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
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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 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

by

John Komlos Jr.

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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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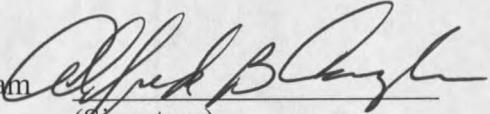
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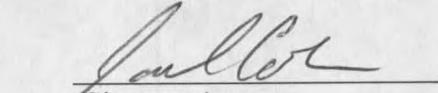
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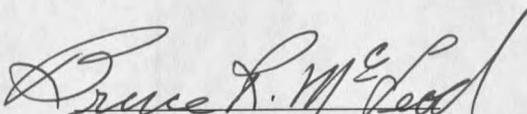
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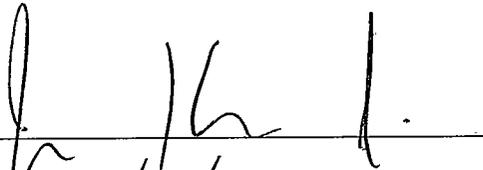
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ABSTRACT

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.

<u>Field-Scale Demonstration of Biofilm Containment Barrier</u>. 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).

<u>Reactive/Reduced Permeability Biofilm Barrier</u>

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 constitutively 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,