substrate

concentrations which was half as long as *B. cepacia's* lag phase (six hours). Exponential growth was defined as the period when the rate of growth remained constant (linear growth on log scale). *K. oxytoca's* exponential growth phase for the 70 and 700 mg/L substrate concentration experiments was eight and ten hours, respectively. This was significantly shorter than the duration of *B. cepacia's* exponential phase (17 and 22 hours, respectively). *B. cepacia's* longer lag and exponential growth phase resulted in *B. cepacia* entering stationary phase 12 and 15 hours after *K. oxytoca* for the 70 and 700 mg/L DOC substrate concentration experiments (Table 21).

Substrate concentration had a slight effect as to when each organism enters stationary phase. For both organisms, increasing the substrate concentration increased the time to the start of stationary phase. The *K. oxytoca* population density starts to level off and appears to enter stationary phase after 11 hours for the 70 mg/L DOC substrate concentration and 13 hours for the 700 mg/L substrate concentrations. The *B. cepacia* populations entered stationary growth phase after 23 hours for the 70 mg/L DOC substrate concentration and 28 hours for the 700 mg/L DOC substrate concentration. A longer exponential growth phase with the 700 mg/L DOC substrate concentration compared to the 70 mg/L DOC substrate concentration explains why the stationary phase population densities were an order of magnitude higher in the 700 mg/L DOC substrate concentration experiment when no change in growth rate was observed for each organism. Results from the dissolved organic carbon (DOC) analysis show substrate utilization to be a function of substrate concentration (more DOC utilized at the higher

substrate concentration) and that a majority of dissolved organic carbon remained in the batch reactor, even when exponential growth ceased.

Table 21. Summary of single-species planktonic growth curves.

	Duration of Lag Phase (hr)		Duration of Exponential Growth Phase (hr)		Time to Late Log/Early Stationary Phase (hr)	
	K.O.	B.C.	K.O.	B.C.	KO	B.C.
70 mg/L	3	6	8	17	11	23
700 mg/L	3	6	10	22	13	28

Dual-Species Population Dynamics

Similar to the single-species experiments, the results of the *K. oxytoca* dual-species batch experiments show an order of magnitude increase in population density over time with an order of magnitude increase in substrate concentration. The stationary phase population densities of *K. oxytoca* for the low and high substrate concentrations are 1.6×10^8 CFU/mL and 1.1×10^9 CFU/mL, respectively. These values are similar to the single-species *K. oxytoca* population densities. Therefore, the presence of *B. cepacia* had no measurable effect on the *K. oxytoca* stationary phase population density. The stationary phase population densities of *B. cepacia* for the low and high substrate concentrations are 1.6×10^6 CFU/mL and 3.8×10^6 CFU/mL, respectively. *B. cepacia's* stationary phase population density was one to two orders of magnitude less in the presence of *K. oxytoca* than by itself. The presence of *K. oxytoca* inhibited the *B. cepacia* population from reaching the population densities observed in the single-species experiments.

Single- and Dual-Species Growth Rates

When the two organisms were combined in one culture, both *K. oxytoca's* and *B. cepacia's* growth rates (0.75/hr and 0.29/hr, respectively) were similar to each organism's single-species growth rates. Stewart et al. (1997) observed this same trend when *Klebsiella pneumonia* KP1 and *Pseudomonas aeruginosa* ERC1 were grown in single- and dual-species batch cultures. For batch conditions, single-species growth rates could be used to describe dual-species interactions.

Effect of Growth Rate on Dual-Species Population Density

In single-species culture, both organisms grew to similar stationary phase population densities, though *B. cepacia* grew at a significantly slower rate than *K. oxytoca*. When the two organisms were combined in a batch culture, the faster growing organism was virtually unaffected by the slower growing organism while the slower growing organism's stationary phase population density was decreased by two to three orders of magnitude. Therefore, growth rate determined the population distribution in a dual-species batch reactor. A similar interaction was observed when *Escherichia coli* and *Staphylococcus aureus* were grown in batch cultures by themselves and combined together in a dual-culture (Oberhofer and Frazier, 1960). In single-species, both organisms grew to similar population densities, but *E. coli* grew measurably faster than *S. aureus*. When the two organisms were combined, *E. coli* grew to its single-species stationary phase population density while *S. aureus* was negatively affected and grew to a significantly lower population density.

Stationary Phase of Growth

A leveling of the planktonic population densities after a period of exponential growth was observed. In the single-species experiments, this leveling was due to the populations reaching stationary phase of growth. In this stage, cell growth is balanced by cell decay and thus no change in the viable population density is observed. Stationary phase in a single-species batch reactor can be caused by utilization of the limiting growth nutrient or build-up of a waste product to an inhibitory level to that organism (Monod, 1949). A majority of the initial dissolved organic carbon (DOC) remained at the end of all batch experiments suggesting that carbon is not the limiting nutrient. Therefore either another limiting nutrient or the production of an inhibitory by-product may be the reason for the cultures entering stationary phase. It is also important to note that the limiting factor that caused each organism to enter stationary phase in a single-species culture may not be the same.

Dual-Species Batch Interactions

In a dual-species batch reactor, stationary phase can be caused by the negative influence of another population. Table 22 contains a summary of the growth parameters (growth rate, stationary phase population density, and time to stationary phase) for the single- and dual-species batch experiments at both substrate concentrations. In the dual-species batch experiments, *B. cepacia* grew at the same rate as when it was in single-species but entered stationary phase much earlier, thus limiting its final population density. *B. cepacia* entered stationary phase after approximately 15 hours of growth in a

dual-species culture compared to entering stationary phase after approximately 25 hours in single species. It is also interesting to note that in a dual-species culture, *B. cepacia* and *K. oxytoca* entered stationary growth at the same time (approximately 15 hours) which was the time *K. oxytoca* entered stationary growth in the single-species experiments. This suggests that the utilization of the same limiting nutrient or production of the same inhibitory byproduct that caused *K. oxytoca* to enter stationary phase in single species also caused both *K. oxytoca* and *B. cepacia* to enter stationary phase in a dual-species culture. This provides an explanation as to why a faster growing organism "out-competes" the slower growing organism in planktonic systems. The organism with the higher growth rate is able to multiply faster and thus will have a higher population density when the limiting variable causes both populations to enter stationary phase. This assumption is true if both organisms have the same limiting nutrient or if both are affected by an inhibitory by-product.

Table 22. Summary of single- and dual-species planktonic experiments.

	Stationary Population Density (CFU/mL) x 10^8		Specific Growth Rate (1/hr)		Time to Stationary Phase (hr)	
	K.O.	B.C.	K.O.	B.C.	K.O.	B.C.
Single-Species						
70 mg/L	1.2	0.67	0.76	0.30	11	23
700 mg/L	11	6.8	0.76	0.30	13	28
Dual-Species						
70 mg/L	1.6	0.038	0.68	0.28	13	13
700 mg/L	11	0.016	0.81	0.29	15	16

Factors that Limit Microbial Growth

As mentioned previously, many factors can influence the growth of microorganisms in a batch culture. In all of the batch experiments, the cultures entered stationary phase even though a significant amount of dissolved organic carbon remained in the reactor. The below section explores the feasibility of other growth factors, such as pH, inhibitory byproducts, nutrient utilization, and dissolved oxygen limitation, causing the batch cultures to enter stationary phase.

To measure the effect of pH in the batch cultures, dual-species batch experiments were performed that monitored pH over time for the 70 and 700 mg/L DOC substrate concentrations (Figure 36). The sampling time points were before, during and after the dual-species populations entered stationary phase in a batch reactor. Negligible change in pH over time was recorded for these dual-species batch reactors.

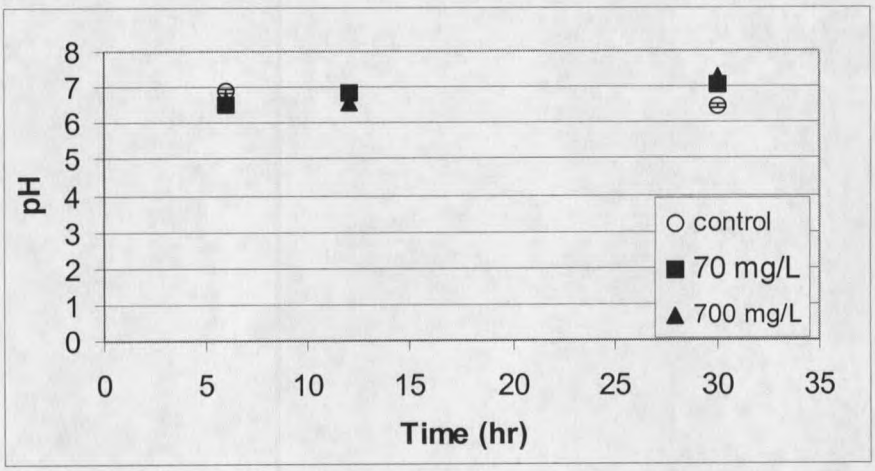

Figure 36. Graph of pH over time for dual-species batch cultures of *K. oxytoca* and *B. cepacia* at substrate concentration of 70 and 700 mg/L DOC. The control was sterile 700 mg/L DOC substrate concentration media. Values are average of duplicate experiments and error bars are +/- the standard deviation.

The accumulation of toxic by-products in the batch reactor could have caused the organisms to enter stationary phase. An example of this would be one or both of the organisms in a dual-species culture producing organic acids, which would lower the pH and inhibit growth in the culture. The limitation of growth through the production of byproducts, however, is contrary to the fact that an increase in substrate concentration resulted in an increase in the stationary phase population density for both single- and dual-species results. Accumulation of a byproduct would presumably be caused when an organism reached a particular density, regardless of substrate concentration. However, it was observed that an order of magnitude increase in substrate causes an order of magnitude increase in the stationary phase population density. This implies that inhibitory byproducts did not cause the bacterial cultures to enter stationary phase (at least at the 70 mg/L DOC substrate concentration).

Utilization of a limiting nutrient when the organisms reach a certain population (10^8 CFU/mL for the 70 mg/L DOC and 10^9 CFU/mL for the 700 mg/L DOC) is a viable reason for the organisms to enter stationary phase. Nutrients essential for microbial growth include carbon, nitrogen, phosphorous, as well as trace nutrients and minerals. For single- and dual-species batch reactors supplied either 70 or 700 mg/L DOC, it appears that carbon was not the limiting growth factor because the population reaches stationary phase with a majority of the dissolved organic carbon (DOC) remaining in the batch reactor. It is important to note that the carbon came from three different sources. The media contained dextrose, yeast extract and tryptone, all of which are sources of

carbon. The organisms could have preferentially utilized one carbon source more than the other two.

To determine if oxygen utilization could cause the batch cultures to enter stationary phase, a series of dual-species batch experiments were performed to monitor oxygen utilization over time for the 70 and 700 mg/L DOC substrate concentrations (Figure 37). The sampling time points were before, during and after the dual-species populations entered stationary phase in a batch reactor. Oxygen concentrations remained constant over time for the 70 mg/L DOC substrate concentration. The oxygen transfer into solution from constant mixing at room temperature compensated for the oxygen demand from the bacterial populations. The higher substrate concentration (700 mg/L DOC) batch culture had a higher oxygen demand than the 70 mg/L DOC substrate concentration experiment due to the order of magnitude higher population density. Oxygen-limiting values were recorded at 12 hours, which is just before the batch culture entered stationary phase and is the period of highest oxygen utilization. Oxygen demand decreased in stationary phase and the oxygen concentration was able to rebound to non-oxygen limiting values. These results suggest that oxygen limitation did not cause the batch cultures to stay in stationary phase.

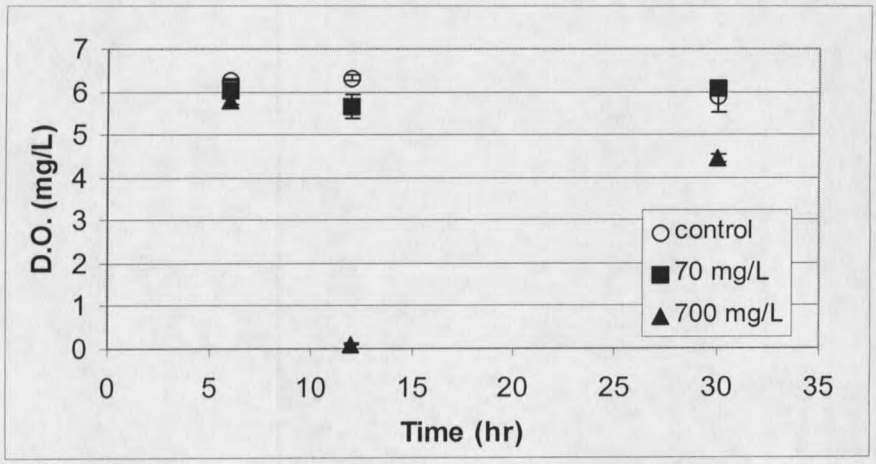

Figure 37. Dissolved oxygen concentrations over time for dual-species batch cultures of *K. oxytoca* and *B. cepacia* at substrate concentration of 70 and 700 mg/L DOC. The control was sterile 700 mg/L DOC substrate concentration media. Values are average of duplicate experiments and error bars are +/- the standard deviation.

The limiting factor that caused stationary phase could have been utilization of some other nutrient. Nitrogen, phosphorus and even trace elements (Mason and Egli, 1993) have been reported to limit growth. Competition is defined as an interaction where two populations compete for a growth-limiting nutrient that is essential to both organisms (James et al. 1995). If growth was limited by a nutrient essential to both organisms, this dual-species batch interaction would be a classic example of competition, with the faster growing organism dominating the population before the limiting nutrient is utilized.

Effect of Biofilm Growth on Planktonic Competition

Two bacterial populations competing for a single limiting nutrient in a spatially homogeneous environment leads to exclusion of one of the competitors if the system is at steady-state (time-invariant) conditions (Fredrickson and Stephanopoulos, 1981). This

assumes that all bacteria are planktonic, but biofilm growth on the surface of a batch reactor is unavoidable. Efforts can be made to minimize the effect of biofilm growth in a batch reactor. These include decreasing the surface-to-volume ratio of the reactor or transferring the batch solution to a sterile reactor periodically throughout the experiment. Chao and Ramsdell (1985) examined this phenomenon in a multi-species batch reactor and Baltzis and Fredrickson (1983) in a multi-species chemostat culture. Both concluded that biofilm on the surface of a reactor prevented the slower growing organism from being completely eliminated from the liquid. In this experiment, growth rate seemed to determine which organism would have a higher population density and biofilm growth is a possible explanation as to why *B. cepacia*, the slower growing organism, was not completely eliminated from the system.

Rotating Disk Reactor Experiments

A series of rotating disk reactor experiments were performed to monitor biofilm growth on the surface of a "well mixed" reactor. The batch experiments demonstrated that growth rate could be used to predict which organism would dominate a dual-species culture. The rotating disk reactor experiments examined if growth rate (or some other variable) could predict population distribution in a biofilm. Steady-state suspended and biofilm population densities, as well as biofilm growth rates, in single- and dual-species rotating disk reactor experiments were compared to the batch experiments.

Effect of Substrate Concentration
on Suspended Population Density

Selective plate counts from the effluent of the "well mixed" rotating disk reactor yielded suspended cell population densities over time for the single- and dual-species experiments. The suspended cell populations of each organism at the 70 mg/L and 700 mg/L substrate concentrations reached steady-state values after approximately 50 hours. With no cells added to the reactor over the course of the experiment, the suspended population density is equal to the net detachment from the biofilm and reproduction of the suspended cells. Measures were taken to minimize suspended cell growth by setting the detention time of the fluid in the completely mixed reactor significantly less than the planktonic growth rate of the faster growing organism. Using the planktonic growth rate values obtained from the batch experiments, the cell doubling time of the faster growing organism was calculated using equation 3 to be 0.91 hours. The detention time of the rotating disk reactor was 0.55 hours.

The leveling of the suspended population density in the rotating disk reactor experiments was due to bacterial growth in the biofilm balanced by bacterial detaching from the biofilm, resulting in steady-state net detachment. This is dependent on the organisms present, the organism's ability to attach to a surface, the surface characteristics, the nutrients supplied, and competition for nutrients and available attachment sites.

Single-Species. In the single-species reactors, the steady-state *K. oxytoca* suspended population density was three times greater than *B. cepacia* for both the 70 mg/L and 700 mg/L DOC substrate concentration. An order of magnitude increase in

substrate concentration (70 to 700 mg/L DOC) resulted in a three-fold increase in *K. oxytoca*, but only a slight increase in *B. cepacia's* suspended population density.

Dual-Species. In the dual-species experiments, *B. cepacia's* steady-state suspended population density was slightly higher than *K. oxytoca's* at both the 30 and 70 mg/L DOC dual-species experiment. At the 700 mg/L DOC substrate concentration dual-species experiment, a shift in the population distribution occurred. *K. oxytoca* had a steady-state population density that was 75 times the *B. cepacia* population density. An increase in substrate concentration resulted in a significant increase in the *K. oxytoca's* population density between the 30 and 700 mg/L DOC substrate concentration (*p-value* < *0.030*). *B. cepacia's* suspended population increased slightly between the 30 and 70 mg/L DOC substrate concentrations and decreased significantly between the 70 and 700 mg/L DOC substrate concentrations (*p-value* < *0.002*).

Single vs Dual Species Suspended Population Density

For the 700 mg/L DOC substrate concentration, the dominant organism in the dual-species culture (*K. oxytoca*) had a similar suspended population density compared to *K. oxytoca's* single-species steady-state population density, while the non-dominant organism (*B. cepacia*) had a suspended population density that was significantly less (*p-value* < *0.015*) than when grown in single-species (Figure 38). This suggests that *K. oxytoca* was not affected by the presence of *B. cepacia* but *B. cepacia* was negatively affected by the presence of *K. oxytoca* at the 700 mg/L DOC substrate concentration. This same trend in population distribution was observed in the dual-species batch

experiments for both substrate concentrations. For the 70 mg/L DOC substrate concentration, the dominant organism in the dual-species culture (*B. cepacia*) had a higher population density compared to *B. cepacia's* steady-state population density when grown in single-species ($p\text{-value} < 0.015$). The non-dominant organism (*K. oxytoca*) had a suspended population density that was comparable to its single-species population density (Figure 38). Similar to the 700 mg/L DOC substrate concentration, *K. oxytoca* was not affected by the presence of *B. cepacia*. The presence of *K. oxytoca* caused *B. cepacia* to have a slight, though statistically significant, increase in suspended population density at the 70 mg/L DOC substrate concentration.

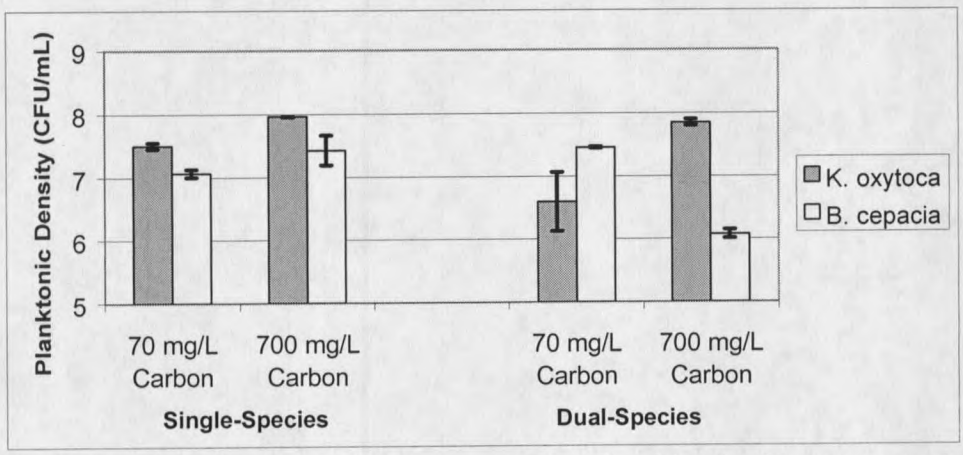

Figure 38. Steady-state suspended population densities for *K. oxytoca* and *B. cepacia* in single- and dual-species rotating disk reactor experiments. Values are an average of duplicate experiments +/- the standard deviation.

The sum of the steady-state suspended population densities of both organisms in the dual-species experiment had values similar to the single-species *B. cepacia's* and *K. oxytoca's* steady-state suspended population for the 700 mg/L substrate concentrations. The sum of the of the dual-species suspended cell population for the 70 mg/L DOC

substrate concentration was similar to the single-species *K. oxytoca* population density at 70 mg/L DOC substrate concentration, but statistically higher than *B. cepacia's* single-species suspended population density at 70 mg/L DOC (*p-value* < *0.020*).

Effect of Substrate Concentration
on Biofilm Population Distribution

Single-Species. Results from the single-species rotating disk reactor show that *K. oxytoca* had a steady-state biofilm population density that was higher than the *B. cepacia* population density at the 70 mg/L substrate concentration. Both organisms had comparable biofilm population densities at the 700 mg/L substrate concentration. An increase in substrate concentration resulted in an increase in the *B. cepacia* population density and a decrease in the *K. oxytoca* population density. All single-species biofilm population densities were within an order of magnitude of each other (Table 4).

Dual-Species. In the dual-species experiments, an increase in substrate concentration resulted in an increase in *K. oxytoca* biofilm population density (Figure 39). The dual-species *B. cepacia* biofilm population density remained constant when the substrate concentration was increased from 30 to 70 mg/L DOC and decreased slightly when the substrate concentration was increased from 70 to 700 mg/L DOC. *B. cepacia* was the dominant organism in the dual-species biofilm at the 30 mg/L DOC substrate concentrations. When the substrate concentration was increased to 700 mg/L DOC, there was a shift in the steady-state biofilm population density with *K. oxytoca* now having a higher population density than *B. cepacia*. This shift in dual-species population dynamics with change in substrate concentration also was observed in the suspended cell

populations of the rotating disk reactors. Varying the substrate concentration provided a mechanism to control the fraction of each organism in the dual-species biofilm.

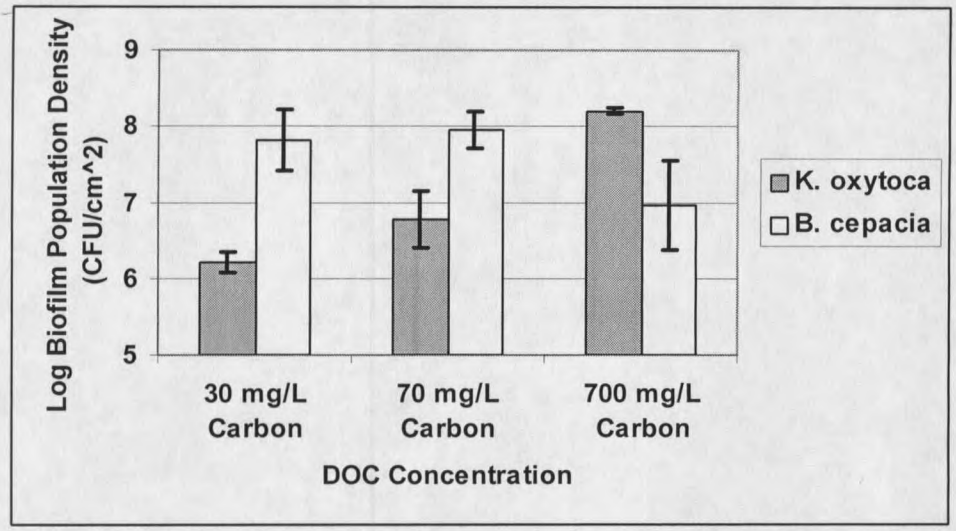

Figure 39. Dual-species biofilm population densities of *K. oxytoca* and *B. cepacia* at steady-state for three different substrate concentrations. Values are the average of duplicate experiments and error bars are +/- standard deviation.

Single- vs. Dual-Species Biofilm Comparison

Single- and dual-species biofilm populations can be compared between the 70 and 700 mg/L DOC substrate concentration. At 70 mg/L DOC, the dual-species population of *B. cepacia* was similar to *B. cepacia's* single-species biofilm population density (Figure 40). *K. oxytoca's* biofilm population in the presence of *B. cepacia* was significantly lower than *K. oxytoca's* single-species population density at the 70 mg/L DOC substrate concentration experiment ($p\text{-value} < 0.025$). The presence of *K. oxytoca* had a neutral affect on the *B. cepacia* biofilm population, but the presence of *B. cepacia* had a negative affect on the *K. oxytoca* population density at the 70 mg/L DOC substrate concentration. At the 700 mg/L DOC substrate concentration, the dual-species population

of *B. cepacia* was lower than *B. cepacia's* single-species biofilm population density (Figure 40). *K. oxytoca's* biofilm population in the presence of *B. cepacia* was similar to *K. oxytoca's* single-species population density. The presence of *B. cepacia* had a neutral affect on the *K. oxytoca* biofilm population, but the presence of *K. oxytoca* had a negative affect on the *B. cepacia* population density at the 700 mg/L DOC substrate concentration.

The sum of the *K. oxytoca* and *B. cepacia* steady-state biofilm population density in dual-culture for both the 70 and 700 mg/L DOC substrate concentration experiments was similar to the *K. oxytoca* and *B. cepacia* single-species steady state biofilm values, regardless of the substrate concentration. This implies that there is a similar maximum biofilm population that can be achieved in this experimental system.

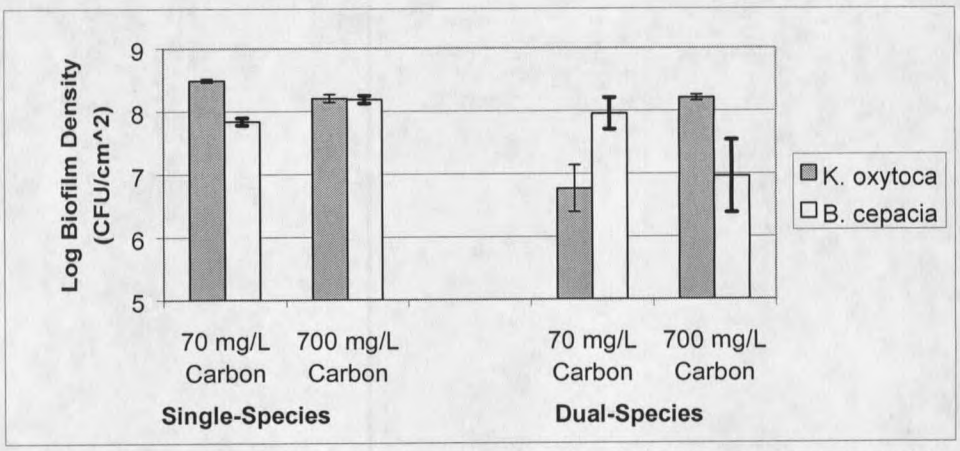

Figure 40. Steady-state biofilm population densities for *K. oxytoca* and *B. cepacia* in single- and dual-species rotating disk reactor experiments. Values are an average of duplicate experiments +/- the standard deviation.

Biofilm Growth Rate Analysis

The rate of biofilm growth is dependent on the organism(s) present, the substrate concentration, mass transfer into the biofilm and competition for available nutrients. At steady-state, growth in the biofilm is equal to detachment in the biofilm.

Single-Species. The single-species biofilm growth rate of *K. oxytoca* increased five-fold when the substrate concentration was increased an order of magnitude (70 to 700 mg/L DOC) while *B. cepacia's* growth rate remained constant with change in substrate concentration (Table 6). At the 70 mg/L DOC substrate concentration, *B. cepacia's* single-species biofilm growth rate was twice *K. oxytoca's* single-species biofilm growth rate. At the 700 mg/L DOC substrate concentration, *K. oxytoca* single-species biofilm growth rate was twice *B. cepacia's* single-species biofilm growth rate. *K. oxytoca* had a higher biofilm population density and a lower biofilm growth rate at the 70 mg/L DOC substrate concentration. Both organisms had comparable biofilm population densities at the 700 mg/L DOC substrate concentration even though their growth rates were different (*B. cepacia's* growth rate was lower than *K. oxytoca's*). Therefore, single-species biofilm growth rate failed to predict which organism would have the greater single-species biofilm population in single-species experiments.

Dual-Species. Dual-species biofilm growth rates were calculated for each organism at three substrate concentrations (Table 10). *K. oxytoca's* growth rate was slightly higher than *B. cepacia's* growth rate for all three dual-species experiments, though not significantly different. Both organisms' growth rate increased slightly from

the 30 mg/L to 70 mg/L DOC substrate concentration experiments and decreased slightly from the 70 mg/L to 700 mg/L DOC substrate concentration experiments, though these changes were not statistically significant.

Single vs. Dual-Species Growth Rates. When *K. oxytoca* was combined with *B. cepacia*, its biofilm growth rate increased significantly at the 70 mg/L DOC substrate concentration and decreased slightly at the 700 mg/L DOC substrate concentration compared to the single-species biofilm growth rates (Figure 41). *B. cepacia's* dual-species growth rate did not significantly change at both substrate concentrations compared to the single-species biofilm growth rates.

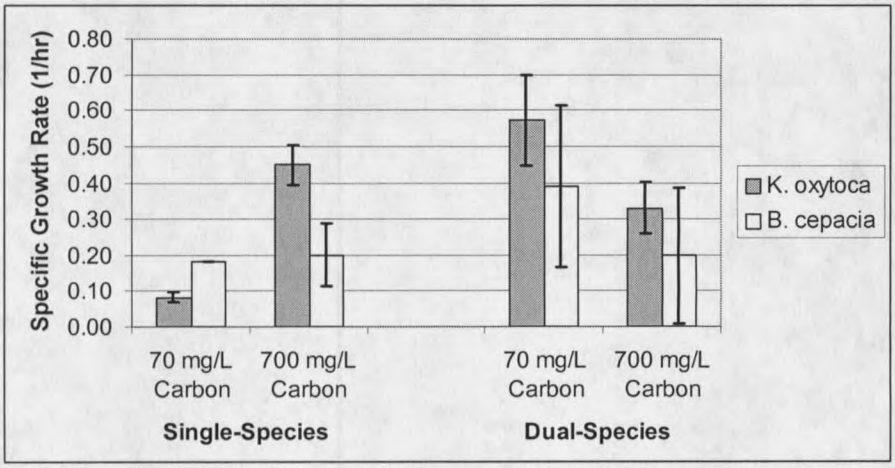

Figure 41. Specific biofilm growth rates for *K. oxytoca* and *B. cepacia* in single- and dual-species rotating disk reactor experiments. Values are an average of duplicate experiments +/- the standard deviation.

Dissolved Organic Carbon

The rate by which nutrients are utilized in a completely mixed stirred tank reactor (CSTR) is dependent on the detention time of the nutrients in the system, the rate of

consumption by planktonic organisms and the diffusion into the biofilm and subsequent utilization by biofilm organisms. All three inoculation scenarios (*K. oxytoca* single-species, *B. cepacia* single-species, and dual-species) resulted in comparable dissolved organic carbon (DOC) utilization at each substrate concentration. More DOC was utilized at the highest substrate concentration (700 mg/L DOC) than the lowest substrate concentration (30 mg/L DOC). For the single- and dual-species rotating disk reactor experiments there was greater than 50% of the dissolved organic carbon remaining in the effluent of each reactor for the entire duration of the five-day experiment.

Effect of Substrate Concentration on
Dual-Species Population Distribution

Substrate concentration could be used to predict the dual-species population density in a biofilm. At the high substrate concentration (700 mg/L DOC), *K. oxytoca* was the dominant organism in the dual-species biofilm. 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. Varying the substrate concentration can regulate the fraction of each organism in this dual-species biofilm. This same trend was also observed by Camper et al. (1996) where slower growing organisms were able to persist at higher cell concentrations in low nutrient (oligotrophic) environments. Therefore, growth rate can predict planktonic dual-species population distribution but cannot be used to predict the fraction of each organism in a dual-species biofilm.

Previous research has observed similar changes in population dynamics from varying substrate concentration. In a chemostat experiment using oral bacterial

populations, Marsh et al. (1983) 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. One of the organisms isolated at higher frequencies at high substrate concentrations had the ability to produce EPS, as does *K. oxytoca*. This suggests that an EPS-producing organism may have a selective advantage at higher substrate concentrations. In addition, Cleland et al. (1997) reported that certain organisms become an "ecological opportunist" when nutrient concentrations are increased. Another possible explanation could be increased oxygen utilization at higher substrate concentrations causing the aerobic bacteria, *B. cepacia*, to be hindered due to oxygen limitation. In a mixed culture biofilm experiment containing a majority of aerobic heterotrophs and a small fraction of NH4-oxidizers and NO2-oxidizers, Okabe et al. (1996) observed that increasing the acetate concentration resulted in retarded accumulation of the slower growing nitrifying bacteria. It was concluded that this decrease in population of one type of organism was due to competition for dissolved oxygen and space in the biofilm. According to r-and K- selection theory (Andrews and Harris, 1986), organisms can either have a higher growth rate or higher affinity to a particular substrate. Organisms that do well at low nutrient concentrations have a low growth rate and high affinity for the substrate. This provides yet another possible explanation. *B. cepacia* could have a higher affinity to the substrate and therefore is able to survive at higher population densities than the faster growing *K. oxytoca* at the lower substrate concentration.

Using substrate concentration to control each organism's population density could provide the desired population density of each organism to perform the desired reaction(s) in an engineered dual-species biofilm.

Porous Media Discussion

Results from the column experiments were used to compare dual-species interactions in porous media. Biofilms grown in porous media differ from the biofilms grown on the surface of a well-mixed reactor. Both types of biofilms could experience substrate limitation deep in the biofilm, but in a porous media system substrate limitation could occur in the bulk fluid. This could have a negative effect on aerobic organisms throughout a porous media reactor if oxygen is utilized at the beginning of the reactor. In addition, hydrodynamic differences could influence the size of the biofilm formed between a rotating disk reactor and a porous media reactor. The effects of these differences on the population distribution and growth rate will be addressed.

Effluent Population Density

Effluent plate counts from the porous media column provided a population distribution of the suspended cells exiting the column. Eight hours into the experiment, *K. oxytoca's* population in the effluent was higher than *B. cepacia* for all experiments. The final effluent concentration of each organism, however, varied with respect to substrate concentration. After 70 hours, *B. cepacia* had a higher effluent population density than *K. oxytoca* at the 30 mg/L DOC substrate concentration while *K. oxytoca* had a higher effluent population density at the 700 mg/L DOC substrate concentration

(Figure 42). An increase in substrate concentration from 30 to 70 mg/L DOC and 70 to 700 mg/L DOC resulted in a significant increase in *K. oxytoca's* effluent population density (*p-value* < *0.050*). A significant decrease in *B. cepacia* effluent population density occurred between the 30 and 700 mg/L DOC substrate concentration (*p-value* < *0.001*). Substrate concentration had an important affect on the dual-species effluent population densities and species composition.

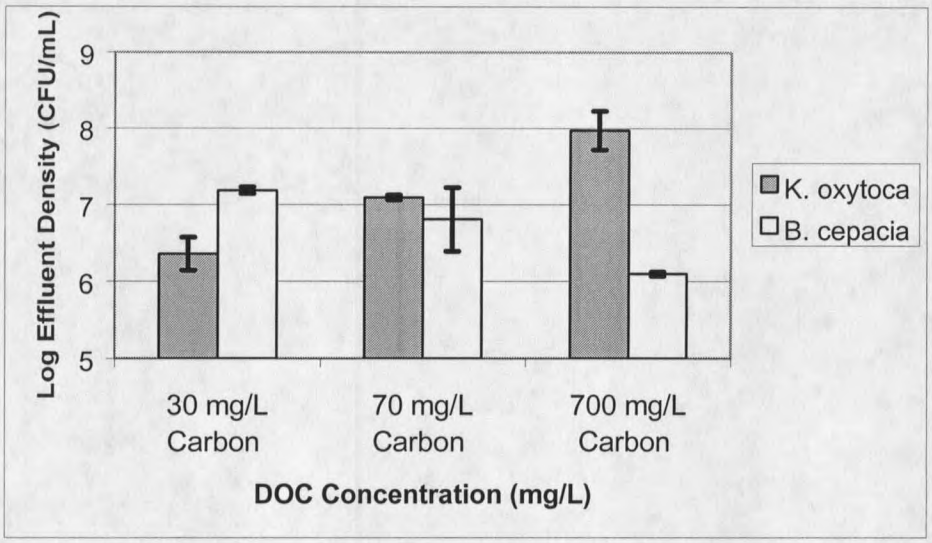

Figure 42. Dual-species effluent population densities of *K. oxytoca* and *B. cepacia* at steady-state in the porous media column for three different substrate concentrations. Values are the average of duplicate experiments and error bars are +/- standard deviation.

Porous Media Population Density

For all three substrate concentration column experiments, *K. oxytoca's* population density decreased from the beginning to the end of the column (Table 12). *B. cepacia's* population density over the length of the column remained constant at the 30 and 70 mg/L DOC substrate concentration and increased at the 700 mg/L DOC substrate

concentrations experiments (Table 12). *B. cepacia*'s presence throughout the column (even during 70mg/L and 700 mg/L DOC substrate loading where oxygen is limited) is interesting due to the fact that *B. cepacia* is a strict aerobe. Whether *B. cepacia* is active or dormant further into the column will be addressed in the TCE degradation discussion. The faster growing organism might preferentially populate the beginning of the column where nutrient concentrations are highest while the slower growing organism is more adapted to sections of the column where nutrient supplies are less. In addition, *B. cepacia* is characterized as having extreme nutritional versatility (Madigan et al. 1997). This may enable *B. cepacia* to scavenge nutrients unusable by *K. oxytoca*.

The porous media biofilm population densities from the beginning and end of the column were averaged and compared for each substrate concentration (Figure 43). As the substrate concentration increased, *K. oxytoca's* population density increased, but *B. cepacia's* population density remained constant between the 30 mg/L and 70 mg/L DOC substrate concentrations and then decreased at the 700 mg/L DOC substrate concentration. *B. cepacia's* biofilm population in porous media was significantly higher than *K. oxytoca* at the 30 mg/L substrate concentration experiment. Both organisms had similar biofilm population densities at the 70 mg/L DOC substrate concentration experiment. At the 700 mg/L DOC substrate concentration experiment there was a shift in population distribution with *K. oxytoca's* population density higher than *B. cepacia's*. These trends are similar to the biofilm population densities recorded in the rotating disk reactor experiments (Figure 39), except the disparity between the two populations at the 30 and 700 mg/L DOC substrate concentrations is greater in the porous media column

than in the rotating disk reactor. Varying substrate concentration provided a mechanism to control the fraction of each organism in dual-species biofilm and porous media environments.

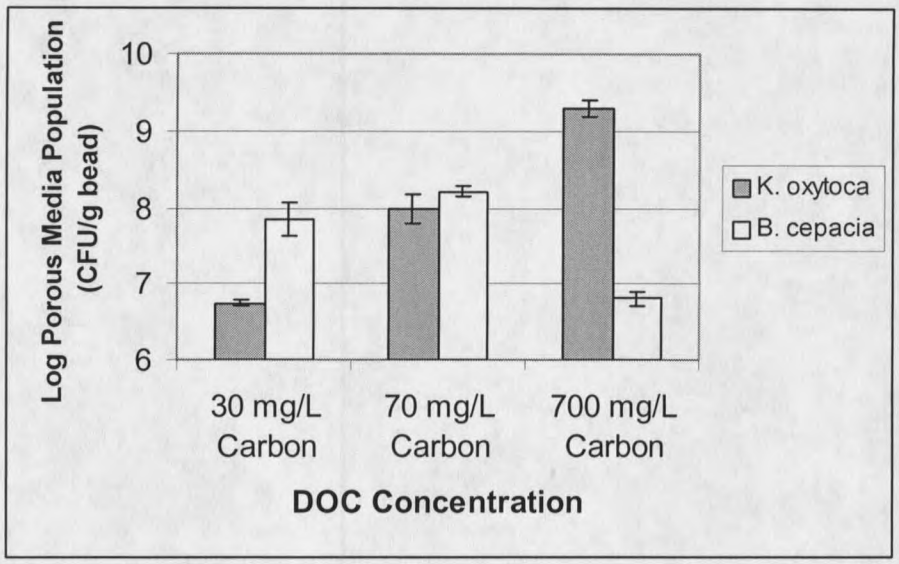

Figure 43. Dual-species porous media population densities of *K. oxytoca* and *B. cepacia* at steady-state for three different substrate concentrations. Values are the average of duplicate experiments and error bars are +/- standard deviation.

Porous Media Growth Rates

Biofilm specific growth rates were obtained for the three substrate concentrations (30, 70 and 700 mg/L DOC) in the porous media column (Figure 44). Increasing the substrate concentration did not cause a significant change in either *B. cepacia's* or *K. oxytoca's* biofilm growth rate in porous media, suggesting zero order growth kinetics between 30 and 700 mg/L DOC substrate concentrations. *K. oxytoca's* growth rate was comparable to *B. cepacia's* growth rate at the 30 and 70 mg/L substrate concentrations. *B. cepacia's* growth rate at the 700 mg/L DOC substrate concentration experiment was

higher than *K. oxytoca's* growth rate. The average biofilm growth rate in the porous media for all three substrate concentration experiments ranged from 0.01/hr to 0.08/hr.

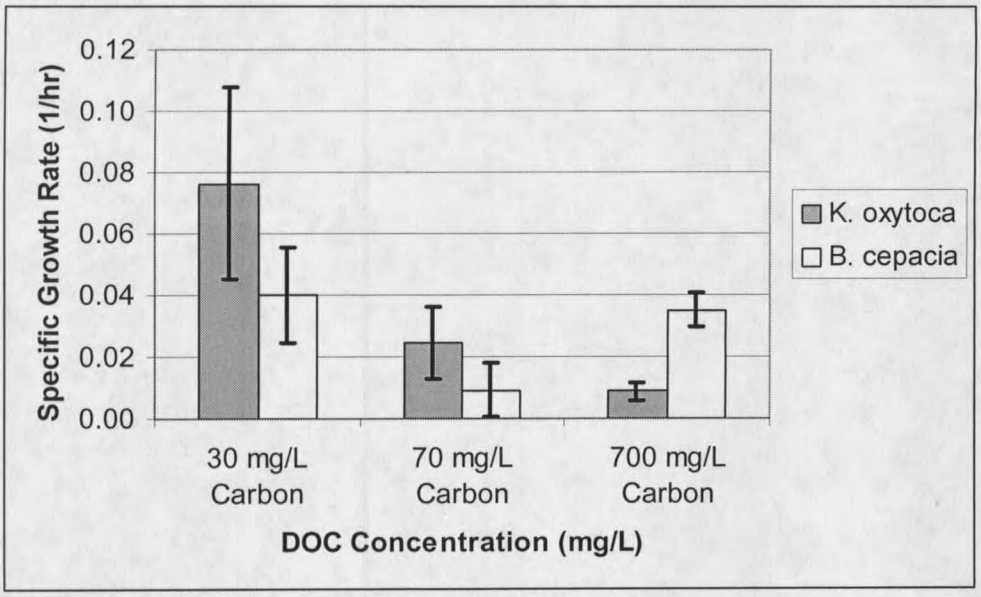

Figure 44. Dual-species porous media specific biofilm growth rates of *K. oxytoca* and *B. cepacia* at steady-state for three different substrate concentrations. Values are the average of duplicate experiments and error bars are +/- standard deviation.

Effect of Oxygen Concentration
on DOC Utilization

The influence of dissolved oxygen on dissolved organic carbon (DOC) utilization was examined by comparing the dissolved oxygen concentrations from the effluent of the porous media column (Figure 33) and the steady-state DOC concentrations from the influent and effluent of the column (Table 15) to the theoretical utilization of carbon under aerobic conditions. The substrate used for all experiments was different dilutions of LBG media, which contains more than one carbon source. Therefore, the DOC concentrations are the sum of carbon from glucose, tryptone and yeast extract. Using the chemical formula for glucose ($C_6H_{12}O_6$), the amount of oxygen utilized per carbon

consumed was estimated to be 1.1 g O_2/g carbon. The amount of DOC utilized over the length of the column increased with increasing substrate concentration (Table 15), though there was only a finite amount of oxygen available. There was 13.5 +/- 0.66 mg/L of dissolved oxygen in the effluent of the column before bacteria were added to each column, but measurable steady-state dissolved oxygen concentrations were recorded only at the lowest (30 mg/L DOC) substrate concentration (9.6 +/- 1.2 mg O_2/L). Using the theoretical estimation of DOC utilization and the difference in initial and steady-state oxygen concentrations, the amount of DOC utilized aerobically was 3.5 mg/L, 12.3 mg/L, and 12.3 mg/L for the three substrate concentrations (30, 70 and 700 mg/L DOC, respectively). These values are lower than what was measured in the column experiments (9.4 mg/L, 21.4 mg/L, 99.0 mg/L for the 30, 70 and 700 mg/L DOC, respectively). This data suggests that when oxygen was depleted in the reactor (or sections of the reactor), the facultative organism (*K. oxytoca*), was able to utilize additional DOC under anaerobic conditions.

Comparison of Population Dynamics in Different Reactor Systems

The single- and dual-species interactions between *K. oxytoca* and *B. cepacia* were compared for three different substrate concentrations in three different reactor systems. The substrate concentrations used in this study (30, 70 and 700 mg/L DOC) were chosen to provide over an order of magnitude variation in dissolved organic carbon concentration. The reactor systems chosen for this research allowed for the examination

of dual-species interactions in planktonic cultures, as well as biofilms grown in well-mixed and porous media environments.

Batch and Rotating Disk Reactor
Planktonic Population Distribution

Planktonic (suspended) population densities were monitored over time for the batch and the rotating disk reactor experiments. A leveling of the planktonic population densities over time in both reactors was observed and this leveling occurred for different reasons. Bacteria growing to stationary phase caused the leveling observed in the batch growth curves, while a balance of growth and detachment in the biofilm with growth and "wash-out" of the suspended culture caused the leveling of the planktonic population in the rotating disk reactor. As discussed in the batch results, the stationary phase was likely caused by either the utilization of a limiting nutrient or production of byproducts (which could be described simply as "overcrowding"). Both of these phenomena would not occur to the same degree in the RDR because nutrients are constantly being re-supplied and waste is constantly being removed. Therefore it would be misleading to compare the bacterial interactions in the batch reactors to that of the bulk fluid region of the rotating disk reactors.

Batch vs Biofilm
Population Distribution

A series of batch and rotating disk reactor experiments were performed to compare dual-species population distributions in a planktonic (batch) culture and a biofilm on the surface of a well-mixed reactor. *K. oxytoca* "outcompeted" *B. cepacia* in a

dual-species batch culture at two different substrate concentrations (70 and 700 mg/L DOC). In a dual-species rotating disk reactor biofilm fed 700 mg/L DOC, *K. oxytoca* had over an order of magnitude greater steady-state population density than *B. cepacia*, but the difference was not significant using a 0.05 level of confidence for the duplicate experiments. At the 70 mg/L DOC substrate concentration, there was a switch in the population distribution. *B. cepacia* had an order of magnitude higher population density than *K. oxytoca*, though the difference was not statistically significant. The results from a lower substrate concentration (30 mg/L DOC) experiment show that *B. cepacia* did have a significantly higher steady-state biofilm population density than *K. oxytoca* in a biofilm. Therefore *K. oxytoca* was the dominant organism in the batch dual-species experiments, but was not always the dominant organism in a dual-species biofilm. Varying substrate concentration did not affect the dual-species population distribution in a batch culture, but did affect the population distribution in a biofilm on the surface of a well-mixed reactor.

Rotating Disk Reactor Biofilm
vs. Porous Media Biofilm

A series of porous media column experiments were performed to compare biofilm population distribution in porous media to that of biofilm population distribution on the surface of a well-mixed reactor. The results show that *K. oxytoca* was the dominant organism in the porous media biofilm at the high (700 mg/L DOC) substrate concentration and *B. cepacia* was the dominant organism at the low (30 mg/L DOC) substrate concentration. The 70 mg/L DOC substrate concentration showed no

significant difference in the biofilm population of the two organisms in dual-species. Therefore, the same shift in biofilm population distribution with change in substrate concentration observed in the well-mixed reactor was observed in the porous media reactor, except the population disparity between the two organisms in the porous media biofilm was significant enough to show a statistical difference at the two extreme substrate concentrations (30 and 700 mg/L DOC). Varying substrate concentration provided a mechanism to control the population density and fraction of total population of *K. oxytoca* and *B. cepacia* in a dual-species biofilm.

The total biofilm population (*B. cepacia* + *K. oxytoca*) of the rotating disk reactor at steady-state was calculated and compared with the total biofilm population of the porous media column at steady-state (converted to CFU/cm^2) (Figure 45). The total biofilm population in the rotating disk reactor remained constant with change in substrate concentration while the porous media biofilm population increased with increasing substrate concentration. The results show that the porous media column had a higher biofilm population per surface area for all three substrate concentrations.

Specific Growth Rate Comparison Between Different Reactor Systems

An important variable of microbial growth is the rate at which an organism multiplies in single cultures or in combination with other species. An understanding as to how an organism will adapt and grow in different environments is essential in determining how that organism will persist in a particular environment.

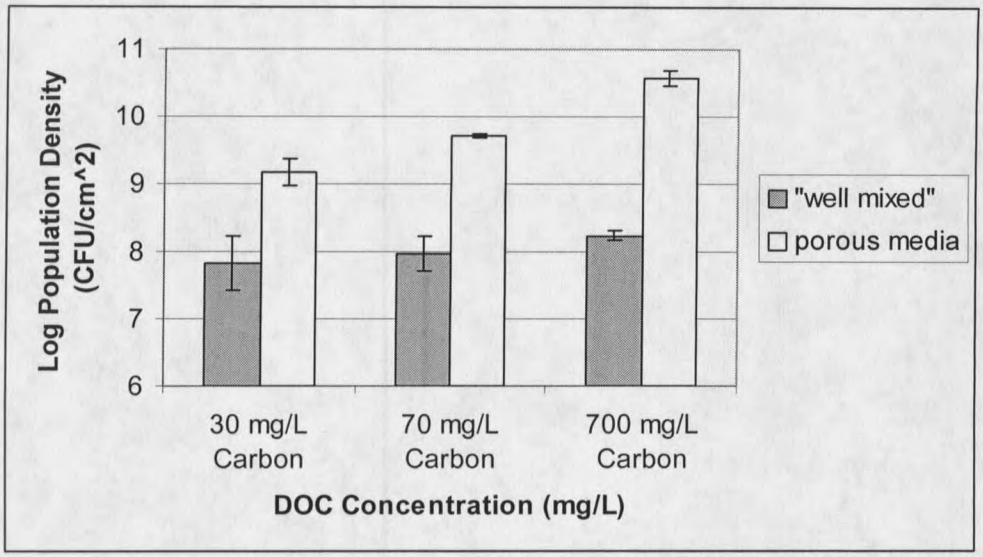

Figure 45. Comparison of total biofilm population density for the dual-species "well mixed" (rotating disk reactor) and porous media biofilm reactors at three different substrate concentrations (30, 70 and 700 mg/L DOC). Values are average of duplicate experiments +/- standard deviation.

The growth rate of *K. oxytoca* and *B. cepacia* in batch systems did not change significantly between single- and dual-species experiments or when grown in 70 and 700 mg/L DOC substrate concentration. In all batch experiments, *K. oxytoca* had a higher growth rate than *B. cepacia*. The single-species biofilm growth rates were lower than the planktonic single-species growth rates for both organisms at the 70 mg/L DOC substrate concentration (*p-values* < *0.030*). At the 700 mg/L DOC substrate concentration, *K. oxytoca's* single-species biofilm growth rate was lower than its batch single-species growth rate (*p-value* < *0.030*), while *B. cepacia's* growth rates did not significantly change. The biofilm growth rates in the dual-species experiments were comparable to the dual-species batch growth rates at the 70 mg/L DOC substrate concentration (Figure 46). At the 700 mg/L DOC substrate concentration, *B. cepacia's* dual-species biofilm growth rate was comparable to its batch dual-species growth rate,

but *K. oxytoca's* was significantly lower (*p-value* < *0.025*). All dual-species porous media biofilm specific growth rates were between 0.01/hr and 0.08/hr for both organisms at all three substrate concentrations (Figure 46). The dual-species porous media growth rates were significantly lower than the batch and biofilm growth rates, except for *B. cepacia* in the 700 mg/L DOC substrate concentration biofilm experiment (Figure 45). Higher porous media biofilm population densities coupled with comparable suspended cell concentrations explain the significantly lower growth rates in the porous media biofilm compared to the rotating disk reactor biofilm. Wentland et al. (1996) reported that biofilm growth rates varied with thickness of *Klebsiella pneumoniae* biofilm colonies. A 70 μm colony had a specific growth rate of 0.3/hr in the center of the colony while a 250 μm colony had a specific growth rate in the center of the colony of <0.05/hr. The data presented in Wentland et al. (1996) suggests that thicker biofilms will have lower biofilm growth rates. In addition, Ellis et al. (2000) reported a negative correlation between growth rate and biofilm cell density. This, combined with the higher biofilm population densities in porous media, provides an explanation for the discrepancies in growth rate between a biofilm in a well-mixed environment and a biofilm in a porous media environment.

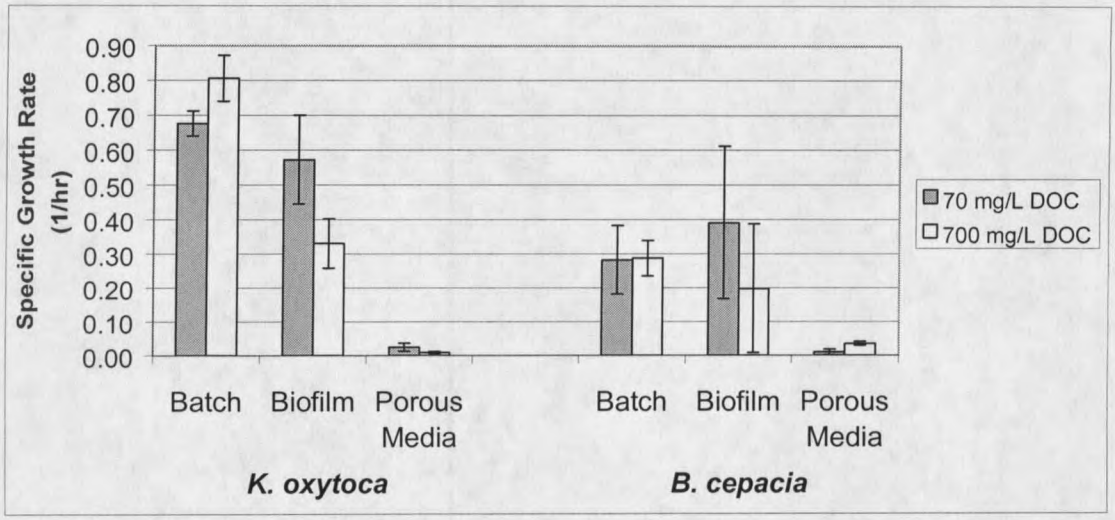

Figure 46. Dual-species growth rates for the three reactor systems. Values are average of duplicate experiments +/- the standard deviation.

Effects of Growth Rate on Population Distribution

The planktonic growth rates, calculated from the single- and dual-species batch growth curves, predicted which organism would have the higher population density in the dual-species planktonic experiments. In addition, little change in each organism's growth rate was recorded with change in substrate concentration (70 to 700 mg/L DOC) or between single- to dual-species cultures. *Burkholderia cepacia* was out-competed by the faster growing *Klebsiella oxytoca* in a batch reactor suggesting that growth rate could be used to predict the fraction of each organism in a dual-species planktonic culture.

B. cepacia's single-species biofilm growth rate was greater than *K. oxytoca's* single-species biofilm growth rate at the 70 mg/L DOC substrate concentration, which coincided with *B. cepacia* being the dominant organism in the dual-species biofilm at that substrate concentration. Likewise, *K. oxytoca's* single-species biofilm growth rate was greater than *B. cepacia's* single-species biofilm growth rate at the 700 mg/L DOC

substrate concentration, which coincided with *K. oxytoca* being the dominant organism in the dual-species biofilm at that substrate concentration. Therefore, a correlation between single-species biofilm growth rates and dual-species biofilm population distributions was observed.

According to existing descriptions of biofilm population dynamics (Wanner and Gujer, 1986), the faster growing organisms should out-compete the slower growing organism. Dual-species biofilm growth rates, however, did not predict dual-species population distributions. *K. oxytoca* and *B. cepacia* had comparable dual-species biofilm growth rates at the 30, 70 and 700 mg/L substrate concentrations, but *K. oxytoca* had a higher population density in the dual-species culture at the 700 mg/L DOC substrate concentration while *B. cepacia* had a higher population density at the 30 mg/L DOC substrate concentration. Therefore, dual-species biofilm growth rates did not predict the dual-species biofilm population density in a biofilm.

TCE Degradation in Porous Media

Burkholderia cepacia and *Klebsiella oxytoca* can be combined together in a porous media column and substrate concentration is an important variable in controlling the fraction of each organism in a dual-species engineered biofilm. TCE degradation in porous media was compared between single-species (*B. cepacia* alone) and dual-species cultures to determine the effect of *K. oxytoca* on *B. cepacia's* ability to degrade TCE. In addition, the effect of substrate concentration on TCE degradation in a dual-species

column was then explored to determine if the activity of a dual-species engineered biofilm could be controlled.

Single- vs Dual-Species TCE Degradation

The results of the *B. cepacia* pure culture and dual-species TCE breakthrough curves for the 30 mg/L DOC substrate concentration were compared. The dual-species columns removed 48.8% of the TCE compared to 79.1% with *B. cepacia* alone. Therefore, *K. oxytoca* had a negative affect on *B. cepacia's* ability to degrade TCE.

Specific TCE Degradation Rate

The specific TCE degradation rate of *B. cepacia* in a pure culture (0.029 μg TCE/min/mg protein), and in combination with *K. oxytoca* (0.024 μg TCE/min/mg protein), was similar for the 30 mg/L DOC substrate concentration experiments. Therefore, the presence of *K. oxytoca* did not affect *B. cepacia's* specific TCE degradation rate. An increase in substrate concentration resulted in a decrease in *B. cepacia's* specific TCE degradation rate in the dual-species experiments.

Biofilm specific TCE degradation rates in porous media were lower than batch TCE degradation rates for *B. cepacia* $PR1_{31c}$ (0.76 μg TCE/min/mg protein) reported by Sharp et al. (1998a) for the same TCE concentration (1.2 mg/L). Lower TCE degradation activity in biofilm cultures compared to batch cultures has been previously reported (Sharp et al. 1998b). One possible reason for the lower rates in porous media is overestimation of the TCE degrading population. The specific TCE degradation rate equation (equation 1) assumes that the entire *B. cepacia* population of the column was

actively degrading TCE. Oxygen utilization along the length of the column (observed in the 70 and 700 mg/L DOC substrate concentration experiments) as well as diffusion limitation of TCE and oxygen into the biofilm on the surface of the glass beads could limit the number of *B. cepacia* cells actively degrading TCE. Lower specific TCE degradation rates corresponded with significantly lower specific growth rates in porous media compared to batch cultures.

Effect of Substrate Concentration on TCE Degradation

Varying substrate concentration manipulated the population distribution in porous media. Substrate concentration also played a very important role in controlling TCE degradation in dual-species porous media columns (Figure 47). Increasing the substrate concentration caused a decrease in TCE degradation. Twice as much TCE was removed from the column supplied 30 mg/L DOC compared to 70 mg/L DOC, yet the TCE-degrading population was slightly higher at the 70 mg/L DOC substrate concentration experiment. The increase in TCE degradation at the 30 mg/L DOC substrate concentration was attributed to oxygen limitation in the middle and end of the column for the 70 mg/L DOC substrate concentration experiments. Oxygen was limited throughout the porous media columns using 700 mg/L DOC substrate concentration. No TCE was degraded at the 700 mg/L DOC substrate concentration.

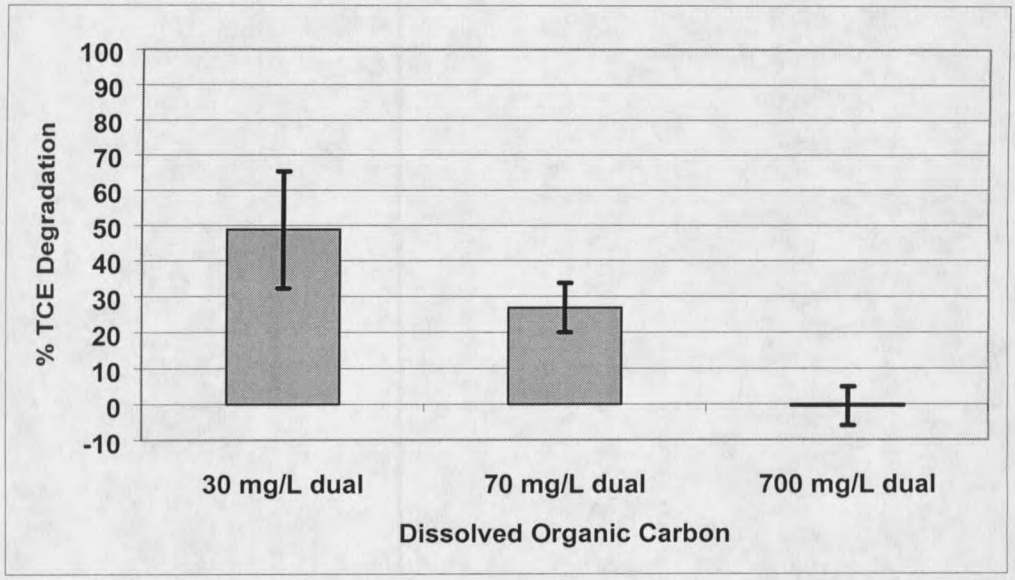

Figure 47. Percent TCE degradation for the dual-species porous media column experiments at three different dissolved organic carbon concentrations (30 mg/L, 70 mg/L and 700 mg/L DOC). Values are an average of duplicate experiments +/- the standard deviation.

Effect of Oxygen Limitation on
B. cepacia Population Density

Dissolved oxygen concentrations from the effluent of the porous media column show that oxygen was below detection limits at the end of the column for the 70 and 700 mg/L DOC substrate concentrations. In addition, lack of TCE degradation in the porous media columns corresponded with the lack of oxygen. Recall that *B. cepacia*, the TCE-degrading organism, is a strict aerobe and the degradation of TCE by *B. cepacia* is an aerobic process. It is interesting to note that no significant change in *B. cepacia's* population density was observed at the end of the column between the 30 and 70 mg/L DOC substrate concentration or between the 70 and 700 mg/L DOC substrate concentration. Though no significant TCE degradation was observed at the end of the porous media column for the 70 and 700 mg/L DOC substrate concentrations (Table 19),

there was still a relatively high *B. cepacia* biofilm population (7.8×10^8 CFU/g bead and 1.1×10^7 CFU/g bead, respectively). This implies that *B. cepacia* is viable but not capable of TCE degradation at the end of the column.

Permeability Reduction in Porous Media

K. oxytoca can reduce porous media permeability through thick biofilm formation (Warwood et al. 1995). Hydraulic conductivity measurements were used to quantify the effect of substrate concentration on permeability reduction in the porous media columns. The hydraulic conductivity was measured by monitoring the hydraulic head across the porous media column and inputting it, along with the flow rate, surface area, and length of column, into Darcy's Law (eq. 8).

The initial hydraulic conductivities for all three experiments ranged from 0.85 to 0.95 cm/min, which is a hydraulic conductivity typical of clean sand (Freeze and Cherry, 1979). At the 30 mg/L DOC substrate concentration, a hydraulic conductivity reduction of less than 10% was recorded. During the 70 mg/L DOC substrate concentration experiment, a steady-state hydraulic conductivity reduction of 39% was recorded. An order of magnitude increase in substrate concentration (70 to 700 mg/L DOC) resulted in a steady-state hydraulic conductivity reduction of 79%. This increase in hydraulic conductivity reduction corresponded with an increase in *K. oxytoca* population density (Figure 42). An increase in substrate concentration also caused oxygen-limiting conditions. *K. oxytoca* is a facultative organism and can survive without oxygen through fermentation (Madigan et al. 1997). This change in metabolism could influence the type

or magnitude of mucoid biofilm produced. Therefore, permeability reduction appears to be a function of *K. oxytoca's* population density and possibly a function of *K. oxytoca's* metabolic changes from oxygen stress.

Warwood et al. (1995) measured hydraulic conductivity reduction by *K. oxytoca* in a column reactor with a slightly higher initial hydraulic conductivity (2.97 cm/min), and fed a sodium citrate medium that had a higher concentration of carbon (1800 mg/L carbon) than the highest substrate concentration used in this experiment (700 mg/L DOC). A steady-state hydraulic conductivity (0.16 cm/min) was recorded that was comparable to the steady-state hydraulic conductivity from the 700 mg/L DOC substrate concentration experiment (0.19 cm/min).

An increase in substrate concentration resulted in an increase in hydraulic conductivity reduction. Varying substrate concentration enabled the control of porous media hydraulic conductivity in an engineered dual-species biofilm system.

CHAPTER 5

CONCLUSION

The goal of this research was to gain a better understanding of the interactions between two microbial species when grown together in an engineered biofilm. The research determined if two organisms would co-exist and what variables influenced the population distribution in batch, rotating disk and porous media reactors. An engineered dual-species biofilm was then manipulated to maximize trichloroethylene (TCE) degradation and permeability reduction in porous media.

Population Distribution

The TCE-degrading organism (*Burkholderia cepacia* PR1-pTOM$_{31c}$) was out-competed by the thick biofilm-forming organism (*Klebsiella oxytoca*) in planktonic experiments. The organism with the higher specific growth rate (*K. oxytoca*) had two to three orders of magnitude higher population density than the slower growing organism (*B. cepacia*) in batch cultures.

In a well-mixed rotating disk reactor, dual-species biofilm growth rates failed to predict the dual-species biofilm population distribution. Substrate concentration, however, could be used to predict the dual-species biofilm population density. At high substrate concentrations (700 mg/L DOC), *K. oxytoca* had a higher fraction of the total population than *B. cepacia* in the dual-species biofilm. At low substrate concentrations (30 mg/L DOC), there was a shift in the population distribution with *B. cepacia*

becoming the dominant organism. Varying substrate concentration regulated the fraction of each organism in the dual-species biofilm. The same trend in population distribution with change of substrate concentration that was observed in the rotating disk reactor experiments was also observed in the porous media column experiments, except disparity in population density at the 30 and 700 mg/L DOC concentration was more profound. Substrate concentration played a major role in controlling the population distribution in biofilm and porous media environments using these two organisms. Further research is needed to understand why this population shift occurs and if similar trends exist when other organisms are combined.

The biofilm growth rates calculated in the porous media column were lower than the planktonic and rotating disk reactor biofilm growth rates. Due to hydrodynamic differences between the well-mixed and porous media reactors, the porous media biofilm density was significantly greater than the biofilm in a well-mixed reactor. It is hypothesized that the increased mass transfer limitation in the thicker biofilm resulted in lower growth rates in porous media.

Trichloroethylene Biodegradation

Burkholderia cepacia degraded approximately 80% of the TCE pumped through the porous media column at the low (30 mg/L DOC) substrate concentration. A dual-species culture of *B. cepacia* and *K. oxytoca* was only able to remove 50% of the TCE added to the column at the same substrate concentration. Therefore, the presence of *K. oxytoca* had a negative effect on *B. cepacia's* ability to degrade TCE in a porous media

column. A possible explanation for this is competition for dissolved oxygen. The importance of oxygen on the aerobic TCE degradation by *B. cepacia* has been reported (Munakata-Marr et al. 1996). TCE degradation decreased with increasing substrate concentrations.

Permeability Reduction

The ability of *K. oxytoca* to reduce porous media permeability in the dual-species column experiments was dependent on substrate concentration. Porous media permeability reduction was increased with increasing substrate concentration. An increase in substrate concentration also caused an increase in *K. oxytoca's* biofilm population density. In addition, higher substrate concentrations (70 and 700 mg/L DOC) caused oxygen-limiting conditions in the porous media column. Anaerobic conditions could influence the type or magnitude of EPS produced by *K. oxytoca*, thus increasing the substrate concentration could affect the structure of the mucoid biofilm matrix. Therefore, permeability reduction appears to be a function of *K. oxytoca's* population density and possibly a function of *K. oxytoca's* metabolic changes from oxygen stress.

Field-Scale Reactive/Reduced Permeability Biofilm Barrier

This research demonstrated the feasibility of creating a TCE reactive/reduced permeability biofilm barrier under laboratory conditions. This research could also describe a possible scenario of both organisms were used for a field-scale biofilm barrier. As the bacteria and nutrients are injected down a well and pumped out through the soil,

the nutrients would be diluted due to mixing with upstream groundwater, thus creating a system with varying substrate concentrations. Higher substrate concentrations a certain radius of influence from the injection well would provide adequate permeability reduction by the thick biofilm-forming organisms, while the lower substrate concentrations upstream of the well (along with dissolved oxygen in the upstream groundwater) could provide an environment for TCE degradation to occur.

This research is just one application of a dual-species engineered biofilm. An engineered multi-species system can be created, but research is imperative to understand which variables control the interaction and activity of the organisms. In this particular scenario, substrate concentration influenced the population distribution and activity of a dual-species engineered biofilm in porous media.

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