This is to certify that the
dissertation entitled

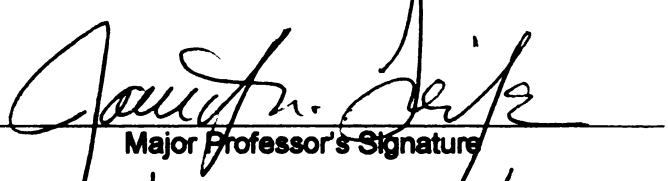
MICROBIAL COMMUNITY STRUCTURE IN A TRICHLOROETHYLENE CONTAMINATED AQUIFER DURING TOLUENE STIMULATED BIOREMEDIATION

presented by

ELICA MONIQUE MOSS

has been accepted towards fulfillment
of the requirements for the

Doctoral	Crop and Soil Science/Environmental Toxicology



Major Professor's Signature

August 19, 2004

Date

Microbial Community Structure in a Trichloroethylene Contaminated Aquifer During Toluene Stimulated Bioremediation

By

Elica Monique Moss

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences

2004

ABSTRACT

Microbial Community Structure in a Trichloroethylene Contaminated Aquifer During Toluene Stimulated Bioremediation

By

Elica Monique Moss

Trichloroethylene (TCE), a common groundwater pollutant, is a major problem facing communities throughout the world. This widespread occurrence has created public demand for technologies to remediate aquifers contaminated with TCE. One such *in situ* technique is the phenol or toluene stimulated co-oxidation of TCE and its dechlorination products. This technique is dependent on the capability of the intrinsic microbial population to produce oxygenases that degrade these chlorinated solvents given the appropriate stimulating conditions. In this work, I evaluated microbial community response to a field test of toluene-stimulated TCE degradation implemented by a Stanford research team at Edwards Air Force Base (AFB). High-throughput sequencing and analysis of 16S rRNA gene libraries from filtered monitoring well water showed that populations taken 1 month and 3 months after toluene additions were statistically different from those found before toluene addition. Furthermore, communities near and down-gradient from the bio-treatment well were different from those up-gradient to the bio-treatment wells. The major trends noted were a decline in the Pseudomonas populations and an increase in Sphingomonas and eventually Legionella populations after toluene feeding. In order to determine the presence of

toluene-degrading populations and to determine whether they co-oxidized TCE, I isolated and characterized dominant populations from the site. While some showed a low level of toluene consumption, none produced rapid growth on toluene and none oxidized TCE. Since both toluene and TCE were removed in the field test, the lack of toluene-degraders in the monitoring wells must be due to the fact that the toluene degraders had not yet reached these wells or the bacteria found there were not the ones oxidizing toluene.

Because of the Bio-Enhanced In-Well Vapor Stripping (BEHIVS) System used at Edwards AFB, I determined if bacteria in the *Burkholderia cepacia* complex (Bcc), were present. This group of opportunistic human pathogens, especially in cystic fibrosis (CF) patients, lives in soil and some grow on toluene. High-throughput 16S rRNA sequencing, isolation and characterization of colonies, and screening larger populations by hybridization with a Bcc specific probe revealed no such populations. But since members of the Bcc can be agents for bioremediation and because the clinical and environmental strains cannot be distinguished, I explored the different patterns of aromatic substrate use between clinical and environmental strains. Cluster analysis indicated that aromatic use by isolates from the environment was much higher than from the clinic, but that no set of one or a few substrates could reliably distinguish an environmental strain from a CF-lung colonizing strain.

To my mother and friend

Yvonne

who is always an inspiration to me.
I Love You!

ACKNOWLEDGEMENTS

The author wishes to express her sincere thanks to:

Dr. James M. Tiedje who has assisted me on my path to academic success with continued patience and words of encouragement. It meant so much to have you as an advisor.

Dr. Stephen A. Boyd, Dr. Michael J. Klug, and Dr. Syed A. Hashsham, members of my guidance committee.

My mother Yvonne, Father Ellis, Grandmother Corine and all members of my family who have been there for me on this long journey. Thank you so much for all your prayers, words of encouragement, and love.

Tomeka, Kerri, Mr. and Mrs. Scott and all my friends who were always there cheering me on.

Dr. James E. Jay and Dr. Eunice Foster for your guidance and kindness.

Dr. Héctor Ayala del Rio, Dr. Joonhong Park, and Dr. Alban Ramette for your knowledge and support throughout my program.

My fellow lab colleagues, Claribel Cruz-Garcia, Benjamin Griffin, Monica Ponder, Xiaoyun Qiu, Veronica Gruntzig, Joel Klappenbach, John Urbance, John Davis, Tamara Tsoi, Jorge Rodriguez Stephan Gantner, Mary Beth Leigh, Jacob Parnell, Peter Bergholz, Vincent Denef, Kostas Konstantinidis, Debra Rodrigues, Carlos Rodriguez, and Baolin Sun, thank you for your friendship.

Center for Microbial Ecology (CME) staff, Lisa Pline, Pat Englehart, Nicole Mulvaney, and Joyce Wildenthal for all your help.

Dr. Perry L. McCarty, Gary Hopkins and the Stanford University team for the Edwards Air Force Base components of this work.

Dr. John LiPuma, University of Michigan Medical School, for providing the strains for the *Burkholderia cepacia* complex analysis.

The Ribosomal Database Project II staff (RDP) James Cole, Benli Chai, and Qiong Wang.

The Genomics Technology Support Facility (GTSF), Annette Thelen, PhD, and Shari Tjugum-Holland.

Darlene Johnson and Rita House for their support since my admission into the graduate program.

The author is indebted to CME, Crop and Soil Science and the Graduate School for providing the opportunity and financial aid during the course of this doctoral work.

Table of Contents

List of Tables

Table **Page**

Chapter II

Chapter III

Chapter IV

List of Figures

Figures **Page**

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter I

Overview

Introduction

Trichloroethene (TCE), a well-known groundwater pollutant, is of great concern throughout the United States, because it and especially its vinyl chloride product are hazardous to humans. Cometabolism of TCE and its dechlorination products 1,2-*cis*-dichloroethene (*c*-DCE) 1,2-*trans*-dichloroethene (*t*-DCE), 1,1-dichloroethene (1,1, DCE), and vinyl chloride, stimulated by primary substrates such as phenol and toluene, has become a successful approach for *in situ* bioremediation of TCE. A pilot-scale study at Moffett Field, CA (McCarty et al., 1998), demonstrated the aerobic cometabolic biodegradation of TCE and evaluated the microbial community composition and succession in the aquifer amended with phenol, toluene, and chlorinated aliphatic hydrocarbons such as TCE and 1,1-dichloroethene (1,1-DCE) (Fries et al., 1997). Following this pilot-scale study, this technology was tested at a TCE contaminated site, Edwards Air Force Base, CA. Prior microcosm studies using Edwards AFB soil mimicked the cometabolic biodegradation observed at Moffett Field and indicated that 87-99% TCE removal could be expected at Edwards AFB (McCarty et al., 1998). Because of the success of the pilot study at Moffett Field and the microcosm studies at Edwards AFB, the opportunity for implementing a full-scale test of TCE remediation by cometabolism was undertaken at Edwards AFB led by Stanford University engineers.

The full-scale study focused only on evaluating the effectiveness of the cometabolic process in removing TCE but with no measures of the microbial population

response during the course of the field trail. I took advantage of this field experiment to study the microbial community succession at three sampling periods: *before toluene injection; one month after the initial injection of toluene; and 3 months after toluene injection* (at the end of the experiment). This study was done to complement their research. Therefore, the **objectives** of this study were to:

Objective 1: Determine which microbial populations are stimulated in the field in this full-scale remediation test. This is important since populations differ in their ability to degrade TCE and hence could explain success or failure. Using this approach, the following questions were addressed:

- *What is the population density of the subset of populations?*

  Using the results from the three (3) sampling periods, i.e., *before toluene was added* (background samples); *one month after the initial injection of toluene*; and *three (3) months after the initial injection of toluene* (the end of the experiment), the samples were analyzed to determine what organisms are present and their portion of the population.

- *What are the similarities in community structures among the different wells?*

  Communities were compared to each other in relation to location of treatment wells and between similar sampling wells.

- *What are the similarities in phylotypes at different wells?*

  The 16S rRNA gene sequences of the different organisms were analyzed to compare how similar they are, especially between replicate wells.

Objective 2: Determine whether *this process selects Burkholderia cepacia, a potential human pathogen, but good TCE degrader.*

The identification of *Burkholderia cepacia complex (Bcc)* is of significance because this group of opportunistic human pathogens, which causes serious infection in patients with Cystic Fibrosis (CF), lives in soil and grows on toluene or phenol (Mahenthiralingam et al., 2000). Therefore, stimulating their growth in the environment may be of concern at sites practicing aerobic TCE cometabolism, such as Edwards AFB. In fact the best-known TCE cometabolizing strain, G4, is a member of the Bcc. The Bio-enhanced In-Well Vapor Stripping (BEHIVS) system used at Edwards AFB, which employs a vapor stripping well, could aerosolize *B. cepacia* cells if they are enriched in the aquifer. If this is the case, this form of bioremediation would most likely be shutdown by public health authorities.

Objective 3: Isolate dominant microorganisms from two wells and determine their toluene and TCE-degrading ability and the type and the diversity of their aromatic oxygenase genes.

Objective 4: Determine ability of various members of the *B. cepacia* complex, both from clinical and environmental isolates to grow on a range of aromatic compounds.

Because Bcc in the soil and lung would be expected to grow on very different resources, for example a variety of aromatics in soil, these substrates might be useful for differentiating between soil and CF-pathogenic strains.

These objectives were addressed by using molecular methods such as 16S rRNA gene analysis, which avoid the limitations of culturability by providing more complete information on community composition. Terminal restriction fragment polymorphism (TRFLP) analysis of amplified total community 16S rRNA genes was used to explore community structure and diversity (Braker et al., 2001). TAP-TRFLP was used; it is a software tool that guides the microbial community analysis as it allows *in silico* restriction digests of the entire 16S rRNA gene sequence database to find fragment sizes of bacterial species that can then be compared to T-RFs of the field data (Osborn et al., 2000). Secondly, clone libraries of 16S rRNA genes were collected from aquifer bacteria using PCR with 16S rRNA gene primers to analyze bacterial diversity among the active microbial communities in the Edwards AFB samples. Finally, I isolated

representative toluene degrading populations present after toluene additions to the site and determined their growth rate on toluene, and their TCE degradation rate. These results contributed to our understanding of the organizational structure of communities within Edwards AFB, thereby conveying to researchers what organisms are stimulated in the field when the primary substrate, toluene, is added. Consequently, this research aided in further understanding the natural populations of microorganisms that metabolize toxic agents such as TCE, and whether there is likely any associated public health risk, especially for CF patients.

Background

What is TCE?

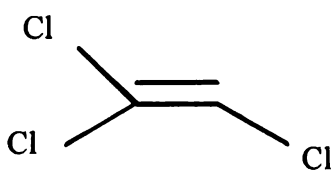


Figure 1.1 Chemical structure of TCE

TCE is a volatile, colorless liquid at room temperature that has been widely used as a solvent to remove oil and grease from metal parts. TCE is an ingredient in adhesives and paint removers and the textile industry uses it as a solvent in dying and finishing operations with cotton, wool, and other fabrics (ATSDR, 1997). It is a

convenient solvent to use because of its low flammability. TCE has a solubility in water of ~1100 ppm, hence, it is mobile in soils and often found as a groundwater contaminant. According to the EPAs Contract Laboratory Program Statistical Database, TCE is reported to occur in 19% of groundwater samples at a geometric mean concentration of 27.3 ppb (individually ranging from 0.1 to 27,300 ppb). It can also migrate through soils and sub-soils and because its density is >1g/cm^3 i.e., it moves to the bottom of aquifers. The presence of such a dense non-aqueous phase liquid (DNAPL) usually serves as a long-term source of groundwater contamination.

One may become exposed to TCE by breathing TCE vapors from the contaminated water (e.g. showers), or drinking the contaminated water. Breathing small amounts of TCE can cause headaches, lung irritation, dizziness, poor coordination, and difficulty concentrating (ATSDR, 1997). Breathing large amounts for short periods may cause impaired heart function, unconsciousness, and death, whereas breathing the TCE for long periods may cause nerve, kidney, and liver damage (ATSDR, 1997). Drinking large amounts of TCE for short periods may cause problems such as nausea, liver damage, unconsciousness, impaired heart function, and death. Additionally, drinking small amounts of TCE for long periods may cause liver and kidney damage, impaired immune system function, and impaired fetal development in pregnant women (ATSDR, 1997). Workers involved in the manufacture or use of TCE degreasers may constitute a group at risk because of the potential for occupational exposure (ATSDR, 1997). The National Occupational Exposure Survey for 1981 to 1983 (NIOSH, 1990) reported that 401,373 employees in the US were exposed to TCE. People located near or downwind of factories involved in vapor degreasing operations,

as well as hazardous waste disposal sites, or landfills, have experienced effects from breathing TCE vapors or drinking TCE contaminated water (ATSDR, 1997).

The Environmental Protection Agency (EPA) has limited the TCE concentration in drinking water to 0.005 ppm (5ppb), and the National Institute for Occupational Safety and Health Administration (OSHA), has set an exposure limit of 100 ppm of air for an 8-hour workday, 40-hr workweek. Evidence of carcinogenicity of TCE in humans suggests that occupational exposure was associated with excess incidences of liver cancer, kidney cancer, and non-Hodgkin's lymphoma (Wartenberg et al, 2000). The results in humans are verified by the presence of tumors at the same sites in experimental animals. The metabolism of TCE in mice, rats, and humans is similar, thus, producing the same primary metabolites such as trichloroacetic acid (NTP, 1990). Consequently, TCE is reasonably anticipated to be a human carcinogen based on limited evidence of carcinogenicity from studies in humans and sufficient studies in animals (NTP, 1990).

What is Toluene?

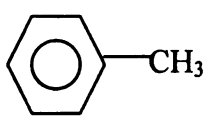


Figure 1.2 Chemical structure of Toluene

Toluene is a clear, colorless liquid with a distinctive smell. It is produced during the gasoline making process from crude oil. It is also used in making paints, fingernail polish, and in some printing and leather tanning processes. Toluene enters surface water

and groundwater from spills of solvents and petroleum products as well as from leaking underground storage tanks at gasoline stations and other facilities (ATSDR, 2000). And when toluene-containing products are placed in landfills or waste disposal sites, the toluene can enter the soil or water near the waste site. However, toluene does not usually stay in the environment long.

One can become exposed to toluene by breathing contaminated air, which includes automobile exhaust, drinking contaminated well water, or living near hazardous waste sites containing toluene products (ATSDR, 2000). Drinking low to moderate levels can cause tiredness, confusion, drunken-type actions, memory loss, and nausea. The inhalation of high levels for short periods causes dizziness, unconsciousness, and sometimes death (ATSDR, 2000). These symptoms usually disappear when exposure is stopped.

Toxicological data suggests that a maximum contamination level (MCL) for toluene in drinking water be 14.3 mg/l (14.3 ppm) (Lederer, 1985). The maximum contamination level goal (MCLG) set by EPA for toluene in drinking water is 1 mg/L (1 ppm), while OSHA has set a limit of 200 ppm of workplace air. Previous studies indicate that toluene does not cause cancer in humans or animals. Therefore, the EPA has determined that the carcinogenicity of toluene cannot be classified (ATSDR, 2000).

Rationale of Research

I. Potential for bioremediation

TCE persists in polluted groundwater because conditions are often not favorable for biodegradation in aerobic subsurface environments (Agency for Toxic Substances, 1997 and Wilson et al., 1985). Biostimulation is a technology used to create subsurface conditions in which naturally occurring TCE-degrading bacteria thrive and grow, resulting in the rapid degradation of the compound.

The widespread occurrence of TCE in groundwater has created public demand for techniques to remediate aquifers contaminated with TCE. *In situ* bioremediation using aerobic cometabolic or anaerobic reductive dechlorination processes is an attractive approach for TCE contaminated aquifers (Semprini, 1995). Potential advantages of *in situ* biological technologies are that TCE is destroyed during the process rather than being transferred to another place for disposal, and there is no above ground treatment system required (McCarty et al., 1998). *In situ* treatment also avoids transporting contaminants to the surface, thereby reducing potential risk exposure to humans. Pilot-scale studies for *in situ* bioremediation have shown that certain substrates such as toluene or phenol injected into aquifer material stimulated indigenous TCE-degrading organisms (Jenal-Wanner et al., 1997).

Aerobic transformation processes

There is potential for the restoration of aquifers to be widely successful due to the *in situ* aerobic bioremediation of contaminated sites with TCE and its anaerobic dechlorination products, 1, 2-*cis*-dichloroethylene (*c-DCE*), 1, 2-*trans*-dichloroethylene

(*t-DCE*), and vinyl chloride (VC) (Hopkins et al., 1993). The likelihood of successful *in situ* bioremediation is dependent on the capability of the intrinsic microbial population to degrade the compounds of interest given the appropriate stimulating conditions (Jenel-Wanner et al., 1997). Previously, Wilson and Wilson (1985) have described successful cometabolism of TCE in soil communities fed with natural gas. In chlorinated aliphatic hydrocarbon (CAH) cometabolism, an oxgenase normally used by the microorganisms for initiating primary substrate oxidation, transforms the pollutant (Hopkins et al., 1995). For example, three soil bacterial cultures that catabolize toluene, *Burkholderia vietnamensis* strain G4, *Pseudomonas putida F1,* and *P. putida* B5, have been demonstrated to cometabolize TCE (Wackett et al., 1988). *P. putida* F1 metabolizes TCE using toluene dioxygenase (Nelson et al., 1987), as seen in Figure 1.3. Other toluene degraders use a monooxygenase to degrade TCE, but by the same pathway. Nelson et al. (1987) used a chloride-specific electrode to show that all three chlorine atoms of TCE are converted to inorganic chloride by strain G4. The initial carbon product of TCE cometabolism, TCE epoxide, (Fig.1.3) further decomposes to form non-chlorinated compounds, which could then be converted to CO_2 by enzymatic or chemical oxidations, or be assimilated into cellular carbon (Nelson et al., 1987).

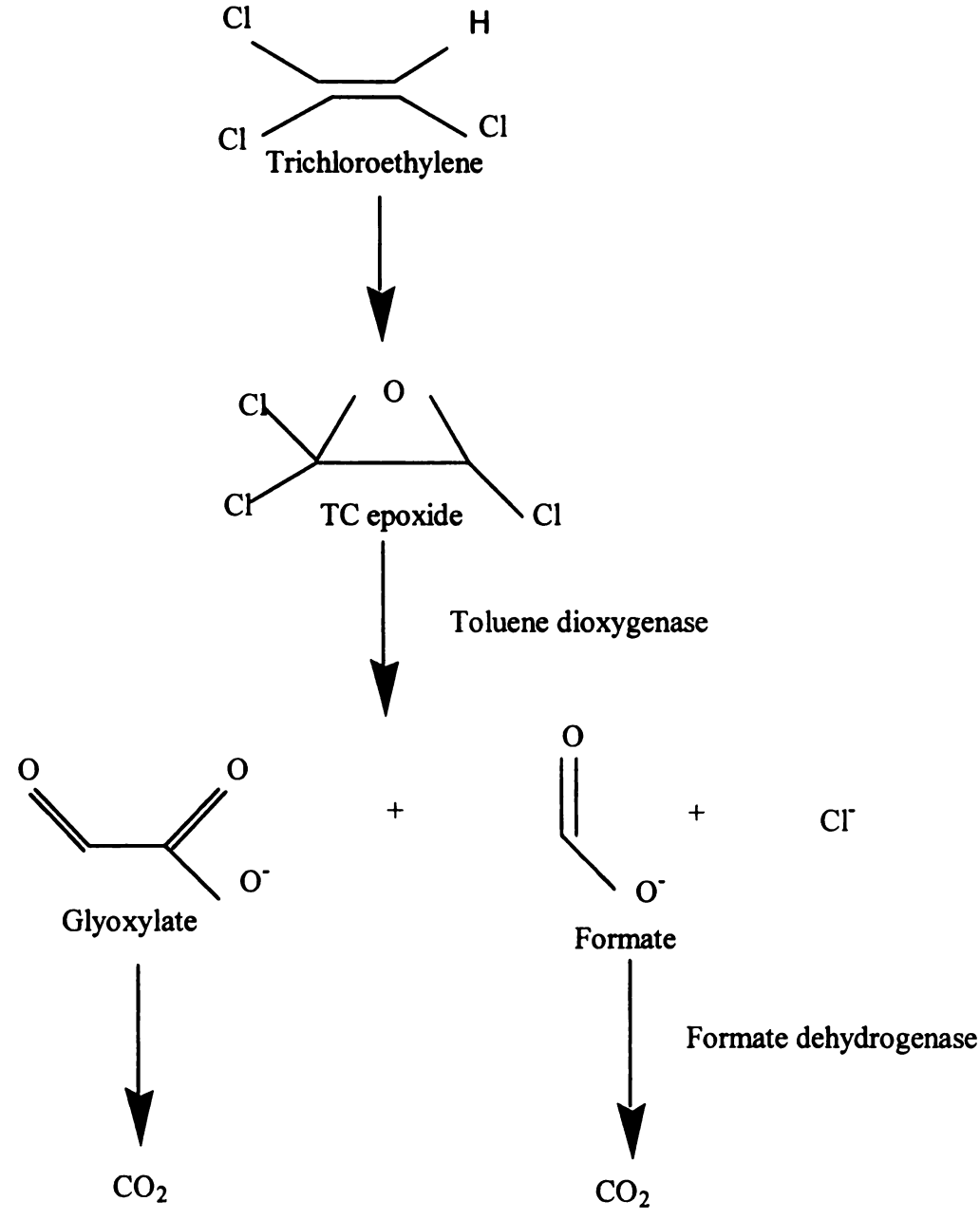


Figure 1.3: TCE Degradation Pathway with *Pseudomonas putida* F1.

McCarty et al. (1998) have completed several small-scale field experiments at Moffett Field, CA to determine how effective phenol and toluene are in stimulating *in situ* bioremediation. Toluene and phenol may stimulate very similar if not the same monooxygenases active in TCE co-oxidation. It is unknown, however, how similar the populations are that are stimulated by these substrates or whether they are equivalent in their TCE-co-oxidizing ability (Fries et al., 1997). The challenge in managing phenol- or toluene-induced cometabolism of TCE *in situ* is to selectively stimulate and maintain active TCE degraders, since in nature many other bacteria grow on the same substrates, but do not cometabolize TCE (Fries et al., 1997). The previous laboratory and field studies, have not established whether phenol or toluene is a better substrate for stimulating TCE bioremediation. Hence, a number of factors are significant in the decision to use toluene as the primary substrate.

Toluene, a naturally produced chemical, is a common groundwater pollutant that is already present at Edwards AFB (McCarty et al., 1998). Even though toluene may already be present, its concentrations are insufficient to induce cometabolic degradation. Laboratory and field studies reveal that toluene concentrations well below 1μg/L result from toluene biodegradation by indigenous aquifer microbes in the Moffett field treatment system (Hopkins et al., 1995). The observed concentrations are several orders of magnitude below the MCLG for drinking water (1mg/L) and an order of magnitude below the taste threshold (120-160 μg/L) (McCarty et al., 1998). Thus, the use of toluene has become the favored substrate for *in situ* cometabolic remediation of TCE (McCarty et al., 1998).

Anaerobic biotransformation processes

Tetrachloroethene (PCE), another potentially toxic chlorinated ethylene can be transformed anaerobically to TCE (Fig. 1.4), then to a series of sequentially lesser chlorinated products, and finally to ethene and Cl^- by anaerobic reductive dehalogenation (Vogel et al. 1987). Tetrachloroethene and trichloroethene are transformed to less chlorinated ethenes by slow, anaerobic, cometabolic processes by methanogenic, homoacetogenic, and sulfate reducing microorganisms or by a faster, anaerobic, halorespiring process (Loeffler et al., 2000). In the latter case, microbes can gain energy for growth by using the chlorinated ethenes as electron acceptors and coupling this exergonic reaction to ATP formation. This process results in a much faster rate of dechlorination. Members of the genera *Desulfuromonas*, *Dehalospirillum*, *Dehalobacter*, *Desulfitobacterium* and *Dehalococcoides* reductively dechlorinate PCE and TCE, and some *Dehalococcoides* can complete the reduction of *cis*-DCE and VC (vinyl chloride) to ethene and chloride (Loeffler et al., 2003). The restoration of some anaerobic polluted environments can be credited to the natural activities of these anaerobic dechlorinating populations in aquifers where this anaerobic process is active (Gerritse et al., 1999). When PCE is present in the contaminating solvent mix, the anaerobic chlororespiring approach is the only feasible bioremediation process since PCE cannot be co-oxidized by natural populations. But if only TCE is present, and the aquifer is primarily aerobic, then the cometabolic approach is feasible and likely faster.

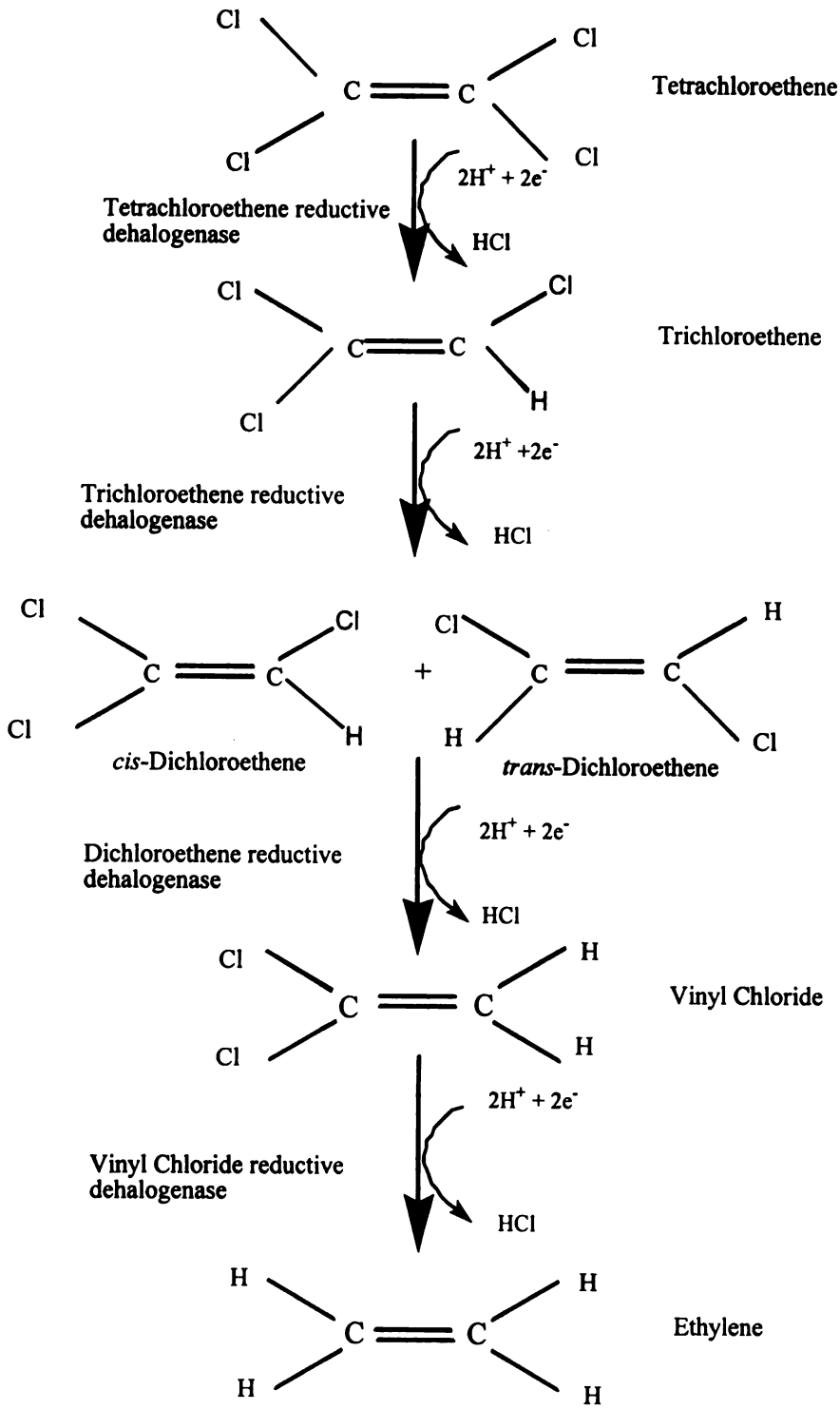


Figure 1.4: Anaerobic Tetrachloroethene Pathway.

Dechlorination may result in the build-up of toxic products that are formed biologically from the primary pollutants. This is particularly a concern for the reductive dechlorination of PCE and TCE since *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) both tend to accumulate. Both are toxic and the later is a human carcinogen (Wackett et al., 1988). In this regard, the use of an aerobic cometabolic approach carries less risk for TCE remediation because of its potential to produce CO_2 and Cl^- as end products, and it does not accumulate toxic intermediates.

The need for remediation at Edwards AFB centers on the potential contact of TCE with soil where it is less easily evaporated than in surface water. Thus, this study will largely focus on understanding how effective the selected indigenous populations are at TCE degradation and how their TCE oxidizing capacity compares to that of well-studied strains, i.e., *Burkholderia vietnamiensis* G4. This study will also enable us to examine the dynamics of the communities in response to toluene injection, such as the extent of diversity among the selected toluene degrading strains and the dominance maintained in the field over time.

II. Field evaluation of toluene-driven cometabolic remediation of TCE

Moffett Field pilot study

A pilot-scale study at Moffett Field demonstrated the aerobic cometabolic biodegradation of TCE and other chlorinated alkenes would be successfully stimulated in the field (McCarty et al., 1998). At Moffett Field, microbial community composition and succession were evaluated in an aquifer amended with phenol, toluene, and chlorinated aliphatic hydrocarbons such as TCE and 1,1-dichloroethene (1,1-DCE)

(Fries et al., 1997). The populations in the aquifer were isolated, identified, and their TCE degrading abilities determined (Fries et al., 1997). Fries et al. (1997) used genomic fingerprints determined by repetitive extragenic palindromic (REP)-PCR as a measure of genetic diversity of the phenol and toluene degrading community. The results indicated that only a few gram-positive isolates were obtained after treatment with phenol +1,1-DCE (~2 rep-PCR groups), compared to the initial isolates with no treatment (~14 rep-PCR groups) (Fries et al., 1997). Although microbial densities increased following phenol-TCE treatment (~5 rep-PCR groups), the original species richness was restored, following toxic 1, 1 –DCE, only after the toluene-TCE treatment (~13 rep-PCR groups). Also, TCE removal efficiency was much greater with either phenol or toluene than with methane (>85% versus 15%). In essence, this study concluded that taxonomically diverse communities of indigenous aquifer phenol and toluene degraders with moderate but satisfactory TCE-degrading ability were stimulated by the addition of the primary substrates. Both gram negative and positive bacteria were found: the former included members of the genera *Burkholderia*, *Variovorax*, and *Azoarcus*, all from the phylum Proteobacteria and the later included members of the genera *Rhodococcus* and *Nocardia,* all from the phylum Actinobacteria of high G+C content (Fries et al., 1997).

Edwards AFB study

Following the pilot-scale study at Moffett Field, this technology was tested at full-scale at Edwards AFB, CA, a TCE contaminated site. From 1958 until 1967 engines for the X-15 rocket plane were stored in facilities at the Edwards AFB

(McCarty et al., 1998). One 55-gallon drum of trichloroethylene (TCE) was used each month to clean the engines, and afterwards, was dumped in the nearby desert creating a large groundwater contaminant plume (McCarty et al., 1998). This resulted in TCE contamination of both the upper and lower aquifers at Edwards AFB.

Edwards Air Force Base (AFB) was originally established in 1933 and occupies 470 square miles in the Mojave Desert. It is the home of the Air Force Flight Test Center (AFFTC), the Air Force Research Laboratory (AFRL), and National Aeronautics and Space Administration (NASA), and involved in aircraft research, development, and testing. It was placed on the National Priorities List (NPL) in 1990. In December 1999, over 460 sites and Areas of Concern (AOC) have been identified on the Base (The Edwards Air Base Installation Restoration Program: An Investment Report, 2000). The Edwards AFB is unique to other Air Force Bases because of the large size of the Base (470 sq. miles), the large number of sites, the extreme temperatures, and rocket engine research missions.

The cometabolic degradation of TCE was tested at site 19 as a cooperative effort among the U.S. Air Force, USEPA, Stanford University, and Oregon State University. The indigenous bacteria use oxygen as "air" and toluene as "food" and produce the toluene monoxygenase enzyme (TMO) which degrades the TCE in the groundwater (The Edwards Air Base Installation Restoration Program: An Investment Report, 2000).

A microcosm study was carried out using Edwards AFB soil to predict treatment performance. The results, which differed with location and varied between 87 and 99% TCE removal, mimicked the cometabolic biodegradation at Moffett Field and

indicated that TCE removal by cometabolism should also work here (Jenal-Wanner al., 1997). Hence, a full-scale remediation experiment involving cometabolism of TCE in the Edwards AFB plume was then attempted by Stanford University engineers. The research focused only on evaluating the effectiveness of the cometabolic process in removing TCE with no measures of the microbial population response. My study focused on the microbial community and was done to complement their research.

References

1. **Agency for Toxic Substances and Disease Registry (ATSDR).** 1997. Toxicological profile for trichloroethylene. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

2. **Agency for Toxic Substances and Disease Registry (ATSDR).** 2000. Toxicological profile for toluene(update). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

3. **Anderson, Eric Elmer and Rikke Granum Andersen.** 1996. In situ bioremediation of TCE. Civil Engineering Dept., Virginia Tech.

4. **Baker, Paul, Kazaya Watanabe, and Shigeaki Hararyama.** Analysis of the microbial community during bioremediation of trichloroethylene (TCE) contaminated soil. Kamaishi Laboratories, Marine Biotechnology Institute Co. Ltd., Kamaishi, Iwate. Japan.

5. **Braker, Gesche, Hector L. Ayala-Del-Rio, Allan H. Devol, and Andreas Fesefeldt, and James M. Tiedje.** 2001. Community Structure of Denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16 S rRNA genes. Appl. Environ. Microbiol. **67**:1893-1901.

6. **Burrell, Paul C., Carol M. Phalen, and Timothy A. Hovanec.** 2001. Identification of bacteria responsible for ammonia oxidation in freshwater aquaria. Appl. Environ. Microbiol. **67**:5791-5800.

7. **Cho, Jang-Cheon, and Sang-Jong Kim.** 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol. **66**:956-965.

8. **EPA.** U.S. Environmental Protection Agency. Contract Laboratory Program Statistical Database. Washington, DC: U.S. EPA, 1989.

9. **Fliermans, C.B., T.J. Phelps, D.Ringelberg, A.T. Mikell, and D.C. White.** 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. Appl. Environ. Microbiol. **54**:1709-1714.

10. **Fries, Marcos R., Gary D. Hopkins, Perry L. McCarty, Larry J. Forney, and James M. Tiedje.** 1997. Microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethene cometabolism. Appl. Environ. Microbiol. **63**:1515-1522.

11. **Fries, Marcos R., Jizhong Zhou, Joanne Chee-Sanford, and James M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802-2810.

12. **Fries, Marcos R., Larry J. Forney, and James M. Tiedje.** 1997. Phenol- and toluene-degrading microbial populations from an aquifer in which successful trichloroethene cometabolism occurred. Appl. Environ. Microbiol. **63:**1523-1530.

13. **Fuller Mark E. and Kate M. Scow.** 1997. Impact of tricholoethylene and toluene on nitrogen cycling in soil. Appl. Environ. Microbiol. **63:**4015-4019.

14. **Glod, Guy, Werner Angst, Christof Holliger, and Rene' P. Schwarzenbach.** 1997. Corrinoid-mediated reduction tetrachloroethene, trichloroethene, and trichlorofluoroethene in homogenous aqueous solution: Reaction kinetics and reaction mechanisms. Environ. Sci. Technol. **31:**253-260.

15. **Gerritse Jan, Oliver Drzyzga, Geert Kloetstra, Mischa Keijmel, Luit P. Wiersum, Roger Hutson, Matthew D. Collins, and Jan C. Gottschal.** 1999. Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene *by Desulfitobacterium frappieri* TCE1.Appl. Environ. Microbiol. **65**:5212-5221.

16. **Holliger, Christof, Gert Wohlfarth, and Gabriele Diekert.** 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Reviews. **22:**383-398.

17. **Hopkins, G.D., L. Semprini, and P.L. McCarty.** 1993. Microcosm and in situ field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms. Appl. Environ. Microbiol. **59:**2277-2285.

18. **Hopkins, Gary D. and Perry L. McCarty.** 1995. Field evaluation of *in situ* aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Sci. Technol. **29:**1628-1637.

19. **Jenal- Wanner, Ursula and Perry L. McCarty.** 1997. Development and evaluation of semicontinuous slurry microcosms to stimulate in situ biodegradation of trichloroethylene in contaminated aquifers. Environ. Sci. Technol. **31:**2915-2922.

20. **Kleopfer, Robert D., Diane M. Easley, Bernard B. Haas, Jr., Trudy G. Deihl, David E. Jackson, and Charles J. Wurrey.** 1985. Anaerobic degradation of trichloroethylene in soil. Environ. Sci. Technol. **19:**277-280.

21. **Lederer, W.H.** Regulatory chemicals of health and environmental concerns. Van Nostrand: New York. 1985.

22. **Löffler, Frank E. Qing Sun, Jieran Li, and James M. Tiedje.** 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* Species. Appl. Envir. Microbiol. **66:** 1369-1374.

23. **Löffler, Frank E., James R. Cole, Kirsti M. Ritalahti, and James M. Tiedje.** 2003. Diversity of Dechlorinating Bacteria. Dehalogenation: Microbial Processess and Environmental Application. M.M. Haggblom and I.D. Bossert, editors. Kluwer Academic Publishers. 53-87.

24. **Magnuson, Jon K., Robert V. Stern, James M. Gossett, Stephen H. Zinder, and David R. Burris.** 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Appl. Environ. Microbiol. **64:**1270-1275.

25. **Mahenthiralingam, Eshwar, Jocelyn Bischof, Sean K. Byrne, Christopher Radomski, Julian E. Davies, Yossef AV-Gay, and peter Vandamme.** 2000. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkhoderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* Genomovars I and III. J. Clin. Microbiol. **38**:3165-3173.

26. **McCarty, Perry L. Mark N. Goltz, and Gary D. Hopkins.** Full-scale evaluation of in situ bioremediation of chlorinated solvent groundwater contamination. Stanford University (www.stanford.edu) Supported by the U.S. Air Force.

27. **McCarty, Perry L., Mark N. Goltz, Gary D. Hopkins, Mark E. Dolan, Jason P. Allan, Brett T. Kawakami, and T.J. Carrothers.** 1998. Full-scale evaluation of in situ cometabolic degradation of trichloroethylene in groundwater through toluene injection. Environ. Sci. Technology. **32:**88-100.

28. **Moran, B.N. and W.J. Hickey.** 1997. Trichloroethylene biodegradation by mesophilic and psychrophilic ammonia oxidizers and methanotrophs in groundwater microcosms. Appl. Environ. Microbiol. **63:**3866-3871.

29. **Munakata-Marr, Junko, Perry L.McCarty, Malcolm S. Shields, Michael Reagin, and Stephen C. Francesconi.** 1996. Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered *Burkholderia* (*Pseudomonas*) *cepacia* G4 and PR1. Environ. Sci. Technol. **30:**2045-2052.

30. **Munakata-Marr, Junko, V. Grace Matheson, Larry J. Forney, James M. Tiedje, and Perry L. McCarty.** 1997. Long- term biodegradation of trichloroethylene influenced by bioaugmentation and dissolved oxygen in aquifer microcosms. Environ. Sci. Technol. **31:**786-791.

31. **Nelson, Michael J.K., Stacy O. Montgomery, William R. Mahaffey and P.H. Pritchard.** 1987. Biodegradation of tricholorethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949-954.

32. **NIOSH**. National Institute for Occupational Safety and Health. National Occupational Exposure Survey (1981-1983). Unpublished provisional data as of 7/1/90. Cincinnati, OH: Department of Health and Human Services, 1990.

33. **NTP**. National Toxicology Program. Carcinogenesis Studies. Trichloroethylene (Without Epichlorohydrin) [CAS No.79-01-6] in F344/N Rats and B6C3F1 Mice (Gavage Studies). Technical Report Series TR-243. Research Triangle Park, NC: NTP, 1990

34. **Nogales, Balbina, Edward R.B. Moore, Enrique Llobet-Brossa, Ramon Rossello-Mora, Rudolf Amann, and Kenneth N. Timmis**. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. **67**:1874-1884.

35. **Osborn, A. Mark, Edward R.B. Moore and Kenneth N. Timmis**. 2000. An evaluation of terminal-restriction fragment polymorphism (TRFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology. **2**:39-50.

36. **Semprini, Lewis.** 1995. In situ bioremediation of chlorinated solvents. Environ. Health Perspectives. **103**:Supp.5.

37. **Sharma, Pramod K. and Perry L. McCarty.** 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenated tetrachloroethene to *cis*-1, 2-Dichloroethene. Appl. Environ. Microbiol. **62**:761-765.

38. **Taylor, R.T.** 1993. In situ bioremediation of trichloroethylene-contaminated water by a resting-cell methanotrophic microbial filter. Hydrolog.Sci. Jour. **38**:323-342.

39. **The Edwards Air Force Base Installation Restoration Program**. 2000. www.edwards.af.mil/penvmng/aboutedwards/cleanup/irppage.htm.

40. **Vogel, Timothy M., Craig Criddle, and Perry L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21:**722-736.

41. **Wackett, Lawrence P. and David T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703-1708.

42. **Wartenberg, D., D. Reyner, and C. Siegel.** Trichloroethylene and cancer: epidemiologic evidence. Environ. Health Perspect., Vol. 108 (Suppl 2), 2000, pp.161-176.

43. **Wilson, John T. and Barbara H. Wilson.** 1985. Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. **49:**242-243.

44. **Zhou, Jizhong, Mary Ann Bruns, and James M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316-322.

Chapter II

Microbial community response during a field evaluation of toluene as the primary substrate for TCE co-oxidation.

Introduction

Trichloroethene (TCE), a well-known groundwater pollutant, is of great concern throughout the United States. It has been widely used as a solvent to remove oil and grease from metal parts. TCE has moderate water solubility (~1100 ppm), hence, it is mobile in soils and often found as a groundwater contaminant. It is denser than water and hence can also migrate through soils and sub-soils as a DNAPL. According to the EPAs Contract Laboratory Program Statistical Database, TCE is reported to occur in 19% of groundwater samples at a geometric mean concentration of 27.3 ppb (individually ranging from 0.1 to 27,300 ppb).

Pilot-scale studies for *in situ* bioremediation have shown that certain substrates such as toluene or phenol injected into aquifer material stimulated co-oxidation of indigenous TCE-degrading organisms (Jenal-Wanner et al., 1997). McCarty et al. (1998) completed several small-scale field experiments at Moffett Field, CA which showed that phenol and toluene stimulated *in situ* TCE bioremediation. The microbial community composition and succession were also evaluated in response to the phenol, toluene, and chlorinated aliphatic hydrocarbon treatments (Fries et al., 1997). The TCE removal efficiency was much greater with either phenol or toluene than with methane (>85% versus 15%) and taxonomically diverse communities of indigenous phenol and toluene degraders with TCE-degrading ability were stimulated. Both gram negative and

positive bacteria were found: the former included members of the genera *Burkholderia*, *Variovorax*, and *Azoarcus*, all from the phylum Proteobacteria and the later included members of the genera *Rhodococcus* and *Nocardia,* all from the phylum Actinobacteria of high G+C content (Fries et al., 1997).

A microcosm and full-scale study done at Edwards AFB, a site contaminated with TCE and amended with toluene, concluded that TCE removal between 87%-99% and 83%-87%, respectively, occurred by cometabolic degradation. Because of the success of their studies at Edwards AFB, another full scale test of TCE remediation by cometabolism was undertaken to test the combination of bioremediation coupled with a vapor stripping system. The Stanford engineering team evaluated the effectiveness of the cometabolic process in removing TCE and I evaluated the microbial community response by measurements at three sampling periods: *before toluene injection (June 2000); one month after the initial injection of toluene (November 2001); and 3 months after toluene injection (late January 2002*, at the end of the experiment).

Materials and Methods

Site Description. Edwards AFB, located about 60 miles north of Los Angeles, was selected by a team of Stanford University scientist and engineers because it presented ideal conditions for the study. The groundwater plume was aerobic and contaminated with approximately 500-1500 μg/L of TCE and no PCE. At the site are two bio-treatment wells, each with two aquifers, upper and lower, separated by a silty clay aquitard of low permeability. The upper aquifer is unconfined while the lower aquifer is confined and lies above weathered bedrock (Fig. 2.1). The sediments are of alluvial origin and lacustrine deposits (McCarty et al., 1998). The aquifer material consists of fine to medium size sand with some silt. Sieve analysis of aquifer material from borings at the evaluation site at four depths indicated that the lower aquifer material is somewhat larger {suter mean diameter d_{sm}=0.4 mm} and better degraded {coefficient of uniformity Cu=7; coefficient of gradient Cg=1} than upper aquifer material {d_{sm}=0.2 mm; Cu=4; Cg=1) (McCarty et al., 1998). The permeability should allow the groundwater to be mixed effectively by pumping.

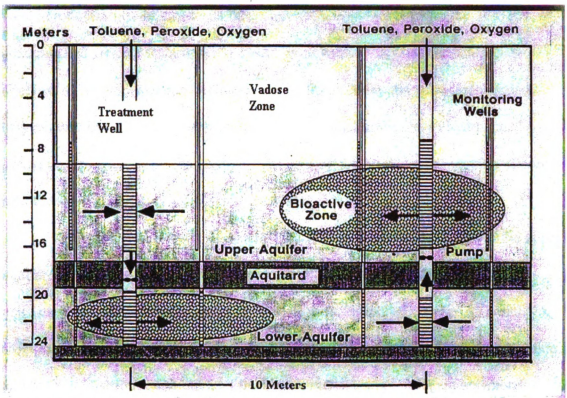


Figure 2.1. A cross-sectional view of a two-well cometabolic TCE biodegradation treatment system spanning two separate aquifers. Also illustrates wells screened in both shallow upper aquifer and deeper lower aquifer. This was the first full-scale test implemented at Edwards AFB (McCarty et al, 1998).

Previous field test design. The Stanford engineering team implemented the following full scale remediation design to treat the groundwater contaminated with 500-1200ug/L TCE *in situ* over a 410-day period by co-metabolic biodegradation. A submersible pump capable of delivering a flow rate of 10 gallons per minute (gpm) was installed between the two screens of each bio-treatment well located 10 m apart to induce the flow field shown in Fig. 2.1 (McCarty et al., 1998). Well 1 withdrew groundwater from the upper aquifer and discharged it into the lower aquifer and well 2 did the reverse. This caused the water to circulate between the two aquifers (Fig. 2.1) (McCarty et al., 1998). Toluene and oxygen, added in pulses for a timed concentration of 7-13.4 mg/L,

were introduced into the two wells through feed lines and mixed with the TCE-contaminated water (McCarty et al., 1998). Following 18 days of periodic toluene injection to develop an active biological population, continuous pulses were added. Oxygen was added because of the potential bactericidal properties of hydrogen peroxide. However, hydrogen peroxide was later used to prevent the clogging of pores near the injection wells (McCarty et al., 1998).

This full-scale test indicated that 87% TCE removal was achieved in the upper aquifer with each pass through the treatment well and 83% in the lower aquifer, consistent with the predicted results of 87-99% removal in the Edwards AFB soil (McCarty et al., 1998). In addition, 99% toluene was removed through biodegradation and biomass distribution throughout the treatment zone was contingent on where toluene consumption was the greatest, which was usually within the first couple of meters from the treatment wells (McCarty et al., 1998).

Design for full-scale test with a vapor-stripping system. This full-scale TCE treatment system, also designed by the Stanford team included a TCE vapor stripping system since preliminary studies suggested it was necessary to reduce TCE to non-toxic levels for the bioremediation. Hence, the Bio-Enhanced In-Well Vapor Stripping (BEHIVS) system was implemented in a different location on the same TCE plume, but much closer to the source of TCE contamination where TCE concentrations were much higher. The system consists of one vapor stripping well (up-flow) pumping at 8 gpm and two bio-treatment wells (down-flow) pumping at 4 gpm each (Fig. 2.2). Each well has two aquifers, upper and lower, separated a clay aquitard of low-permeability. The

figure below (Fig. 2.2) shows that the vapor stripping well creates a raised water gradient that aids the water cycling in the upper aquifer. TCE-contaminated water is pumped up through the lower aquifer to the upper aquifer of the vapor-stripping well and then dispersed out to the upper aquifer of the bio-treatment wells pumping down-flow to the lower aquifer where toluene, O_2 and less TCE-contaminated water flowed. The vapor stripping well (DO4) and bio-treatment wells (Bio 1, Bio 2) (Fig. 2.3a), created the groundwater flow shown in Fig. 2.3b. Also included in this BEHIVS system are 20 nested (deep and shallow) monitoring wells in the test zone (Fig. 2.3a and 2.3b). The monitoring wells could be easily installed because the groundwater surface lies about 9 m below the ground surface.

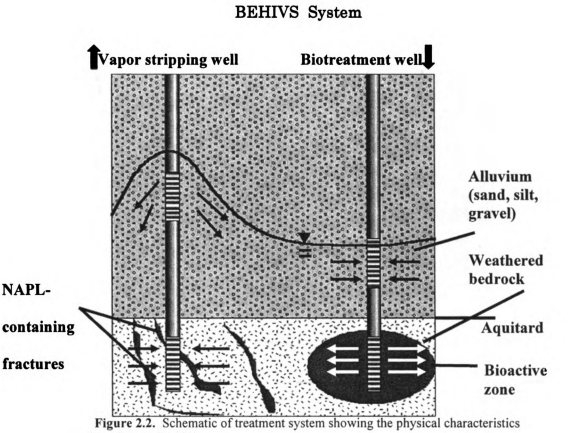


Figure 2.2. Schematic of treatment system showing the physical characteristics (Gandhi, et al, 2000).

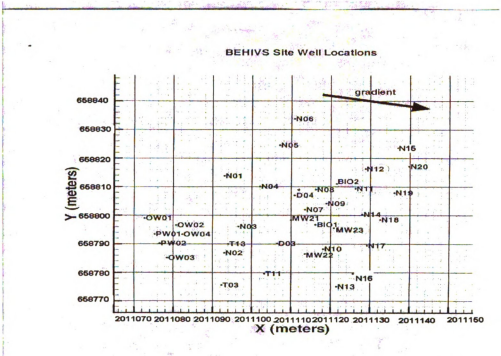


Figure 2.3a. Aerial view of the site locations within the BEHIVS system. The BEHIVES system includes the vapor stripping well (D04) and the bio-treatment wells (Bio). The other wells are monitoring wells (MW) and (T) and nested wells (N). The remaining wells were not examined.(Gandhi,, et al, 2000).

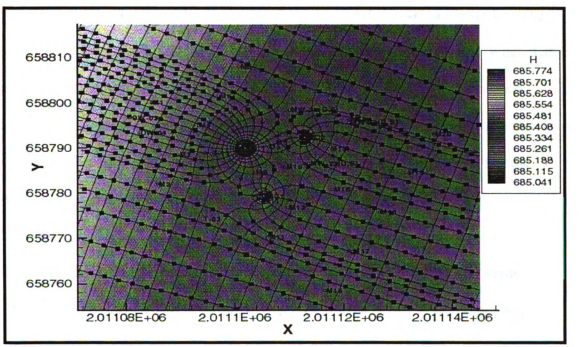


Figure 2.3b. Simulated Flow Field at Edwards AFB created by one up-flow well and two down-flow bio-treatment wells. (Gandhi, et al, 2000).

Collection of microbes from groundwater samples. High throughput filters, which had 50 gallons of monitoring well water run through them, were used to collect the microbial biomass from the site. The commercial filters (Parker Process Filtration, Lebanon, IN) were made of thick layers of yarn raveled together to create a hollow cylinder. The yarn cores were removed from their holder, placed in plastic bags along with some of the water contained in the filter housing, and transported on dry ice in coolers overnight to Michigan State University (MSU). On arrival, unfrozen samples were kept at 4°C until analysis, which was within 7 to 10 days.

Biomass collection. The filters were unraveled with the aid of a sterile scalpel and forceps, and all contents placed in a sterile 2 L beaker. Sterile 1M sodium phosphate buffer (pH 7) was then added to cover the filter parts and the beaker was covered with aluminum foil. Each beaker was placed on a rotary shaker in a 4°C room and operated at medium speed overnight. The buffer was then poured off into 4L sterile flasks. The beakers were filled again with sterile buffer and placed on the shaker for 2h. When that buffer was poured off, additional buffer was used to rinse the parts. The combined collection solution was centrifuged at 5000 rpm in sterile centrifuge bottles to collect cell pellets. A pellet was noticeable in some of the samples. A filter control sample was processed in the same way except no water was passed through the filter.

Terminal Restriction Fragment Length Polymorphism (TRFLP)

In order to determine the initial community structure of the site at Edwards AFB, 16S rRNA TRFLP was done on water samples taken before toluene injection (***background***). A 1 mL solution of the cell pellet was resuspended in 50 mL of sodium phosphate buffer. DNA was extracted using the MO BIO Ultra-Clean Soil DNA

Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA). DNA recovery was assessed on a 0.8% agarose electrophoresis gel, and quantified at 260 nm by UV spectrophotometry. Total DNA was amplified using the Polymerase Chain Reaction (PCR) with the fluorescently labeled primer, 8F, (*E. coli* numbering) {8 forward --5' AGAGTTTGATCMTGGCTCAG 3'} and the reverse primer 1392{1392 reverse --5' ACGGGCGGTGTGTACA 3'}(Amann, et al, 1995). The primers along with the DNA were added to a master mix containing ReadyMix™ RedTaq™ PCR Reaction Mix with $MgCl_2$ (Sigma, St. Louis, MO). This PCR generated a mixture of amplicons of the same size and with a fluorescent label at the 5' end. The PCR cycle used for amplification was: 95°C 3 min-Hot Start/Denaturation, 30 cycles of {94°C 30 sec denaturation, 55°C 45 sec annealing, 72°C 1 min elongation} followed by 72°C at 7 min-final extension held at 4°C.

After three (3) replications of PCR, the amplicons were combined and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified DNA product was quantified by UV spectrophotometry and by gel visualization. The samples were then digested with restriction enzyme *Hpa11*. The master mix consisted of 1 µl 10X buffer, 1 µl BSA, 0.5 µl restriction enzyme (10 U/µl), and 2.5 µl water. Digestion occurred for 2-3 h at 37°C using 5 µl of PCR product. The enzyme was inactivated by heating the digest to 65°C for 15 min.

Two micro liters of TAMRA GS 2500 marker (Perkin Elmer) was added to 2 µl of the digested PCR products. The combination was separated in a 6% polyacrylamide gel in a DNA sequencer (373 ABI Stretch) for 14 h at 168 volts. The data was viewed using Gene Scan software version 3.1.

Community similarity. Community similarity before toluene injection (TRFLP) was calculated using the Morisita index, as described by Dollhopf et al (2001). The Morisita index of community similarity, which is based on Simpson's dominance index, was calculated using the formula

$$I_M = \frac{2\Sigma n_{1i} n_{2i}}{(l_1 + l_2) N_1 N_2}$$

where n_1 is the number of individuals of species i, N is the total number of individuals sampled and l is Simpson's dominance index for each community. The formula for Simpson's index is

$$l = \frac{\sum_{n=1}^{s} (ni(ni-1))}{N(N-1)}$$

where s is the total number of species in the community. The index ranges from 0 to 1, with 0 designating that no species are shared between the two communities and 1 meaning there is complete identity. Because the index takes species abundance into account, communities that contain the same species but have different species abundance will have an n index value less than 1. The data will be reflective of recognizing each terminal restriction fragment as a separate species and peak height as a measure of species abundance.

Burkholderia cepacia analysis. Samples were analyzed for *Burkholderia cepacia* complex (Bcc) by using Bcc-specific *recA* primers. Edwards' samples taken three months after toluene was added to the wells (*end of the experiment*) were analyzed for the presence of the *recA* gene. DNA was extracted using the MO BIO UltraClean Soil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA). DNA recovery was assessed on a 0.8% agarose electrophoresis gel, and quantified at 260 nm by UV spectrophotometry. The RFLP pattern or the nucleotide sequence is a rapid and reproducible means of identifying the genomovars within the *B. cepacia* complex. BCR1 (5'- TGACCGCCGAGAAGAGCA-3') and BCR2 (5'-CTCTTCTTCGTCCATCGCCTC-3') were designed by Mahenthiralingam et. al (2000) from homologous sequences at the 5' and 3' end of the *recA* open reading frame and amplify a single 1-kb amplicon from all strains representative of the Bcc.

The DNA samples were analyzed by PCR using a master mix of ReadyMix™ RedTaq™ PCR Reaction Mix with $MgCl_2$ (Sigma, St. Louis, MO). The positive control was *Burkholderia vietnamiensis* G4. The PCR cycle used for amplification was: 95°C 3 min-Hot Start/Denaturation, 30 cycles of {94°C 30 sec denaturation, 58°C 45 sec annealing, 72°C 1 min elongation} followed by 72°C at 10 min-final extension held at 4°C. The amplicons generated were then purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified DNA product was quantified by UV spectrophotometry and by gel visualization. Finally, the products were taken to GTSF at Michigan State University to be sequenced in order to determine if the samples that were selected using the *recA* primers were indeed members of the *Burkholderia cepacia* complex.

High-Throughput Sequencing of Clone Libraries

To better determine the active microbial communities in the Edwards AFB well samples, I prepared clone libraries of 16S rRNA genes in the aquifer water before and after substrate addition. The wells analyzed for the subsequent analysis were chosen in relation to their location to the Bio-treatment wells (BIO) (Fig. 2.4). I used: N1L and N5L-wells upstream of BIO; MW23L and N11L-wells nearest BIO; and N16L and N18L-wells downstream of BIO. The sampling times were: before toluene injection (***background***); one month after toluene injection (***initial***); and three months after toluene injection (***end of the experiment***). DNA was extracted from all samples using the MO BIO UltraClean Soil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA). DNA recovery was assessed on a 0.8% agarose electrophoresis gel, and quantified at 260 nm on an UV spectrometer. DNA was amplified using the Polymerase Chain Reaction (PCR) with the universal primer (*E. coli* numbering) 8-27F {8 forward --5' AGAGTTTGATCMTGGCTCAG 3'} and the reverse primer 1392-1406R {1392 reverse --5' ACGGGCGGTGTGTACA 3'}(Amann, et al, 1995). The primers along with the DNA were added to a master mix containing ReadyMix™ RedTaq™ PCR Reaction Mix with $MgCl_2$ (Sigma, St. Louis, MO). The PCR cycle used for amplification was: 95°C 3 min-Hot Start/Denaturation, 30 cycles of {94°C 30 sec denaturation, 55°C 45 sec annealing, 72°C 1 min elongation} followed by 72°C at 7 min and a final extension held at 4°C. The amplicons generated were then purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified DNA product was quantified by UV spectrophotometry and by gel visualization.

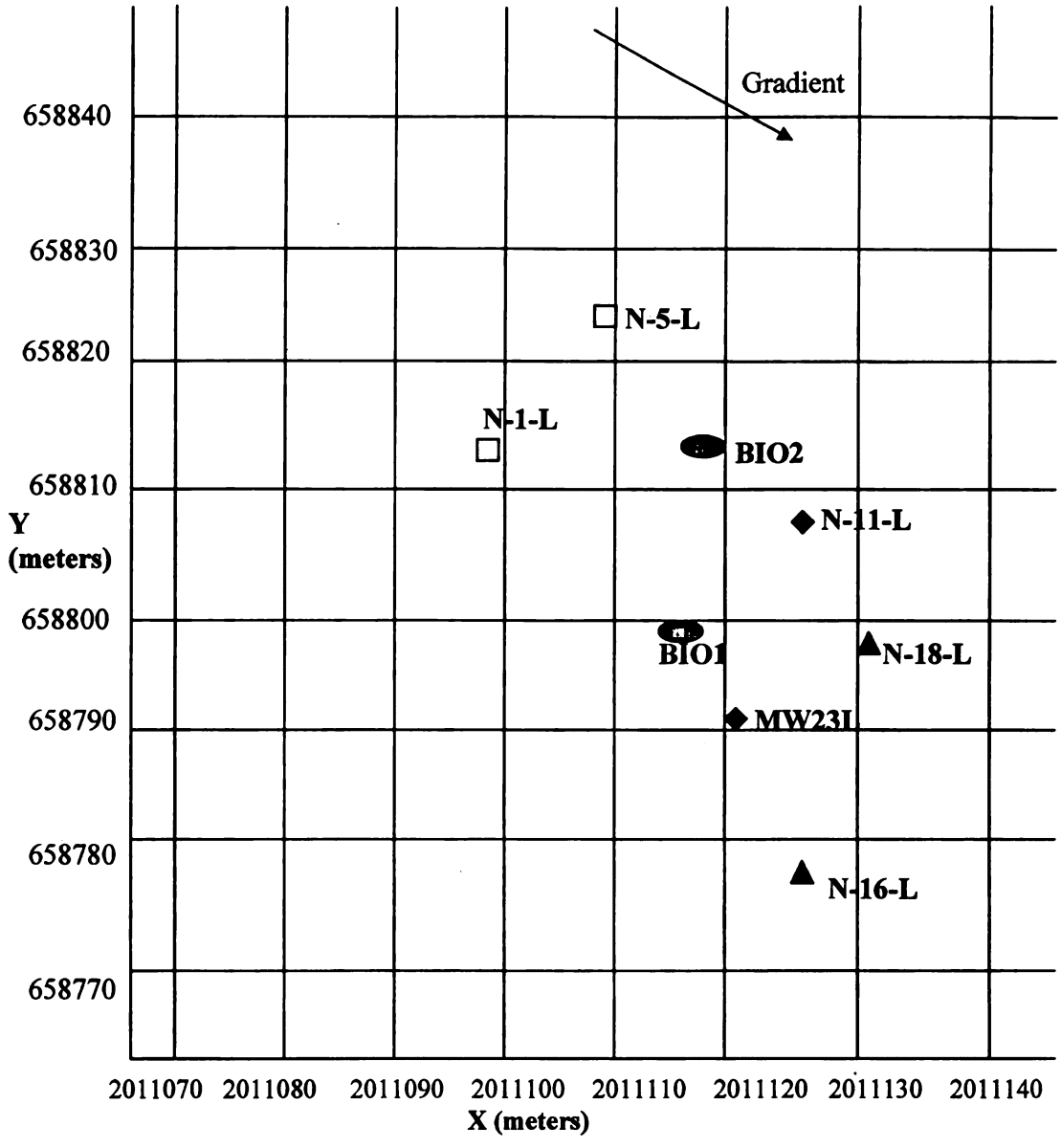


Figure 2.4: Well diagram of only the samples that were analyzed. ⬭ represents the biotreatment wells (BIO1 and BIO2) amended with toluene, □ represents samples upstream of BIO1 and BIO2, ◆ represents samples nearest BIO1 and BIO2, and ▲ represents samples downstream of BIO1 and BIO2. The images seen here as well as several other images in this dissertation are presented in color.

Cloning. Purified PCR products were cloned using the TOPO-TA cloning kit with the PCR 2.1 Vector according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). To set up the reaction, the following components were needed: 2 µl of fresh PCR product; 2 µl sterile water; 1 µl salt solution and 1 µl TOPO vector (each from the TOPO TA Cloning Kit). The reagents were mixed gently and then incubated for 5 min at room temperature. The reaction was immediately placed on ice or at -20°C overnight before the One Shot Chemical Transformation. Two micro liters of the cloning reaction was added into a vial of one shot chemically competent *E. coli* and mixed gently. The cells were incubated on ice for 30 min and then heat shocked for 30 sec. at 42°C without shaking and again transferred to ice. Then, 250 µl of the SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added and the vials were placed horizontally on a shaker (200 rpm) at 37°C for 1 h. Afterwards, 10-150 µl of each transformation was spread on a pre-warmed Luria Bertani (LB) plates containing 250 µl ampicillian, 500 µl IPTG (4%), and 500 µl XGAL (4%), and incubated overnight at 37°.

For analysis, LB Freezing Buffer (LBFB) was used. The pH was corrected to 7.5 with NaOH and the following was added: K_2HPO_4 12.6 g; KH_2PO_4 3.6 g; sodium citrate, dehydrate 1.0 g; $MgSO_4 \cdot 7H_2O$ 2.0 g; ammonium sulfate 1.8 g; and glycerol 88.0 ml. After the volume was adjusted to 2 L, 500 µl was transferred to individual bottles and autoclaved. Once cooled, 83 µl of ampicillian (300 ng /ml) was added to each bottle.

Sterile toothpicks were used to pick up well-defined white colonies from the LB plates and place them into each well of a growth block containing 600 µl of the LBFB. When completed, air pore paper was used to cover the blocks. The growth blocks were then placed on a shaker at 37°C overnight.

Sequencing. One hundred micro liters was taken from each well of the growth blocks and placed in the same position in the 96-well micro-plates. Once completed, aluminum foil was used to cover the plate to avoid contamination and the plate taken to the Genomic Technology Support Facility (GTSF) at Michigan State University, East Lansing, MI 48824. DNA from *E. coli* clones was purified at GTSF using Qiagen 3000 robots. Once the fluorescent-labeled sequencing products were generated by PCR amplification, the products were separated by capillary electrophoresis on an ABI Prism 3700 DNA Analyzer. The sequence from GTSF was piped to a Finch-Server (copyright 2000 Geospiza, Inc., Seattle, WA. http://www.geospiza.com), a web-based interface for retrieving sequencing data. The data were then filtered for quality and classified by the Ribosomonal Database Projects (RDP) classifier according to Bergey's Hierarchy. This output was compared among wells and sampling times.

Determination of significant differences within communities. The difference in occurrence of microbial taxa was analyzed based on the Bayesian method. Given the usual community complexity, observing a given taxon qualifies as a rare event, as the abundance of most individuals are of the order of a few percents or less (Audic et al, 1997). The probability of a given 16S rRNA gene sequence to be picked up x times when the sampling size was N_1, and y times when the sampling size was N_2 is given by the formula:

$$P(y/x)=\left(\frac{N_2}{N_1}\right)^y \quad \frac{(x+y)}{x!y!\left(\frac{1+N_2}{N_1}\right)^{(x+y+1)}}$$

whereas significant differences between x (from one library) and y (from the other) will characterize different communities, i.e., the relative abundance of which is unlikely to be the same in the two libraries, each having different sampling sizes. If the probability (P) is less than 0.01 (10^{-2}), the difference in x and y is significant (Audic et al, 1997). The probability was calculated by the Ribosomal Database Project (RDP) at MSU.

Results

Community composition. DNA was extracted from the cotton filters (4°C) that had recovered microbes from six sampled wells at Edwards AFB. A PCR amplification using 16S rRNA primers 8F and 1392R showed that bacteria were present in all samples (Fig. 2.5). Once the samples were purified the DNA concentrations were measured to find out how much of the DNA remained in those samples (Fig. 2.6). A blank sterile filter extracted in the same way produced no detectable DNA.

T-RFLP analysis was used to assess the similarity in community structure among the six wells before toluene was added. The digested fragments ranged from 30 bp to 565 bp in length. Some of the communities within the different wells (Fig. 2.7) were quite similar in that they contained several of the same fragments but not in the same quantity. For instance, all have the presence of a dominant 207 bp fragment. The species that represent this fragment have densities comprising more than 90% of the community (N-11-L), but only 40% of N-5-L. Thus, these communites had a similarity index of 0.74 (Table 2.1). The next dominant T-RF has 93 bp. It is also prevalent in almost all of the samples. Although it is not as dominate as 207 bp, it does constitute almost 40% of the total profile for the N-5-L well community, but as low as 10% for others such as N-18-L. These two communities when compared to each other have a similarity index of 0.66 (Table 2.1). Most of the remaining fragments are distributed in lesser percentages within each community.

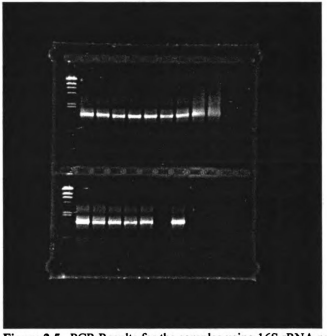
While most of the profiles contain T-RFs 143 bp, 137 bp, and 127 bp, they are not prevalent in all the communities. The amount of DNA recovered from each well varied considerably (Fig. 2.6). There was no apparent correlation between the purified

DNA concentrations (Fig. 2.6) and the community profile (Figure 2.7). The TRFLP analyses indicated that even though the same fragments (representing different taxa) were present in a majority of the well samples, they were not as evenly distributed in the well communities before the toluene injection as evidenced by the average community similarity indices of 0.77, 0.81, 0.83, 0.81, 0.72, and 0.78 among the wells i.e., N-1-L, N-5-L, MW23-L, N-11-L, N-16-L and N-18-L, respectively (Table 2.1).

Lane 1: Marker
Lane 2: N9L
Lane 3: MW23D
Lane 4: T11
Lane 5: N10L

Lane 1: Marker
Lane 2: N5L
Lane 3: N6L

Lane 4: N16L
Lane 5: N7L



Lane : N18L
Lane 7: N19L
Lane 8: N4L
Lane 9: N12L
Lane10: N17L

Lane 6: N13L
Lane 7: Blank
Lane 8:
*Positive Control
Lane 9: Blank
Lane 10:
*Negative Control

Figure 2.5. PCR Results for the samples using 16S rRNA primers.
Positive control=*E. coli*; Negative control=blank filter

Table 2.1. Morisita community similarity indices for the six well communities, N-1-L, N-5-L, MW23-L, N-11-L, N-16-L, and N-18-L.

	Sample Well Communities					
	N-1-L	**N-5-L**	**MW23L**	**N-11-L**	**N-16-L**	**N-18-L**
N-1-L	**1**	**0.76**	**0.80**	**0.88**	**0.66**	**0.73**
N-5-L	**0.76**	**1**	**0.96**	**0.74**	**0.66**	**0.66**
MW23-L	**0.80**	**0.96**	**1**	**0.80**	**0.81**	**0.77**
N-11-L	**0.88**	**0.74**	**0.80**	**1**	**0.67**	**0.92**
N-16-L	**0.66**	**0.91**	**0.81**	**0.67**	**1**	**0.80**
N-18-L	**0.73**	**0.66**	**0.77**	**0.92**	**0.80**	**1**
Average	***0.77***	***0.81***	***0.83***	***0.81***	***0.72***	***0.78***

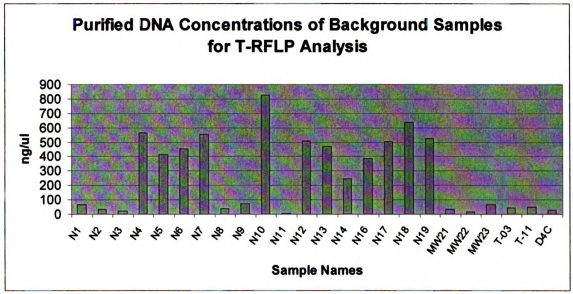


Figure 2.6. Purified DNA concentrations for the various wells taken before the toluene treatment began.

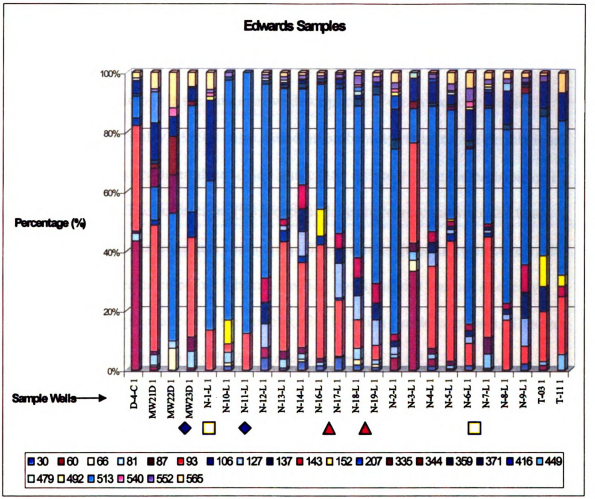


Figure 2.7. TRFLP analysis of background communities taken from 24 different wells. Sizes of colored bars reflect peak heights of different T-RFs (terminal restriction fragment). □ N-1-L and N-5-L, ◆ N-11-L and MW23L, and ▲ N-16-L and N-18-L all reflect wells that will be further analyzed.

Temporal analysis of populations. A clone library of the 16s rRNA genes was constructed and clones sequenced in order to indicate potential community members with a matching TRF. Comparative analyses of the populations present in the Edwards AFB samples before and after toluene injection were evaluated. The results are based on RDPs classifier analysis which is based on Bergey's Taxonomy. Bergey's Manual of Systematic Bacteriology is the authoritative descriptions of all prokaryotic species validly described (Staley, 1989). Also, based on probability statistics, the populations in the communities are determined to be significantly different when the probability (P) is less than 10^{-2}. The percentages (%) identified here are representative of the total number of populations from each community. These results show that collectively, all six (6) well samples taken before toluene injection (***background***) was not as evenly represented by all populations as the samples after injection (***initial and end***). This was further examined by comparing the ***background vs. initial*** (Fig. 2.8-2.10). For example, the genus Pseudomonas occupied 65% of the total community before toluene injection and only 24% of the total community after the initial injection. The probability test shows that the likelihood of the Pseudomonas populations representing the same percentage of both communities is highly unlikely and is thus significantly different ($P=10^{-10}$). These results further show that as you add toluene, the Pseudomonas population was not as abundant, as other populations were stimulated. In contrast, the Sphingomonas population was enriched (16%) as toluene was added over its background (3%) population. The probability that Sphingomonas would occupy the same fraction of the background and initial communities was low ($P=10^{-3.5}$), which

means this particular population is significantly different in the number of members represented in both communities

When comparing ***background vs. end*** samples (Fig. 2.11-2.13), there was also a significant difference with Pseudomonas, Sphingomas, and Legionella populations. Pseudomonas first occupied 65% of the total ***background*** community and then only 27% of the ***end*** community resulting in a decreased probability of occurrence ($P=10^{-13}$) in both communities. Sphingomonas represented 3% of the ***background*** community but 28% of the ***end*** community so there is a very high chance that both communities will not be represented by the same quantity of Sphingomonas species as is evident by the probability of occurrence ($P=10^{-13}$). Legionella on the other hand was not present in the ***background*** community, but was significantly enriched (10%) in the ***end*** community, and thus significantly different ($P=10^{-8}$). Whereas there was a difference in Legionella when ***background*** samples were compared to ***end*** samples, there was no difference when comparing ***background*** to ***initial*** samples, zero and two sequences, respectively. Because of this uneven distribution, no significant difference between the two earlier samples was noted ($P=10^{-1}$). This could indicate that the longer toluene is in the system, the greater the Legionella population. These results also indicate that Sphingomonas and Legionella had similar trends as it relates to their abundance when toluene was added.

Comparison of the ***initial*** samples vs. ***end*** samples (Fig. 2.14-2.16) indicate that there is no difference ($P=10^{-1.5}$) in the Pseudomonas population, which represents 24% of the initial community, and 27% of the end community. These analyses also show a trend of increasing frequency of the Sphingomonas and Legionella populations.

Sphingomonas was significantly different ($P=10^{-3}$) occupying 16% of the initial community composition and then had a shift to 28% of the end community. And it is also not likely that Legionella would occur in both communities($P=10^{-3.5}$) embodying 2% of the initial composition and 10% of the end community.

The results of the changing community profiles of the background, initial, and end samples are summarized in Fig. 2.17.

Spatial analysis of populations. The same data was compared to determine any spatial patterns of populations present in the Edwards AFB samples. The two (2) wells located ***upstream*** of the bio-treatment wells, N-1-L and N-5-L were tested against wells ***nearest*** the treatment wells, MW23L and N11L and wells ***downstream*** of the treatment wells N16L and N18L. Data from all three sampling wells are included in this analysis. Well samples ***nearest*** and ***downstream*** of the treatment wells show a richer composition than the wells ***upstream*** of the treatment wells. This is demonstrated in Figures 2.18-2.20 where again Pseudomonas was not as evenly distributed in the well community ***nearest*** the bio-treatment wells (26%) as it was in the ***upstream*** community (51%). Thus the probability of Pseudomonas representing the same fraction of both communities was low ($P=10^{-2.1}$). This spatial analysis also showed a significant reduction in the Sphingomonas populations for the ***upstream*** to ***nearest*** wells with Sphingomonas representing 21% and 11%, respectively of each well community. Legionella, however, was much more plentiful in the wells ***nearest*** the treatment wells representing 11% of the community than it was in the ***upstream*** community where 0% of the population was represented and thus the probability of occurring in both wells was low ($P=10^{-7}$).

In the evaluation of the ***upstream*** well samples to the well samples ***downstream*** of the treatment wells, again there was a significant decline in the Pseudomonas population from 51% to 23%, respectively. However the Sphingomonas populations in these two systems were not significantly different representing 21% and 24% of the two communities. But, the Legionella population was significantly more abundant, from not being represented in the ***upstream*** well community and then representing 9% of the ***end*** community.

The well communities ***nearest*** the bio-treatment wells compared to the communities ***downstream*** of the treatment show that Pseudomonas basically represented similar fractions of both community (26% and 23%, respectively), so the probability of occurring was relatively high ($P=10^{-1.4}$). Also, the Legionella populations were not significantly different with a slight downward shift from 11% to 9% of the communities. Sphingomonas, however, was significantly more abundant representing 11% of the well community ***nearest*** the bio-treatment wells and 24% of the well community downstream of the bio-treatment wells.

The population profiles are summarized in Figure 2.27 and indicate a trend of Pseudomonas becoming less abundant moving from ***upstream*** to ***nearest*** to ***downstream*** of the treatment wells. Alternatively, Legionella showed a greater species richness from the well community upstream to the community nearest the bio-treatment wells, but then a reduction in number further downstream of the groundwater flow. Additionally, Sphingomonas declined from the well communities upstream of the bio-treatment wells to the communities nearest the treatment wells, but a significant rise in number further downstream of the groundwater flow.

Detection of Burkholderia cepacia complex* (Bcc).** Several samples from Edwards AFB that were taken three months after the injection of toluene (end of experiment***) were used in an attempt to amplify the *recA* gene of the Bcc. About ¼ of the samples produced visible bands and those that did were not strong. Those well samples were N-8-L, N-9-L, N-12-L, N-16-L, N-17-L, and N-18-L (Fig. 2.28a and 2.28b). Sequencing those amplicons produced no results, i.e., they did not have a passing score when analyzed by GTSF, therefore, they could not be sequenced. The result is consistent with finding no members of the genus *Burkholderia* in the analysis of the 16S clones (Fig. 2.8-2.16). Bacteria of the order Burkholderiales and the family Burkholderace were found, however (Table 2.2). The weak bands found using the *recA* primers that were not sequenced could be attributed to miss priming, hence not detecting the *Burkholderia cepacia* complex.

Clone Library results of all six (6) wells and their significance to each other

Figures 2.8-2.9: Composite analysis of samples taken **before toluene injection** and samples taken **one month after toluene injection.**

Figure 2.10: Significant difference of samples taken **before toluene injection** and samples taken **one month after toluene injection.**

Figures 2.11-2.12: Composite analysis of samples taken **before toluene injection** and samples taken **three months after injection.**

Figure 2.13: Significant difference of samples taken **before toluene injection** and samples taken **three months after injection.**

Figures 2.14-2.15: Composite analysis of samples taken **one month after toluene injection** and samples taken **three months after injection.**

Figure 2.16: Significant difference of samples taken **one month after toluene injection** and samples taken **three months after injection.**

Figure 2.17: Profiles of samples taken **before toluene injection (background), one month after injection (initial),** and samples **taken at the end of the experiment (end)** samples.

Figures 2.18-2.19: Composite analysis from all three sampling periods of samples **upstream** and **nearest** the BIO.

Figure 2.20: Significant difference of the composite analysis from all three sampling periods of samples **upstream** and **nearest** the BIO.

Figures 2.21-2.22: Composite analysis from all three sampling periods of samples **upstream** and **downstream** of BIO.

Figure 2.23: Significant difference of the composite analysis from all three sampling periods of samples **upstream** and **downstream** of BIO.

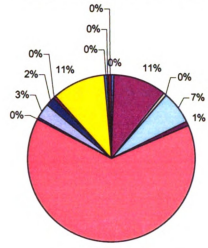
Figures 2.24-2.25: Composite analysis from all three sampling periods of samples **downstream** and **nearest** of BIO.

Figure 2.26: Significant difference of the composite analysis from all three sampling periods of samples **downstream** and **nearest** of BIO.

Figure 2.27: Profiles of the composite analysis from all three sampling periods of samples **upstream, nearest, and downstream** of BIO.

2.8

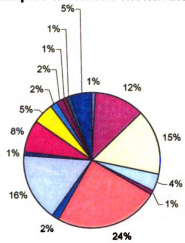
Samples taken before toluene injection (background)



- ACINETOBACTER(1)
- ALISHEWANELLA(24)
- FLAVOBACTERIUM(1)
- FLEXIBACTER(15)
- PROPIONIBACTERIUM(2)
- PSEUDOMONAS(148)
- RHIZOBIUM(1)
- SPHINGOMONAS(7)
- CORYNEBACTERIUM(4)
- FINEGOLDIA(1)
- JANTHINOBACTERIUM(24)
- METHYLOBACTERIUM(1)
- SHIGELLA(1)
- THIOCAPSA(1)

2.9

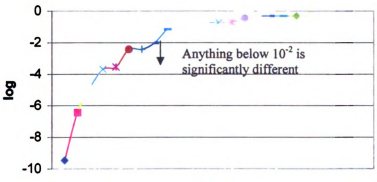
Samples taken one month after toluene injection (initial)



- SINORHIZOBIUM (1)
- ALISHEWANELLA(14)
- FLAVOBACTERIUM(18)
- FLEXIBACTER(5)
- ARENIBACTER(1)
- PSEUDOMONAS(29)
- RHIZOBIUM (2)
- SPHINGOMONAS(19)
- BREVUNDIMONAS (1)
- DYADOBACTER(9)
- GELIDIBACTER(6)
- HYDROGENOPHAGA(2)
- LEGIONELLA(2)
- DEVOSIA (1)
- MAGNETOSPIRILLUM (1)
- OPITUTUS(6)

2.10

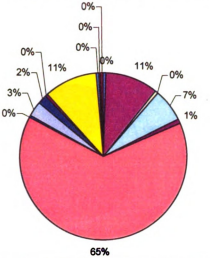
log_sign of (background vs. intial)



- PSEUDOMONAS
- FLAVOBACTERIUM
- JANTHINOBACTERIUM
- DYADOBACTER
- SPHINGOMONAS
- OPITUTUS
- GELIDIBACTER
- RHODOBACTER
- BREVUNDIMONAS
- FLEXIBACTER

2.11

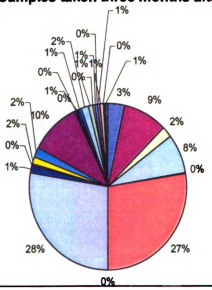
Samples taken before toluene injection (background)



- ACINETOBACTER(1)
- ALISHEWANELLA(24)
- FLAVOBACTERIUM(1)
- FLEXIBACTER(15)
- PROPIONIBACTERIUM(2)
- PSEUDOMONAS(148)
- RHIZOBIUM(1)
- SPHINGOMONAS(7)
- CORYNEBACTERIUM(4)
- FINEGOLDIA(1)
- JANTHINOBACTERIUM(24)
- METHYLOBACTERIUM(1)
- SHIGELLA(1)
- THIOCAPSA(1)

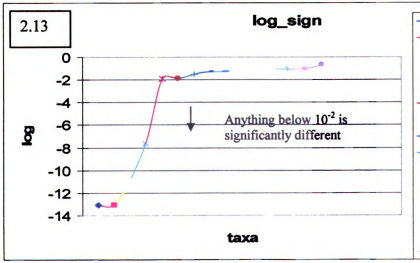
2.12

Samples taken three months after toluene injection (end)

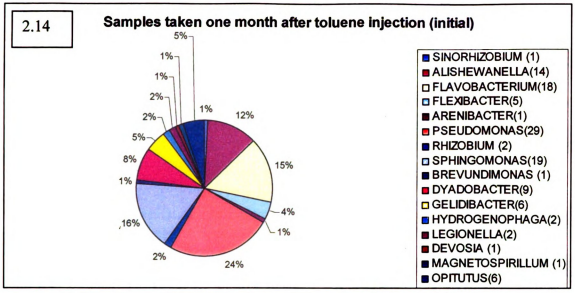


- ACINETOBACTER(13)
- ALISHEWANELLA(35)
- FLAVOBACTERIUM(8)
- FLEXIBACTER(29)
- PROPIONIBRIC(1)
- PSEUDOMONAS(105)
- RHIZOBIUM(1)
- SPHINGOMONAS(106)
- BREVUNDIMONAS (5)
- DYADOBACTER(1)
- GELIDIBACTER(6)
- HYDROGENOPHAGA(7)
- LEGIONELLA(39)
- AMINOBACTER(1)
- ASTICCACAULIS (1)
- AZOSPIRILLUM (2)
- CAULOBACTER (2)
- LIMNOBACTER(6)
- MICROBACTERIUM (3)
- NOVOSPHINGOBIUM (1)
- PARACOCCUS (2)
- PSYCHROBACTER(1)
- RHODOBACTER(2)
- RHODOCOCUS (3)
- SHEWANELLA(1)
- SPHINGOBACTERIUM(2)

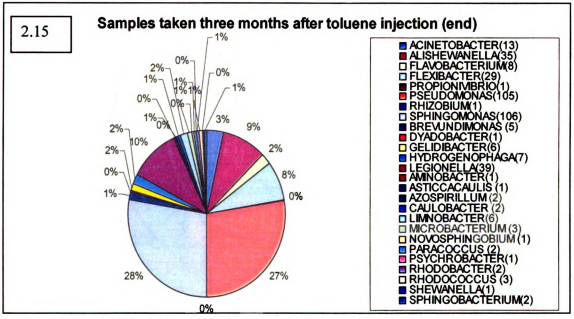
2.13



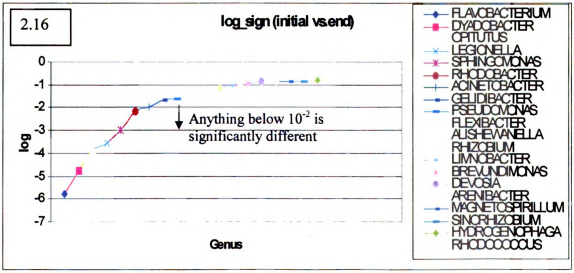
2.14

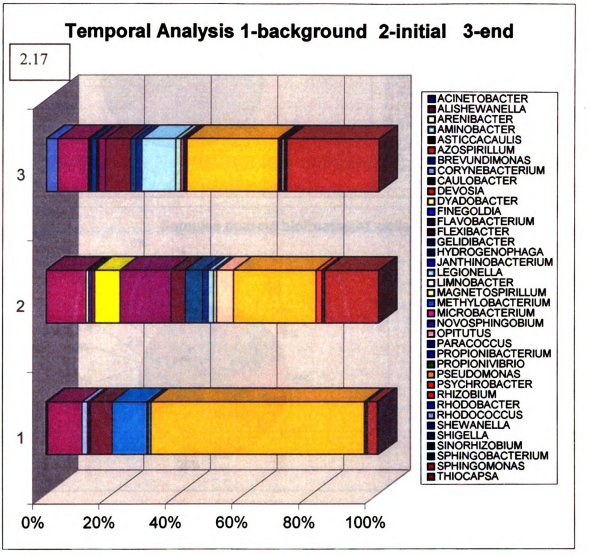

Samples taken one month after toluene injection (initial)
5%
1%
1%
2%
2%
5%
8%
1%
16%
2%
1%
12%
15%
4%
1%
24%
SINORHIZOBIUM (1)
ALISHEWANELLA(14)
FLAVOBACTERIUM(18)
FLEXIBACTER(5)
ARENIBACTER(1)
PSEUDOMONAS(29)
RHIZOBIUM (2)
SPHINGOMONAS(19)
BREVUNDIMONAS (1)
DYADOBACTER(9)
GELIDIBACTER(6)
HYDROGENOPHAGA(2)
LEGIONELLA(2)
DEVOSIA (1)
MAGNETOSPIRILLUM (1)
OPITUTUS(6)

2.15

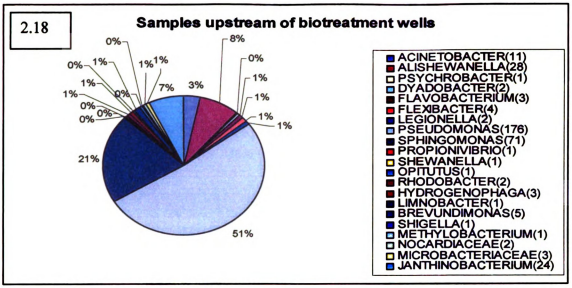

Samples taken three months after toluene injection (end)
1%
0%
2%
1%
1%
1%
0%
0%
0%
1%
1%
0%
3%
2%
10%
2%
9%
2%
0%
8%
1%
0%
28%
27%
0%
ACINETOBACTER(13)
ALISHEWANELLA(35)
FLAVOBACTERIUM(8)
FLEXIBACTER(29)
PROPIONIVIBRIO(1)
PSEUDOMONAS(105)
RHIZOBIUM(1)
SPHINGOMONAS(106)
BREVUNDIMONAS (5)
DYADOBACTER(1)
GELIDIBACTER(6)
HYDROGENOPHAGA(7)
LEGIONELLA(39)
AMINOBACTER(1)
ASTICCACAULIS (1)
AZOSPIRILLUM (2)
CAULOBACTER (2)
LIMNOBACTER(6)
MICROBACTERIUM (3)
NOVOSPHINGOBIUM (1)
PARACOCCUS (2)
PSYCHROBACTER(1)
RHODOBACTER(2)
RHODOCOCCUS (3)
SHEWANELLA(1)
SPHINGOBACTERIUM(2)

2.16

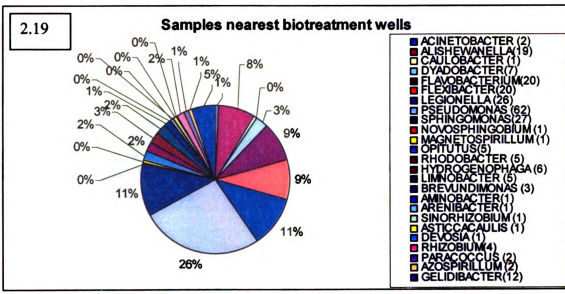

log_sign (initial vs.end)
0
-1
-2
-3
-4
-5
-6
-7
log
Anything below 10-2 is
significantly different
Genus
FLAVOBACTERIUM
DYADOBACTER
OPITUTUS
LEGIONELLA
SPHINGOMONAS
RHODOBACTER
ACINETOBACTER
GELIDIBACTER
PSEUDOMONAS
FLEXIBACTER
ALISHEWANELLA
RHIZOBIUM
LIMNOBACTER
BREVUNDIMONAS
DEVOSIA
ARENIBACTER
MAGNETOSPIRILLUM
SINORHIZOBIUM
HYDROGENOPHAGA
RHODOCOCCUS


Temporal Analysis 1-background 2-initial 3-end
2.17
3
2
1
0%
20%
40%
60%
80%
100%
ACINETOBACTER
ALISHEWANELLA
ARENIBACTER
AMINOBACTER
ASTICCACAULIS
AZOSPIRILLUM
BREVUNDIMONAS
CORYNEBACTERIUM
CAULOBACTER
DEVOSIA
DYADOBACTER
FINEGOLDIA
FLAVOBACTERIUM
FLEXIBACTER
GELIDIBACTER
HYDROGENOPHAGA
JANTHINOBACTERIUM
LEGIONELLA
LIMNOBACTER
MAGNETOSPIRILLUM
METHYLOBACTERIUM
MICROBACTERIUM
NOVOSPHINGOBIUM
OPITUTUS
PARACOCCUS
PROPIONIBACTERIUM
PROPIONIVIBRIO
PSEUDOMONAS
PSYCHROBACTER
RHIZOBIUM
RHODOBACTER
RHODOCOCCUS
SHEWANELLA
SHIGELLA
SINORHIZOBIUM
SPHINGOBACTERIUM
SPHINGOMONAS
THIOCAPSA

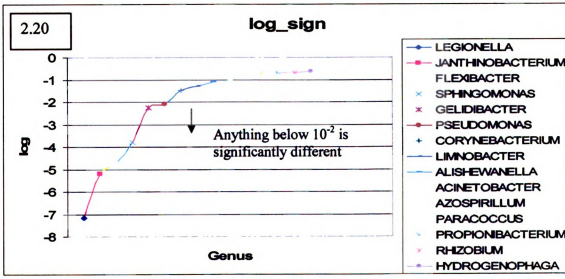
2.18


Samples upstream of biotreatment wells
0%
8%
0%
1%
1%
1%
1%
3%
1%
1%
0%
0%
0%
0%
1%
1%
21%
51%
ACINETOBACTER(11)
ALISHEWANELLA(28)
PSYCHROBACTER(1)
DYADOBACTER(2)
FLAVOBACTERIUM(3)
FLEXIBACTER(4)
LEGIONELLA(2)
PSEUDOMONAS(176)
SPHINGOMONAS(71)
PROPIONIVIBRIO(1)
SHEWANELLA(1)
OPITUTUS(1)
RHODOBACTER(2)
HYDROGENOPHAGA(3)
LIMNOBACTER(1)
BREVUNDIMONAS(5)
SHIGELLA(1)
METHYLOBACTERIUM(1)
NOCARDIACEAE(2)
MICROBACTERIACEAE(3)
JANTHINOBACTERIUM(24)

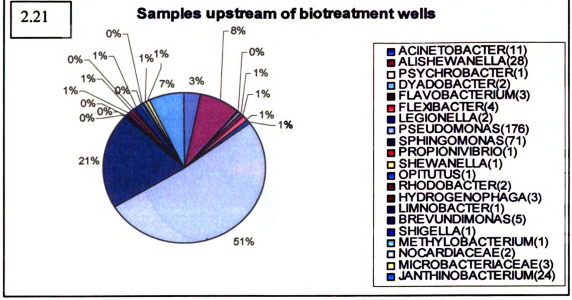
2.19


Samples nearest biotreatment wells
0%
0%
1%
1%
8%
0%
0%
2%
5%
1%
0%
1%
0%
3%
2%
3%
9%
2%
0%
2%
0%
9%
11%
11%
26%
ACINETOBACTER (2)
ALISHEWANELLA(19)
CAULOBACTER (1)
DYADOBACTER(7)
FLAVOBACTERIUM(20)
FLEXIBACTER(20)
LEGIONELLA (26)
PSEUDOMONAS (62)
SPHINGOMONAS(27)
NOVOSPHINGOBIUM (1)
MAGNETOSPIRILLUM (1)
OPITUTUS(5)
RHODOBACTER (5)
HYDROGENOPHAGA (6)
LIMNOBACTER (5)
BREVUNDIMONAS (3)
AMINOBACTER(1)
ARENIBACTER(1)
SINORHIZOBIUM (1)
ASTICCACAULIS (1)
DEVOSIA (1)
RHIZOBIUM(4)
PARACOCCUS (2)
AZOSPIRILLUM(2)
GELIDIBACTER(12)

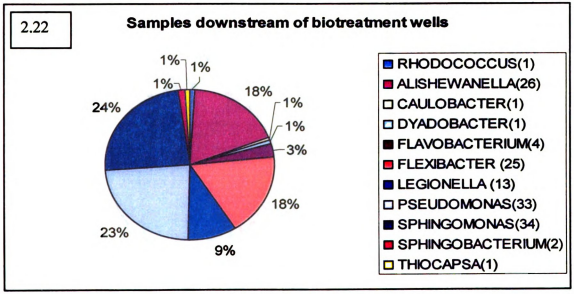
2.20


log_sign
0
-1
-2
-3
-4
-5
-6
-7
-8
log
Anything below 10-2 is
significantly different
Genus
LEGIONELLA
JANTHINOBACTERIUM
FLEXIBACTER
SPHINGOMONAS
GELIDIBACTER
PSEUDOMONAS
CORYNEBACTERIUM
LIMNOBACTER
ALISHEWANELLA
ACINETOBACTER
AZOSPIRILLUM
PARACOCCUS
PROPIONIBACTERIUM
RHIZOBIUM
HYDROGENOPHAGA

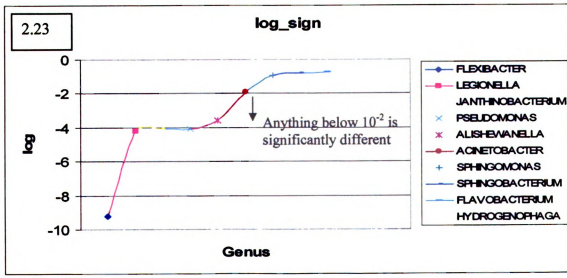
2.21


Samples upstream of biotreatment wells
0%
8%
0%
1%
1%
1%
0%
1%
7%
3%
1%
1%
0%
1%
1%
0%
0%
1%
21%
51%
ACINETOBACTER(11)
ALISHEWANELLA(28)
PSYCHROBACTER(1)
DYADOBACTER(2)
FLAVOBACTERIUM(3)
FLEXIBACTER(4)
LEGIONELLA(2)
PSEUDOMONAS(176)
SPHINGOMONAS(71)
PROPIONIVIBRIO(1)
SHEWANELLA(1)
OPITUTUS(1)
RHODOBACTER(2)
HYDROGENOPHAGA(3)
LIMNOBACTER(1)
BREVUNDIMONAS(5)
SHIGELLA(1)
METHYLOBACTERIUM(1)
NOCARDIACEAE(2)
MICROBACTERIACEAE(3)
JANTHINOBACTERIUM(24)

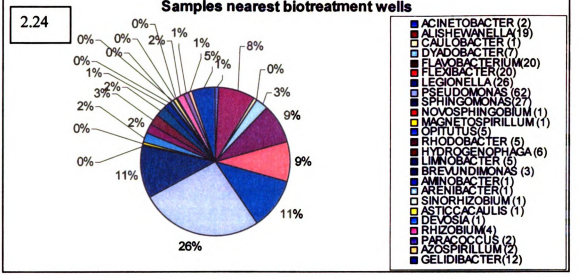
2.22


Samples downstream of biotreatment wells
1%
1%
1%
18%
24%
1%
1%
3%
18%
23%
9%
RHODOCOCCUS(1)
ALISHEWANELLA(26)
CAULOBACTER(1)
DYADOBACTER(1)
FLAVOBACTERIUM(4)
FLEXIBACTER (25)
LEGIONELLA (13)
PSEUDOMONAS(33)
SPHINGOMONAS(34)
SPHINGOBACTERIUM(2)
THIOCAPSA(1)

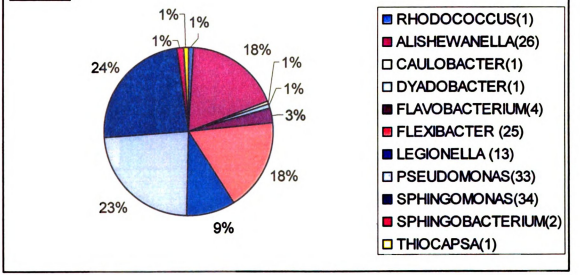
2.23


log_sign
0
-2
-4
-6
-8
-10
log
Anything below 10^{-2} is significantly different
Genus
FLEXIBACTER
LEGIONELLA
JANTHINOBACTERIUM
PSEUDOMONAS
ALISHEWANELLA
ACINETOBACTER
SPHINGOMONAS
SPHINGOBACTERIUM
FLAVOBACTERIUM
HYDROGENOPHAGA

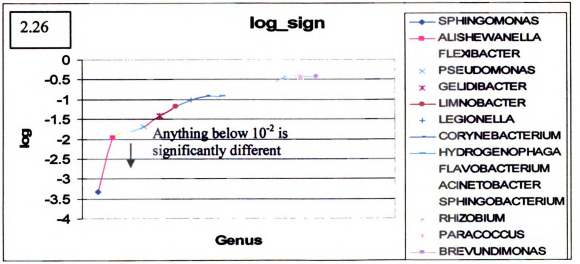
2.24


Samples nearest biotreatment wells
0%
0%
2%
1%
1%
8%
5%
1%
0%
0%
0%
1%
2%
3%
3%
9%
2%
0%
2%
0%
9%
11%
11%
26%
ACINETOBACTER (2)
ALISHEWANELLA(19)
CAULOBACTER (1)
DYADOBACTER(7)
FLAVOBACTERIUM(20)
FLEXIBACTER(20)
LEGIONELLA(26)
PSEUDOMONAS (62)
SPHINGOMONAS(27)
NOVOSPHINGOBIUM(1)
MAGNETOSPIRILLUM(1)
OPITUTUS(6)
RHODOBACTER (5)
HYDROGENOPHAGA(6)
LIMNOBACTER (5)
BREVUNDIMONAS (3)
AMINOBACTER(1)
ARENIBACTER(1)
SINORHIZOBIUM (1)
ASTICCACAULIS (1)
DEVOSIA (1)
RHIZOBIUM(4)
PARACOCCUS (2)
AZOSPIRILLUM (2)
GELIDIBACTER(12)

2.25

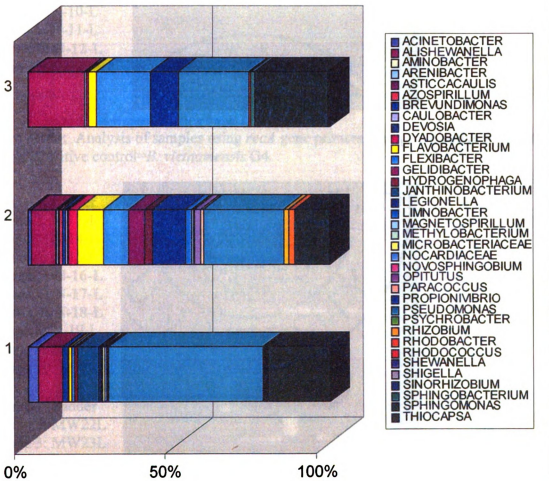

Samples downstream of biotreatment wells
1%
1%
1%
18%
24%
1%
1%
3%
18%
23%
9%
RHODOCOCCUS(1)
ALISHEWANELLA(26)
CAULOBACTER(1)
DYADOBACTER(1)
FLAVOBACTERIUM(4)
FLEXIBACTER (25)
LEGIONELLA (13)
PSEUDOMONAS(33)
SPHINGOMONAS(34)
SPHINGOBACTERIUM(2)
THIOCAPSA(1)

2.26


log_sign
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
log
Anything below 10-2 is
significantly different
Genus
SPHINGOMONAS
ALISHEWANELLA
FLEXIBACTER
PSEUDOMONAS
GELIDIBACTER
LIMNOBACTER
LEGIONELLA
CORYNEBACTERIUM
HYDROGENOPHAGA
FLAVOBACTERIUM
ACINETOBACTER
SPHINGOBACTERIUM
RHIZOBIUM
PARACOCCUS
BREVUNDIMONAS

2.27

Spatial Analysis 1-upstream 2-nearest 3-downstream



Lane 1: Ladder
Lane 2: N-1-L
Lane 3: N-2-L
Lane 4: N-3-L
Lane 5: N-4-L
Lane 6: N-5-L
Lane 7: N-6-L
Lane 8: N-7-L

Lane 1: Ladder
Lane 2: N-8-L
Lane 3: N-9-L
Lane 4: N-10-L
Lane 5: N-11-L
Lane 6: N-12-L
Lane 7: Pos. control

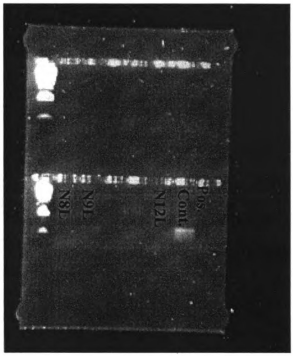


Figure 2.28a: Analysis of samples using *recA* gene primers. Samples positive for *recA* are bold. Positive control=*B. vietnamensis* G4.

Lane 1: Ladder
Lane 2: N-13-L
Lane 3: N-14-L
Lane 4: N-15-L
Lane 5: N-16-L
Lane 6: N-17-L
Lane 7: N-18-L
Lane 8: N-19-L
Lane 9: N-20-L
Lane 10: MW21L

Lane 1: Ladder
Lane 2: MW22L
Lane 3: MW23L
Lane 4: D4L
Lane 5: Pos. control
Lane 6: Empty
Lane 7: Neg. control

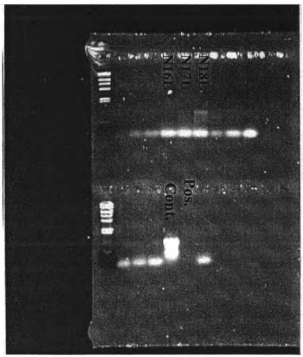


Figure 2.28b: Additional analysis of samples using *recA* gene primers. Samples positive for *recA* are bold. Positive control=*B. vietnamensis* G4.

Table 2.2. Taxonomy of well samples showing their hierarchical structure to show the presence or lack there of for members of the genus Burkholderia.

Well sample name	*Phylum*	*Class*	*Family*	*Order*	*Genus*
N-8-L	Proteo bacteria	Beta proteobacteria	Comamonadaceae Comamonadaceae Burkholderiaceae	Burkholderiales Burkholderiales Burkholderiales	Acidovorax Hydro genophaga Limnobacter
N-9-L	Proteo bacteria	Beta proteobacteria	Comamonadaceae	Burkholderiales	Hydro genophaga
N-17L	Proteo bacteria	Beta proteobacteria	Comamonadaceae	Burkholderiales	Acidovorax

16S rRNA Clone Library results of all six (6) well samples taken at the end of the experiment that were selected for analysis of Bcc using the *recA* gene primers

Figures 2.29: Composite analysis of well ***N-8-L***

Figures 2.30: Composite analysis of well ***N-9-L***

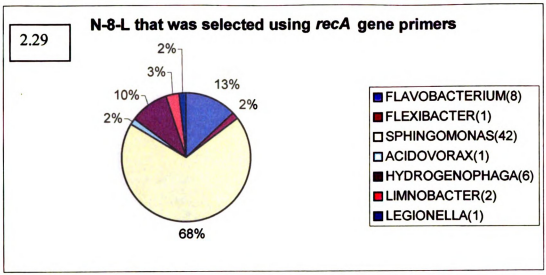
Figures 2.31: Composite analysis of well ***N-12-L***

Figures 2.32: Composite analysis of well ***N-16-L***

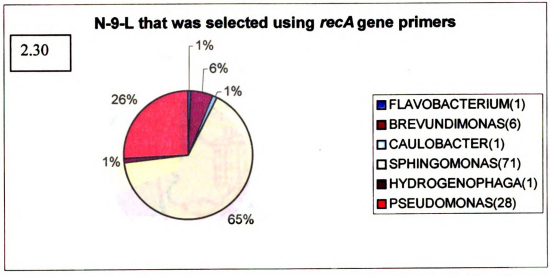
Figures 2.33: Composite analysis of well ***N-17-L***

Figures 2.34: Composite analysis of well ***N-18-L***

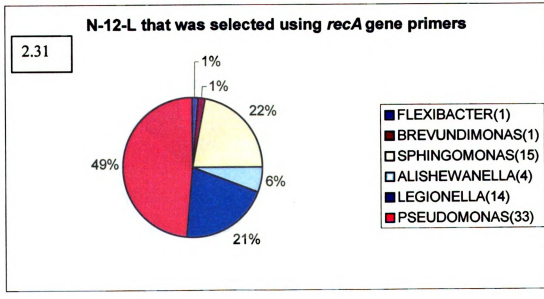
2.29


N-8-L that was selected using recA gene primers
2%
3%
13%
10%
2%
2%
68%
FLAVOBACTERIUM(8)
FLEXIBACTER(1)
SPHINGOMONAS(42)
ACIDOVORAX(1)
HYDROGENOPHAGA(6)
LIMNOBACTER(2)
LEGIONELLA(1)

2.30

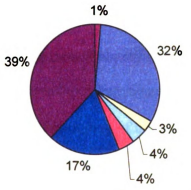

N-9-L that was selected using recA gene primers
1%
6%
1%
26%
1%
65%
FLAVOBACTERIUM(1)
BREVUNDIMONAS(6)
CAULOBACTER(1)
SPHINGOMONAS(71)
HYDROGENOPHAGA(1)
PSEUDOMONAS(28)

2.31


N-12-L that was selected using recA gene primers
1%
1%
22%
49%
6%
21%
FLEXIBACTER(1)
BREVUNDIMONAS(1)
SPHINGOMONAS(15)
ALISHEWANELLA(4)
LEGIONELLA(14)
PSEUDOMONAS(33)

2.32

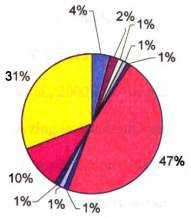
N-16-L that was selected using *recA* gene primers



- RHODOCOCCUS(1)
- FLEXIBACTER(24)
- SPHINGOBACTERIUM(2)
- SPHINGOMONAS (3)
- ALISHEWANELLA(3)
- LEGIONELLA(13)
- PSEUDOMONAS(29)

2.33

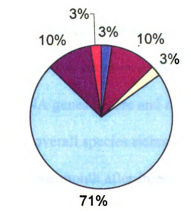
N-17L that was selected by recA primers



- FLEXIBACTER(4)
- BREVUNDIMONAS(2)
- NOVOSPHINGOBIUM(1)
- RHIZOBIUM(1)
- SPHINGOBIUM(1)
- SPHINGOMONAS(45)
- ACIDOVORAX(1)
- ACINETOBACTER(1)
- ALISHEWANELLA(1)
- LEGIONELLA(10)
- PSEUDOMONAS(30)

2.34

N-18-L that was selected using *recA* gene primers



- DYADOBACTER(1)
- FLAVOBACTERIUM(4)
- CAULOBACTER(1)
- SPHINGOMONAS(28)
- PSEUDOMONAS(4)
- ALISHEWANELLA(1)

Discussion

The detection, identification, and characterization of microbial populationsand their activities in environments are quite challenging because of their diversity and difficulty in culturing (Qiu et al., 2001). PCR targeting the 16S rRNA gene has become an important culture-independent means of characterizing microbial communities and has advanced our understanding of microbial diversity and evolutionary relationships (Speksnijder et al., 2001). Several 16S rRNA approaches have been used, including TRFLP and the construction of clone libraries to be analyzed by high-throughput sequencing.

One of the best methods to analyze bacterial diversity is the use of clone libraries of 16S rRNA genes collected from bacteria using PCR with 16S rRNA gene primers (Cottrell et al., 2000). In the past, bacterial communities have normally been compared by analyzing isolates cultivated on plates (Dunbar et al., 1999). Methods of analysis and direct amplification of 16S rRNA genes are replacing the cultivation of isolates when a more comprehensive sampling of the community is desired (Dunbar et al., 1999). There have been several studies that reflect the advantages and significance of constructing these clone libraries (Cottrell et al., 2000, Nogales et al., 2001, and Dunbar et al., 1999). Hence, to better determine the active microbial communities in the Edwards AFB samples taken from the full-scale BEHIVS site, I prepared clone libraries of 16S rRNA genes before and after substrate addition. These results indicated that there is greater overall species richness once toluene is added to the samples. The well samples taken one month after injection (***initial***) and those three months after injection (***end***) have less difference from each other because both of these groups had

new substrate whereas the ***background*** had only very low levels of indigenous substrate. Temporal profile analysis of the Edwards samples indicate populations such as Pseudomonas declined as toluene was added and Sphingomonas and Legionella were much more abundant.

Spatial analysis was also done on the well samples in relation to their location to the treatment wells. The TRFLP analysis prior to treatment showed that several well communities regardless of location are similar in the fragments (representative of different taxa) they contain, but have an uneven distribution of those fragments as was evidenced by the similarity indices. ***Upstream*** samples should not have been affected because the flow of the groundwater with toluene injection was down gradient. Hence, the well samples ***nearest*** and ***downstream*** of the treatment wells are the ones expected to be affected by substrate addition. The clone libraries show that the upstream wells are somewhat reflective of the ***background*** samples. This could suggest that the population shift seen between the ***background*** vs. ***initial*** and ***end*** communities is due to the samples ***nearest*** and ***downstream*** of the bio-treatment wells.

Further evaluation of the spatial profiles show that populations of Pseudomonas were reduced with the groundwater/toluene flow and the Sphingomonas population was larger with the groundwater /toluene flow. Populations of Legionella, however, were greater initially with the groundwater/toluene flow, but smaller at the ***downstream*** wells. This suggests that the further the flow of groundwater, the more evidence there is of a population shift in the communities.

Legionella, the genus that causes Legionnaires disease, was enriched once toluene was added to the bio-treatment wells at Edwards AFB. The bacterium easily

breeds in warm, moist conditions and thus most sources of outbreaks come from water or air conditioning systems in large public buildings. The presence of Legionella via the RDP classifier does not necessarily constitute the detection of a pathogenic species because the RDP hierarchy includes all bacteria with very similar 16S rRNA gene sequences but is not further classified to the species level. More work is needed to evaluate the occurrence of pathogenic Legionella in the Edwards samples.

Evaluation of both spatial and temporal analysis of samples from Edwards AFB however, rendered no evidence that species of the genus *Burkholderia* were present. The identification of this group of opportunistic human pathogens, which causes infection in patients with Cystic Fibrosis (CF), has become increasingly important in the past few years. Cystic Fibrosis (CF) is the most common inherited lethal disorder of Caucasian populations, with more than 500,000 individuals worldwide (30,000 in US) affected and many others carrying the gene that causes CF (Govan et al., 1996). Mortality in CF patients is often caused by long-term microbial colonization of the airways that result in crippling pulmonary infections (Govan et al., 1996). In the 1980s, concern over the emergence of these opportunistic human pathogens developed in the CF community (Govan et al., 1996). Thus, the source of Burkholderia in strains infecting patients with CF became a focal point of CF research.

Patients with CF are often infected with a group of diverse opportunistic human pathogens, but *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex (Bcc) are the most problematic (Mahenthiralingam et al., 2000). The Bcc are a culmination of nine species-like groups, i.e., genomovars I through IX. All are now described as species (in numerical order): *Burkholderia cepacia, B. multivorans, B. cenocepacia, B.*

stabilis, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia*. Strains of all nine are responsible for infection in humans with CF. The original *Burkholderia cepacia (genomovar I)* strain is a plant pathogen and nonpathogenic for healthy humans (Li Puma et al., 1999). The outcome of Bcc infected CF patients range from fatal pneumonia "the *cepacia* syndrome", to an unmodified respiratory status (Segonds et al., 1999). By better identifying some of the bacteria of the *B. cepacia* complex and understanding their source, we may be able to improve the poor outcome associated with these infections (Mahenthiralingam et al., 2000).

Because some of the *B. cepacia* complex bacteria live in soil and grow on toluene or phenol (Mahenthiralingam et al., 2000), stimulating their growth in the environment may be of concern at sites practicing aerobic TCE cometabolism, such as Edwards AFB. In fact the best-known TCE co-metabolizing strain, G4, is a member of the complex. By identifying the organisms that are selected by the treatment at the site, I would be able to determine if any are from the Bcc. I used the Bcc *recA* gene primers to determine if there were any Bcc genomovars present in the extracted DNA. A single copy of the *recA* gene resides on each of the two large chromosomes of *B. cepacia* (Mahenthiralingam et al., 2000). Rec A is a protein necessary for repair and recombination of DNA (Mahenthiralingam et al., 2000). Use of Bcc specific *recA* typing by RFLP, and confirmation by sequencing is currently the standard in the medical field for rapid classification of infectious strains. I found only weak amplification products that did not produce useable sequences and I did not find any clones of the Burkholderia genus in the 16S rRNA gene clone library. Hence, members of the Bcc did not appear to be present or selected by toluene feeding at the Edwards

site as was observed by the methods used. The BEHIVS system, with the vapor stripping well, could aerosolize Bcc cells if they were enriched in the aquifer. But since this appeared not to be the case, there is no evidence for a public health concern for using the BEHIVES system at Edwards AFB.

References

1. **Agency for Toxic Substances and Disease Registry (ATSDR).** 1997. Toxicological profile for trichloroethylene. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

2. **Agency for Toxic Substances and Disease Registry (ATSDR).** 2000. Toxicological profile for toluene (update). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

3. **Amann, R.I., Wolfgang Ludwig, and Karl-Heinz Schleifer.** 1995. Phylogentic identification and in situ detection of individual microbial cells without cultivation. Microbiological Review. **59**:143-169.

4. **Anderson, Eric Elmer and Rikke Granum Andersen.** 1996. In situ bioremediation of TCE. Civil Engineering Dept., Virginia Tech.

5. **Audic, Stéphane and Jean-Michel Claverie.** 1997. The Significance of Digital Gene Expression Profiles. Genome Res. **7**:986-995.

6. **Baker, Paul, Kazaya Watanabe, and Shigeaki Hararyama.** Analysis of the microbial community during bioremediation of trichloroethylene (TCE) contaminated soil. Kamaishi Laboratories, Marine Biotechnology Institute Co. Ltd., Kamaishi, Iwate. Japan.

7. **Braker, Gesche, Hector L. Ayala-Del-Rio, Allan H. Devol, and Andreas Fesefeldt, and James M. Tiedje.** 2001. Community Structure of Denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16 S rRNA genes. Appl. Environ. Microbiol. **67**:1893-1901.

8. **Burrell, Paul C., Carol M. Phalen, and Timothy A. Hovanec.** 2001. Identification of bacteria responsible for ammonia oxidation in freshwater aquaria. Appl. Environ. Microbiol. **67**:5791-5800.

9. **Cho, Jang-Cheon, and Sang-Jong Kim.** 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol. **66**:956-965.

10. **Cottrell, Matthew T., and David L. Kirchman.** 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl. Environ. Microbiol. **66**:5116-5122.

11. **Dollhopf S.A., S.A. Hashsham, and J.M. Tiedje.** 2001. Interpreting 16S rDNA T-RFLP Data: Application of Self-Organizing Maps and Principal Component Analysis to Describe Community Dynamics and Convergence. Microb. Ecol. **42**:495-505.

12. **Dunbar, John, Shannon Takala, Susan M. Barns, Jody A. Davis, and Cheryl R. Kuske.** 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65**:1662-1669.

13. **EPA.** U.S. Environmental Protection Agency. Contract Laboratory Program Statistical Database. Washington, DC: U.S. EPA, 1989.

14. **Fliermans, C.B., T.J. Phelps, D.Ringelberg, A.T. Mikell, and D.C. White.** 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. Appl. Environ. Microbiol. **54**:1709-1714.

15. **Fries, Marcos R., Gary D. Hopkins, Perry L. McCarty, Larry J. Forney, and James M. Tiedje.** 1997. Microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethene cometabolism. Appl. Environ. Microbiol. **63:**1515-1522.

16. **Fries, Marcos R., Jizhong Zhou, Joanne Chee-Sanford, and James M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802-2810.

17. **Fries, Marcos R., Larry J. Forney, and James M. Tiedje.** 1997. Phenol- and toluene-degrading microbial populations from an aquifer in which successful trichloroethene cometabolism occurred. Appl. Environ. Microbiol. **63:**1523-1530.

18. **Fuller Mark E. and Kate M. Scow.** 1997. Impact of tricholoethylene and toluene on nitrogen cycling in soil. Appl. Environ. Microbiol. **63:**4015-4019.

19. **Gandhi, R., M.N. Goltz, S.M. Gorelick, G.D. Hopkins, and P.L. McCarty.** 2000. Design of a Field Demonstration of Bioenhanced In-well Vapor Stripping (BEHIVS) to treat Trichloroethylene-Contamination. Stanford University and Air Force Institute of Technology, Palo Alto, California.

20. **Glod, Guy, Werner Angst, Christof Holliger, and Rene' P. Schwarzenbach.** 1997. Corrinoid-mediated reduction tetrachloroethene, trichloroethene, and trichlorofluoroethene in homogenous aqueous solution: Reaction kinetics and reaction mechanisms. Environ. Sci. Technol. **31:**253-260.

21. **Gerritse Jan, Oliver Drzyzga, Geert Kloetstra, Mischa Keijmel, Luit P. Wiersum, Roger Hutson, Matthew D. Collins, and Jan C. Gottschal.** 1999. Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene *by Desulfitobacterium frappieri* TCE1.Appl. Environ. Microbiol. **65**:5212-5

22. **Govan, J.R.W. and V. Deretic.** 1996. Microbial Pathogenesis in Cystic Fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia.* Microbiological Review. **60**:539-574.

23. **Holliger, Christof, Gert Wohlfarth, and Gabriele Diekert.** 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Reviews. **22:**383-398.

24. **Hopkins, G.D., L. Semprini, and P.L. McCarty.** 1993. Microcosm and in situ field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms. Appl. Environ. Microbiol. **59:**2277-2285.

25. **Hopkins, Gary D. and Perry L. McCarty.** 1995. Field evaluation of *in situ* aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Sci. Technol. **29:**1628-1637.

26. **Jenal- Wanner, Ursula and Perry L. McCarty.** 1997. Development and evaluation of semicontinuous slurry microcosms to stimulate in situ biodegradation of trichloroethylene in contaminated aquifers. Environ. Sci. Technol. **31:**2915-2922.

27. **Kleopfer, Robert D., Diane M. Easley, Bernard B. Haas, Jr., Trudy G. Deihl, David E. Jackson, and Charles J. Wurrey.** 1985. Anaerobic degradation of trichloroethylene in soil. Environ. Sci. Technol. **19:**277-280.

28. **LiPuma, John J., Betty Jo Dulaney, Jennifer D. McMenamin, Paul W. Whitby, Terrence L. Stull, Tom Coenye, and Peter Vandamme.** 1999. Development of rRNA-Based PCR Assays for Identification of *Burkholderia cepacia* Complex Isolates Recovered from Cystic Fibrosis Patients. J. Clin. Microbiol. **37**:3167-3170

29. **Löffler, Frank E. Qing Sun, Jieran Li, and James M. Tiedje.** 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* Species. Appl. Envir. Microbiol. **66:** 1369-1374.

30. **Löffler, Frank E., James R. Cole, Kirsti M. Ritalahti, and James M. Tiedje.** 2003. Diversity of Dechlorinating Bacteria. Dehalogenation: Microbial

Processess and Environmental Application. M.M. Haggblom and I.D. Bossert, editors. Kluwer Academic Publishers. 53-87.

31. **Magnuson, Jon K., Robert V. Stern, James M. Gossett, Stephen H. Zinder, and David R. Burris.** 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Appl. Environ. Microbiol. **64:**1270-1275.

32. **Mahenthiralingam, Eshwar, Jocelyn Bischof, Sean K. Byrne, Christopher Radomski, Julian E. Davies, Yossef AV-Gay, and peter Vandamme.** 2000. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkhoderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* Genomovars I and III. J. Clin. Microbiol. **38**:3165-3173.

33. **Mahenthiralingam, Eshwar, David A. Simpson, and David P. Speert.** 1997. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. J. Clin. Microbiol. **35**:808-816.

34. **Marsh, Terence L., Paul Saxman, James Cole, and James Tiedje.** 2000. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl. Environ. Microbiol. **66**:3616-3629.

35. **McCarty, Perry L. Mark N. Goltz, and Gary D. Hopkins.** Full-scale evaluation of in situ bioremediation of chlorinated solvent groundwater contamination. Stanford University (www.stanford.edu) Supported by the U.S. Air Force.

36. **McCarty, Perry L., Mark N. Goltz, Gary D. Hopkins, Mark E. Dolan, Jason P. Allan, Brett T. Kawakami, and T.J. Carrothers.** 1998. Full-scale evaluation of in situ cometabolic degradation of trichloroethylene in groundwater through toluene injection. Environ. Sci. Technology. **32:**88-100.

37. **Moran, B.N. and W.J. Hickey.** 1997. Trichloroethylene biodegradation by mesophilic and psychrophilic ammonia oxidizers and methanotrophs in groundwater microcosms. Appl. Environ. Microbiol. **63:**3866-3871.

38. **Munakata-Marr, Junko, Perry L.McCarty, Malcolm S. Shields, Michael Reagin, and Stephen C. Francesconi.** 1996. Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered *Burkholderia* (*Pseudomonas*) *cepacia* G4 and PR1. Environ. Sci. Technol. **30:**2045-2052.

39. **Munakata-Marr, Junko, V. Grace Matheson, Larry J. Forney, James M. Tiedje, and Perry L. McCarty.** 1997. Long- term biodegradation of trichloroethylene influenced by bioaugmentation and dissolved oxygen in aquifer microcosms. Environ. Sci. Technol. **31**:786-791.

40. **Nelson, Michael J.K., Stacy O. Montgomery, William R. Mahaffey and P.H. Pritchard.** 1987. Biodegradation of tricholorethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53**:949-954.

41. **Nogales, Balbina, Edward R.B. Moore, Enrique Llobet-Brossa, Ramon Rossello-Mora, Rudolf Amann, and Kenneth N. Timmis.** Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. **67**:1874-1884.

42. **Osborn, A. Mark, Edward R.B. Moore and Kenneth N. Timmis.** 2000. An evaluation of terminal-restriction fragment polymorphism (TRFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology. **2**:39-50.

43. **Qui, Xiaoyun, Liyou Wu, Heshu Huang, Patrick E. McDonel, Anthony V. Palumbo, and James Tiedje.** 2001. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl. Environ. Microbiol. **67**:880-887.

44. **Rantakokko-Jalava, K. and J. Jalava.** 2001. Development of conventional real-time PCR assays for detection of *Legionella* DNA in respiratory specimens. J. Clin. Microbiol. **39**:2904-2910.

45. **Segonds, Christine, Thierry Heulin, Nicole Marty, and Gerard Chabanon.** 1999. Differentiation of Burkholderia Species by PCR-Restriction Fragment Length Polymorphism Analysis of the 16S rRNA Gene and Application to Cystic Fibrosis Isolates. J. Clin. Microbiol. **37**:2201-2208.

46. **Semprini, Lewis.** 1995. In situ bioremediation of chlorinated solvents. Environ. Health Perspectives. **103**:Supp.5.

47. **Sharma, Pramod K. and Perry L. McCarty.** 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenated tetrachloroethene to *cis*-1, 2-Dichloroethene. Appl. Environ. Microbiol. **62**:761-765.

48. **Speksnijder, Arrjen G.C.L., George A. Kowalchuk, Sander De Jong, Elizabeth Kline, John R. Stephen, and Hendrikus J. Laanbroek.** 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Appl. Environ. Microbiol. **67**:469-472.

49. **Staley, James T.** ed. Bergey's Manual of Systematic Bacteriology. 3rd ed. Williams and Wilkens. Baltimore. 1989.

50. **Taylor, R.T.** 1993. In situ bioremediation of trichloroethylene-contaminated water by a resting-cell methanotrophic microbial filter. Hydrolog.Sci. Jour. **38**:323-342.

51. **Vogel, Timothy M., Craig Criddle, and Perry L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722-736.

52. **Wackett, Lawrence P. and David T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54**:1703-1708.

53. **Wartenberg, D., D. Reyner, and C. Siegel.** Trichloroethylene and cancer: epidemiologic evidence. Environ. Health Perspect., Vol. 108 (Suppl 2), 2000, pp.161-176.

54. **Wilson, John T. and Barbara H. Wilson.** 1985. Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. **49**:242-243.

55. **Yeager, Chris M., Peter J. Bottomley, and Daniel J. Arp.** 2001. Cytotoxicity Associated with Trichloroethylene Oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. **67**:2107-21185.

56. **Zhou, Jizhong, Mary Ann Bruns, and James M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62**:316-322.

Chapter III

Isolation, characterization, and distribution of toluene degraders from soil and aquifer habitats.

Introduction

Trichloroethene (TCE), a common groundwater pollutant is of significant importance throughout the world, and specifically at Edwards AFB, where TCE was used to clean the engines of the X-15 planes housed there in the late 50's and 60's. Workers at the Air Force Base used 55 gallons of TCE each month to clean the engines and afterwards dumped the remainder into a nearby desert creating a large groundwater plume. A more detailed explanation of the site and its history is in Chapter 2.

The widespread occurrence of TCE in groundwater has created public demand for techniques to remediate aquifers contaminated with TCE. While aerobic microorganisms cannot use TCE as a carbon and energy source for growth, some can co-metabolize TCE making this an attractive approach for cleanup of TCE contaminated aquifers (Semprini, 1995). Pilot-scale studies for *in situ* bioremediation have shown that certain substrates such as toluene or phenol injected into aquifer material stimulated indigenous TCE- degrading organisms (Jenal-Wanner et al., 1997). Previously, Wilson and Wilson (1985) described successful cometabolism of TCE in soil communities fed with natural gas. Certain oxgenases normally used by the microorganisms for initiating primary substrate oxidation can transform chlorinated ethenes (Hopkins et al., 1995). For example, three soil bacterial cultures that catabolize toluene, *Burkholderia vietnamensis* strain G4, *Pseudomonas putida F1,* and *P. putida* B5, have been demonstrated to cometabolize TCE (Wackett et al., 1988).

A microcosm study demonstrated that indigenous microbes in the Edwards aquifer would co-metabolize TCE when fed toluene as the primary substrate. This exercise resulted in 87% and 99% TCE removal. Following this, a full-scale test was implemented which resulted in between 83-87% TCE removal (McCarty et al, 1998). The full-scale showed a similarity between predicted results and hence the opportunity for another full-scale test using a vapor stripping system to measure the microbial population response was initiated. My previous work based on DNA analysis indicated that there was a shift in community structure following toluene treatment and that wells nearby and down-gradient from the bio-treatment well had different communities than the upstream wells (Chapter 2).

In this study I wanted to determine which culturable toluene-degrading populations were present, whether they co-oxidized TCE, and to further explore whether any isolates from Edwards AFB and other sites were *Burkholderia cepacia* complex.

Materials and Methods

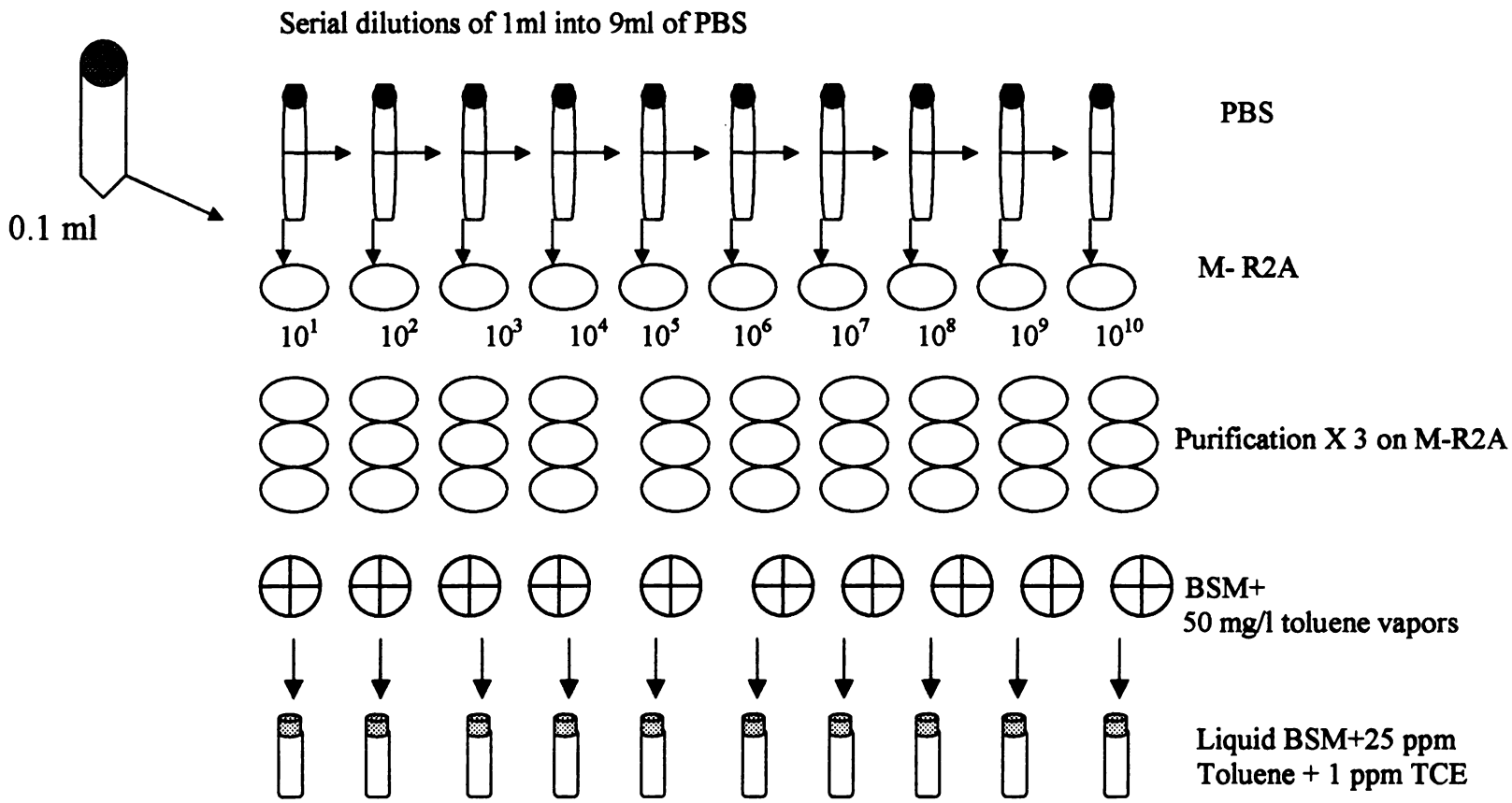
Isolation of dominant populations. The isolates were obtained from the resuspended cells extracted from the filters from 4L of Edwards AFB well water. The filters analyzed were N-11-L and MW-23-L taken three months after toluene injection and both representing communities nearest the bio-treatment wells (Chapter 2). Isolations were performed by a 10X fold dilution of 1 ml of the sample into 9 ml of phosphate-buffered saline (PBS) at pH 7.3 (Fig. 3.1). PBS was made of 80 g NaCl; 2 g KCl; 11.5 g $Na_2HPO_4 \cdot 7H_20$; and 2g KH_2PO_4. Next, 100 µl of the solution was spread on modified-R2A agar (Fries, 1995). Modified R2A (M-R2A) is based on the original carbon composition provided by Difco (Detroit, MI), combined with a low phosphate plus trace salts mixture (Fries, 1995). The plates were incubated under aerobic conditions. After initial growth on M-R2A, one colony of each morphology, color, and size were taken from each plate and streaked onto another M-R2A plate for purification (this was done for a total of three times). Once the isolates were purified, they were streaked on a low salt media, Basal Salt Media (BSM), and placed in a desiccators with a small vial of toluene vapors to determine their relative growth on the aromatic substrate. The amount of toluene added was calculated based on the total volume of the desiccators to provide a final concentration of 50 ppm. The toluene concentration was kept low since Fries et al (1995) had found higher concentrations were inhibitory to much of the toluene-degrading aquifer communities. Basal Salt Media consisted of 40 ml/L Na/KPO_4 buffer; 5 ml/L $MgSO_4$; 5 ml/L $CaCl_2$; 5ml/L $FeSO_4$; 5 ml/L $NaMoO_4$; 1 ml/L Metal 44 (MMO); 10 ml/L 10% $(NH_4)_2$ SO_4; and 10 g Nobel Agar (used to solidify medium).

Burkholderia vietnamiensis strain G4 , *Pseudomonas putida* F1, *Burkholderia pickettii* PKO1, all of which are toluene and TCE degraders were used as positive controls.

Characterization of isolates. After the DNA analysis, the isolates were measured for toluene degradation and TCE co-oxidation. Each colony that grew on toluene vapors was inoculated into 160 ml bottles with 12 ml of liquid BSM. Then 2 ml was distributed into 20 ml bottles, spiked with 25 ppm toluene and 1 ppm TCE, and sealed with teflon-lined stoppers. Those bottles were incubated at 25°C on a shaker until they were measured for growth at days 1 and 5. Each isolate was incubated in triplicates. Controls from the same batch of medium without cells were incubated at the same time to determine any non-biological loss. After determining the growth by optical density, cultures were sacrificed by adding 0.2 ml of 5 N HCl and stored at 4°C until analyzed by gas chromatography. A vial was assumed to be positive for biodegradation of the primary substrate and TCE co-oxidation when more than 50 and 95% of the TCE and toluene, respectively, had disappeared (Fries, 1995). The amounts of toluene and TCE remaining in the bottles were then measured by flame ionization gas chromatography (Varian model 3700) using an auto-sampler (Hewlett Packard).

Figure 3.1 Experimental protocol for the determination of toluene degraders, TCE co-oxidizers, and for isolation of the most dominant populations from the aquifer.

•Biomass collected by shaking samples in Sodium Phosphate Buffer followed by centrifugation of supernatant



•Different morphotypes were selected and purified three times on M-R2A medium

•Isolates were then transferred on BSA medium and incubated for 2 weeks with 50 mg/l toluene vapors

Identification of toluene degraders. To determine which isolates successfully consumed toluene and co-oxidized TCE, their 16S rRNA genes were sequenced. Purified colonies were taken from the plates of those isolates which showed the most substrate consumed, and added to 100 µl 0.05 NaOH. The solution was incubated at 99°C for 20 min to break the cells. A PCR was then run by taking 1µl of that lysate and adding it to a master mix containing ReadyMix™ RedTaq™ PCR Reaction Mix with $MgCl_2$ (Sigma, St. Louis, MO) and using the primers 8F and 1392R (Amann, et al, 1995). The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and separated by electrophoresis to determine the size of the PCR product. The concentration of cDNA was measured by UV spectrophotometry and custom sequencing was performed by MSU's Genomics Technology Support Facility (GTSF). The sequences were analyzed using Sequence Match on the RDP website to determine to which group they had the greatest similarity.

Determining substrates consumed. To determine the amount of toluene degraded and TCE co-oxidized, the results obtained from the GC were calculated by subtracting the area of the peak of the sample from Day 5 from the area of the peak from the control of Day 5, dividing by the control of Day 1 and then multiplying by 100. An example of the percent of toluene degraded in a vial containing the G4 strain is shown:

$$\frac{\text{Control (Day 5)-G4(Day 5)}}{\text{Control (Day 1)}} * 100: \frac{44066056-81405.5}{43584282} * 100 = 100\%$$

Of my 65 isolates, only 48 grew on plates on toluene vapors and were further tested for growth in liquid BSM with 25 ppm toluene and 1 ppm TCE. None of these isolates co-

oxidized TCE, and only a few minimally degraded toluene (Table 3.1). The most active isolates showed only 14.4 to 17.6 % toluene removed. Importantly, known strains, G4, PKO1, F1, did show essentially complete toluene degradation and TCE co-oxidation with 100% & 98.4%; 93.8% & 100%; and 93.7% & 30.7% removal, respectively.

Soil Samples. Soil samples used in this experiment were from a variety of locations including Schoolcraft PW-1 63-64'- a Michigan site contaminated with TCE; Wexford I 43-48' and BearLake 10 83-88'-both sites contaminated with petroleum form oil production wells; Merredin-a soil of Southwestern Australia; Lago Penuelas-a soil of Central Chile; and Brittern- a soil from Saskatchewan (Fulthorpe et al., 1996). These samples were kept at 4°C until analysis.

Isolation of dominant soil populations. Five grams of each soil was placed in bottles with 160 ml of Basal Salt Medium (BSM) and 200 ppm of toluene. The bottles were incubated at 25°C. After 4 days, another 200 ppm of toluene was added to each of the sample bottles. Aliquots of each sample was then spread on BSM plates and placed in a desiccators with toluene vapors to provide a final concentration of 50 ppm.

Determination of Burkholderia cepacia complex (Bcc). In order to determine if the colonies were of the Bcc, we performed colony hybridization using a Bcc specific probe provided by Dr. Alban Ramette @ Michigan State University. This probe targets the V3 variable region of 16S gene. I followed the following protocol for colony hybridization provided by Dr. Alban Ramette:

A. Labeling of the probe:

1) Adjust the probe concentration to 10 ng /µl
2) Denature for 5 minutes in boiling water
3) Cool on ice for 5 minutes
4) Prepare 1:4 (v:v, cross-linker:water) dilution of the cross-linker solution [Alkphos labeling and detection kit (RPN3691, Amersham)]

5) Add to the tube containing the probe the following reaction(mix gently by pipetting each time): [All from Alkphos labeling and detection kit (RPN3691, Amersham]
   ·Probe 7µl; Reaction buffer 7µl; Labeling reagent 1.4µl; and Cross-Linker 7µl
6) Incubate at 37°C for at least 2 hours

B. Fixation of the DNA to the membranes

Colony lift- Place a sterile precut membrane on the surface of a dry micro liter plate. Press the membrane (Hybond N+ membranes (RPN303B, Amersham) using the lid of the plate, to ensure a homogenous contact for all colonies

0) Prepare 9 sheets of filter paper (Whatman #1). Use them as pads (2 sheets/pad). One pad is soaked with denaturing solution, two pads with neutralizing and one with 2 x SSC. The 9th sheet is to carry the membranes in the drying step in the oven
1) Denaturing solution: 7 min
2) Neutralizing solution: 3 min (x2)
3) 2x SSC: 2 min
4) Cross-link (UV autocross linker, Biorad)

C. Prehybridization

1) Prehybridize the membrane at 40°C in 10 ml of Hybridization buffer (Provided in Alkphos labeling and detection kit (RPN3691, Amersham) for 1hour in a shaking water bath.

D. Hybridization

1) Discard the prehybridization buffer
2) Add 20 µl of labeled probe to 10 ml of fresh hybridization buffer in a separate tube. Mix well and add the hybridization buffer containing the probe to the bag containing the membrane
3) Seal and hybridize overnight

E. Washes

1) *Primary (stringent) wash.* Wash with pre-warmed (40°C) Wash Buffer I: 40 ml/membrane for 10 minutes by shaking. Repeat once with fresh Buffer I

Wash Buffer I (500 ml): Urea 60 g; 10% SDS 5 ml; 0.5 M Sodium Phosphate Buffer pH 7.0 50 ml; NaCl 4.35 g; 1 M $MgCl_2$ 0.5 ml; blocking agent (provided in Alkphos labeling and detection kit [RPN3691, Amersham])

2) *Secondary wash* (to get rid of urea) by shaking at room temperature, twice at 5 minutes

   Wash Buffer II (250 ml): 20x stock 12.5 ml; 1 M $MgCl_2$ 0.5 ml; and water to 250 ml. **20x stock** (1L)-Tris base 121 g; NaCl 112 g pH 10

F. Detection

1) Place the membrane on a Saran wrap and cover with CDP-Star reagent (Alkphos labeling and detection kit (RPN3691, Amersham)
2) Wrap in Saran wrap avoiding any leaking form the bag or any drop on the outside of the wrap
3) Tape the wrapped membranes in a detection cassette by using the frame of an already-exposed film
4) In a dark room, under a safe light, place a film on the membrane and expose for 20 min to an hour.
5) Develop the film, allowing 20 min to warm up machine (Xomat, Kodak) prior to developing the film

Table 3.1 Results of toluene degradation and TCE co-oxidation by isolates from Edwards AFB and three positive control strains. Data are means of triplicate samples.

Isolates MW23-L	%Toluene Degradation	%TCE Degradation	Isolates N11-L	%Toluene Degradation	%TCE Degradation
EAFB-A-1	0	0.46	EAFB-B-1	8.87	7.69
EAFB-A-2	0	0.43	EAFB-B-2	-	-
EAFB-A-3	1.95	0.46	EAFB-B-3	-	-
EAFB-A-4	7.63	0.47	EAFB-B-4	13.8	6.45
EAFB-A-5	8.15	0.40	EAFB-B-5	1.59	4.31
EAFB-A-6	2.19	0	EAFB-B-6	-	-
EAFB-A-7	12.5	1.99	EAFB-B-7	7.09	0
EAFB-A-8	0	0.50	EAFB-B-8	0.22	3.83
EAFB-A-9	6.30	0.46	EAFB-B-9	-	-
EAFB-A-10	4.47	0.44	EAFB-B-10	-	-
EAFB-A-11	9.47	0.49	EAFB-B-11	8.39	25.9
EAFB-A-12	-	-	EAFB-B-12	-	-
EAFB-A-13	-	-	EAFB-B-13	17.6	7.41
EAFB-A-14	6.92	2.21	EAFB-B-14	7.27	14.2
EAFB-A-15	3.59	2.33	EAFB-B-15	6.93	17.87
EAFB-A-16	8.24	0	EAFB-B-16	-	-
EAFB-A-17	9.26	4.05	EAFB-B-17	-	-
EAFB-A-18	8.72	5.07	EAFB-B-18	4.86	0
EAFB-A-19	4.94	0	EAFB-B-19	4.37	8.05
EAFB-A-20	1.23	0	EAFB-B-20	-	-
EAFB-A-21	0	11.0	EAFB-B-21	0	0
EAFB-A-22	6.78	4.03	EAFB-B-22	4.70	0
EAFB-A-23	0	0	EAFB-B-23	0.82	0
EAFB-A-24	13.3	8.04	EAFB-B-24	6.57	0
EAFB-A-25	-	-	EAFB-B-25	4.98	0.47
EAFB-A-26	-	-	EAFB-B-26	4.81	0
EAFB-A-27	10.2	0	EAFB-B-27	7.48	1.56
EAFB-A-28	13.6	0	EAFB-B-28	2.38	3.84
EAFB-A-29	14.4	0.66	EAFB-B-29	11.2	0.08
EAFB-A-30	12.7	1.75	EAFB-B-30	11.5	1.22
EAFB-A-31	17.2	1.03	EAFB-B-31	-	-
			EAFB-B-32	-	-
			EAFB-B-33	-	-
			EAFB-B-34	-	-
			G4	100	98.4
			F1	93.7	30.7
			PKO1	93.8	100

Identification of isolates. Because none of the isolates effectively degraded toluene or co-oxidized TCE, I chose only a few of the isolates that were in the median range of toluene oxidation to determine their identity. Four of the isolates (EAFB-A-24; EAFB-A-29; EAFB-A-31; and EAFB-B-4) were highly similar to *Brevibacillus parabrevis* and two (EAFB-A-27; EAFB-B-29) were highly similar to *Bacillus anthracis* (Table 3.2). Because these two isolates had a high similarity to *Bacillus anthracis*, we needed to further resolve their identity. I performed ClustalW analysis (***http://clustalw.genome.ad.jp/***) to determine how similar those isolates were to *Bacillus cereus*, *Bacillus thuringenis*, and both reference and outbreak strains of *Bacillus anthracis*, since it is often quite difficult to distinguish one species from the other (Table 3.3).

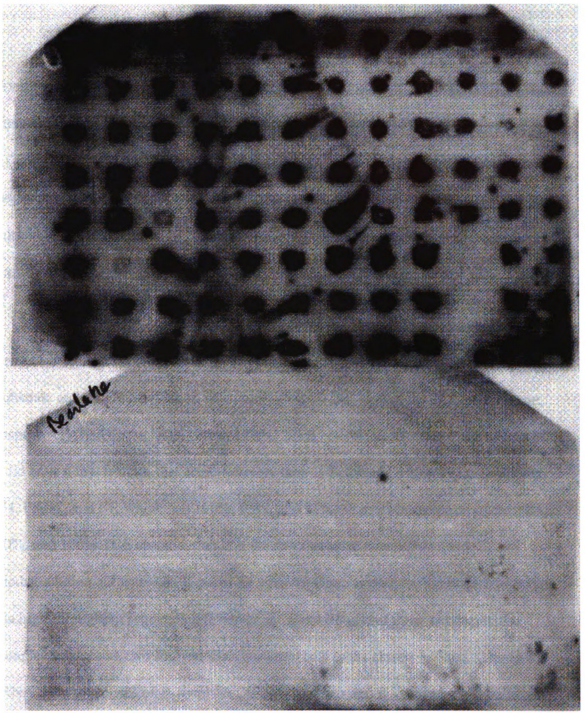
Table 3.2. Closest phylogenetic relative of sequenced isolates and their toluene and TCE degrading abilities.

Strain Number	Closet Relatives	% Similarity	Consumption of Aromatics%	
			Toluene	TCE
EAFB-A-7	Bacillus sp. No.61	0.985	12.5	1.99
	Bacillus sp. No.49	0.985		
EAFB-A-24	*Brevibacillus parabrevis*	0.769	13.3	8.04
	Brevibacillus sp. Riau	0.818		
EAFB-A-27	*Bacillus anthracis*	0.982	10.2	0
	Bacillus anthracis	0.982		
EAFB-A-29	*Brevibacillus parabrevis*	<.750	14.4	0.66
	Brevibacillus sp. Riau	<.750		
EAFB-A-31	*Brevibacillus parabrevis*	0.745	17.3	1.03
	Brevibacillus sp. Riau	0.809		
EAFB-B-4	*Brevibacillus parabrevis*	<.750	13.8	6.45
	Brevibacillus sp. Riau	<.750		
EAFB-B-13	*Bacillus thuringiensis*	0.895	17.6	7.41
	Bacillus anthracis	0.893		
EAFB-B-29	*Bacillus anthracis*	0.985	11.2	0.08
	Bacillus anthracis	0.985		
EAFB-B-30	unidentified bacterium	0.895	11.5	1.22
	Bacillus thuringiensis	0.906		

Table 3.3. ClustalW analysis of isolates against different Bacillus species.

Isolates	*Bacillus anthracis*-outbreak strain	*Bacillus anthracis*-reference strain	*Bacillus cereus*	*Bacillus thuringiensis*
MW #27	99.7	99.7	99.7	99.1
N11 #29	99.6	99.6	99.6	99.5

Colony hybridization. The soil samples incubated in the desiccators with the toluene vapors produced only small white colonies, not the distinctive type of colonies of the *Burkholderia cepacia* complex (Bcc). After performing colony hybridization with the Bcc specific probes, none of the colonies from any of the six soil samples were Bcc (Fig. 3.2 a & b). The distinction between the positive control and the colonies from the samples, as illustrated for the Bear Lake samples, is clear cut. Approximately 50 colonies were screened but none were positive, thus indicating Bcc is rare in these environments.



Figures 3.2a and 3.2b. Detection of bacteria of the *Burkholderia cepacia* complex(Bcc) as determined by colony hybridization. 3.2a is the positive control 3.2b is the Bearlakesample and is representative of all soil samples tested.

Discussion

I analyzed toluene degradation, TCE co-oxidation, and the diversity of semi-dominant toluene-degrading bacteria isolated from an aquifer at Edwards AFB. I used conventional microbiological techniques to quantify specific toluene degraders and TCE co-oxidizers. For this evaluation, the amount of the primary substrate used was important. I used 25 ppm of toluene because previous studies have shown that samples enriched with 25 ppm are the lowest concentration feasible for evaluation of TCE co-oxidizers (Fries, 1995).

By using toluene at 25 ppm, I was unable to recover some toluene degraders. Fries (1995) used the criteria that a strain was positive for biodegradation of the primary substrate and TCE co-oxidation when more than 50 and 95% of the TCE and toluene, respectively, had disappeared (Fries, 1995). I had no isolates that met these criteria but I did have a few isolates that achieved more than 10% toluene degradation, excluding G4, PKO1, and F1, which had 100%, 93%, and 93% toluene biodegradation and 98%, 30%, and 100% TCE co-oxidation. Of the few that were over 10% toluene biodegradation, all were Gram-positives whereas Gram-negatives dominated the results via high-throughput sequencing (Chapter 2). Surprisingly, of those Gram-positive samples sequenced, two showed *Bacillus anthracis* as the closest relative. The 16S rRNA gene sequences of *B. anthracis*, *B. thuringiensis* and *B. cereus* have high levels of sequence similarity (>99%), which hampers their identification and differentiation (Sacchi, et al, 2002). After alignment in CLUSTALW, my isolates were found to have approximately 99% similarity to each of the reference species. Sacchi et al, reported that all true *B. anthracis* were identical only to each other (Sacchi, et al, 2002). Hence,

my isolates are not *B. anthracis*. Since these isolates have no further value, I autoclaved all of them.

Colony hybridization was performed on a variety of soil samples from around the world to see how often isolates from toluene enrichments from Bcc are found. *B. cepacia* was examined because it is a common and widely distributed species with tremendous intraspecies diversity (Leff et al., 1995). Many species of bacteria which can be cultivated cannot be easily identified by conventional methods, thus colony hybridization was used to detect diagnostic DNA sequences. I found no bacteria of the Bcc present in any of the six soil samples examined in this work. This confirms the colony observations since only small white colonies were noted when incubated with the toluene vapors, which was not indicative of colonies of the Bcc. Bcc colonies, as illustrated by the growth of G4, are larger, milky, pale colored colonies. Hence, toluene-degrading Bcc do not seem to be widespread in the environments as was evident with this colony hybridization protocol.

The absence of readily growing, toluene-degrading strains in the Edwards AFB full-scale BEHIVS water samples after toluene treatment in the field is surprising. The positive control strains indicate the method should be reliable. The most likely explanation is that toluene degraders, which obviously grew in the field since both toluene and TCE were consumed, had not yet reached the sampling wells or just could not be detected in the analysis of the data collected. Changes in community structure seen via high-throughput sequencing (Chapter 2) may be due to products from toluene degraders selecting new populations

References

1. **Agency for Toxic Substances and Disease Registry (ATSDR).** 1997. Toxicological profile for trichloroethylene. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

2. **Agency for Toxic Substances and Disease Registry (ATSDR).** 2000. Toxicological profile for toluene(update). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

3. **Amann, R.I., Wolfgang Ludwig, and Karl-Heinz Schleifer.** 1995. Phylogentic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Review. **59**:143-169.

4. **Anderson, Eric Elmer and Rikke Granum Andersen.** 1996. In situ bioremediation of TCE. Civil Engineering Dept., Virginia Tech.

5. **Baker, Paul, Kazaya Watanabe, and Shigeaki Hararyama.** Analysis of the microbial community during bioremediation of trichloroethylene (TCE) contaminated soil. Kamaishi Laboratories, Marine Biotechnology Institute Co. Ltd., Kamaishi, Iwate. Japan.

6. **Braker, Gesche, Hector L. Ayala-Del-Rio, Allan H. Devol, and Andreas Fesefeldt, and James M. Tiedje**. 2001. Community Structure of Denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16 S rRNA genes. Appl. Environ. Microbiol. **67**:1893-1901.

7. **Burrell, Paul C., Carol M. Phalen, and Timothy A. Hovanec**. 2001. Identification of bacteria responsible for ammonia oxidation in freshwater aquaria. Appl. Environ. Microbiol. **67**:5791-5800.

8. **Cho, Jang-Cheon, and Sang-Jong Kim.** 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol. **66**:956-965.

9. **Cottrell, Matthew T., and David L. Kirchman**. 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl. Environ. Microbiol. **66**:5116-5122.

10. **Dunbar, John, Shannon Takala, Susan M. Barns, Jody A. Davis, and Cheryl R. Kuske.** 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. **65**:1662-1669.

11. **EPA.** U.S. Environmental Protection Agency. Contract Laboratory Program Statistical Database. Washington, DC: U.S. EPA, 1989.

12. **Fliermans, C.B., T.J. Phelps, D.Ringelberg, A.T. Mikell, and D.C. White.** 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. Appl. Environ. Microbiol. **54**:1709-1714.

13. **Fries, Marcos R., Gary D. Hopkins, Perry L. McCarty, Larry J. Forney, and James M. Tiedje.** 1997. Microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethene cometabolism. Appl. Environ. Microbiol. **63:**1515-1522.

14. **Fries, Marcos R., Jizhong Zhou, Joanne Chee-Sanford, and James M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802-2810.

15. **Fries, Marcos R., Larry J. Forney, and James M. Tiedje.** 1997. Phenol- and toluene-degrading microbial populations from an aquifer in which successful trichloroethene cometabolism occurred. Appl. Environ. Microbiol. **63:**1523-1530.

16. **Fries, Marcos.** 1995. Diversity of Mono Aromatic Hydrocarbon Degrading Bacteria and Their TCE Co-Oxidation Potential. Ph.D Dissertation. Michigan State University. East Lansing, MI.

17. **Fuller Mark E. and Kate M. Scow.** 1997. Impact of tricholoethylene and toluene on nitrogen cycling in soil. Appl. Environ. Microbiol. **63:**4015-4019.

18. **Fulthorpe, Roberta R., Albert N. Rhodes, and James M. Tiedje.** 1996. Pristine Soils Mineralize 3-Chlorobenzoate and 2,4-Dichlorophenoxyacetate via Different Microbial Populations. Appl. Environ. Microbiol. **62**:1159-1166.

19. **Glod, Guy, Werner Angst, Christof Holliger, and Rene' P. Schwarzenbach.** 1997. Corrinoid-mediated reduction tetrachloroethene, trichloroethene, and trichlorofluoroethene in homogenous aqueous solution: Reaction kinetics and reaction mechanisms. Environ. Sci. Technol. **31:**253-260.

20. **Gerritse Jan, Oliver Drzyzga, Geert Kloetstra, Mischa Keijmel, Luit P. Wiersum, Roger Hutson, Matthew D. Collins, and Jan C. Gottschal.** 1999. Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene *by Desulfitobacterium frappieri* TCE1.Appl. Environ. Microbiol. **65**:5212-5221.

21. **Holliger, Christof, Gert Wohlfarth, and Gabriele Diekert.** 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Reviews. **22:**383-398.

22. **Hopkins, G.D., L. Semprini, and P.L. McCarty.** 1993. Microcosm and in situ field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms. Appl. Environ. Microbiol. **59:**2277-2285.

23. **Hopkins, Gary D. and Perry L. McCarty.** 1995. Field evaluation of *in situ* aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Sci. Technol. **29:**1628-1637.

24. **Jenal- Wanner, Ursula and Perry L. McCarty.** 1997. Development and evaluation of semicontinuous slurry microcosms to stimulate in situ biodegradation of trichloroethylene in contaminated aquifers. Environ. Sci. Technol. **31:**2915-2922.

25. **Kleopfer, Robert D., Diane M. Easley, Bernard B. Haas, Jr., Trudy G. Deihl, David E. Jackson, and Charles J. Wurrey.** 1985. Anaerobic degradation of trichloroethylene in soil. Environ. Sci. Technol. **19:**277-280.

26. **Lechner Sabine, Ralf Mayr, Kevin P. Francis, Birgit M. Prüβ, Thomas Kaplan, Elke Weiβner-Gunkel, Gordon S.S.B. Stewart and Siegfried Scherer.** 1998. *Bacillus wiehenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int. J. Syst. Bacteriol. **48**:1373-1382

27. **Leff, L.G., R.M. Kernan, J.V. McArthur and L.J. Shimkets.** 1995. Identification of Aquatic *Burkholderia (Pseudomonas) cepacia* by hybridization with species-specific rRNA gene probes. Appl. Environ. Microbiol. **61**:1634-1636.

28. **Löffler, Frank E. Qing Sun, Jieran Li, and James M. Tiedje.** 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* Species. Appl. Environ. Microbiol. **66:** 1369-1374.

29. **Löffler, Frank E., James R. Cole, Kirsti M. Ritalahti, and James M. Tiedje.** 2003. Diversity of Dechlorinating Bacteria. Dehalogenation: Microbial Processess and Environmental Application. M.M. Haggblom and I.D. Bossert, editors. Kluwer Academic Publishers. 53-87.

30. **Magnuson, Jon K., Robert V. Stern, James M. Gossett, Stephen H. Zinder, and David R. Burris.** 1998. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Appl. Environ. Microbiol. **64:**1270-1275.

31. **Mahenthiralingam, Eshwar, David A. Simpson, and David P. Speert.** 1997. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. J. Clin. Microbiol. **35**:808-816.

32. **Marsh, Terence L., Paul Saxman, James Cole, and James Tiedje.** 2000. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl. Environ. Microbiol. **66**:3616-3629.

33. **McCarty, Perry L. Mark N. Goltz, and Gary D. Hopkins.** Full-scale evaluation of in situ bioremediation of chlorinated solvent groundwater contamination. Stanford University (www.stanford.edu) Supported by the U.S. Air Force.

34. **McCarty, Perry L., Mark N. Goltz, Gary D. Hopkins, Mark E. Dolan, Jason P. Allan, Brett T. Kawakami, and T.J. Carrothers.** 1998. Full-scale evaluation of in situ cometabolic degradation of trichloroethylene in groundwater through toluene injection. Environ. Sci. Technology. **32:**88-100.

35. **Moran, B.N. and W.J. Hickey.** 1997. Trichloroethylene biodegradation by mesophilic and psychrophilic ammonia oxidizers and methanotrophs in groundwater microcosms. Appl. Environ. Microbiol. **63:**3866-3871.

36. **Munakata-Marr, Junko, Perry L.McCarty, Malcolm S. Shields, Michael Reagin, and Stephen C. Francesconi.** 1996. Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered *Burkholderia* (*Pseudomonas*) *cepacia* G4 and PR1. Environ. Sci. Technol. **30:**2045-2052.

37. **Munakata-Marr, Junko, V. Grace Matheson, Larry J. Forney, James M. Tiedje, and Perry L. McCarty.** 1997. Long- term biodegradation of trichloroethylene influenced by bioaugmentation and dissolved oxygen in aquifer microcosms. Environ. Sci. Technol. **31:**786-791.

38. **Nelson, Michael J.K., Stacy O. Montgomery, William R. Mahaffey and P.H. Pritchard.** 1987. Biodegradation of tricholorethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949-954.

39. **Nogales, Balbina, Edward R.B. Moore, Enrique Llobet-Brossa, Ramon Rossello-Mora, Rudolf Amann, and Kenneth N. Timmis.** Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. **67**:1874-1884.

40. **Osborn, A. Mark, Edward R.B. Moore and Kenneth N. Timmis**. 2000. An evaluation of terminal-restriction fragment polymorphism (TRFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology. **2**:39-50.

41. **Qui, Xiaoyun, Liyou Wu, Heshu Huang, Patrick E. McDonel, Anthony V. Palumbo, and James Tiedje.** 2001. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl. Environ. Microbiol. **67**:880-887.

42. **Rantakokko-Jalava, K. and J. Jalava**. 2001. Development of conventional real-time PCR assays for detection of *Legionella* DNA in respiratory specimens. J. Clin. Microbiol. **39**:2904-2910.

43. **Sacchi, Claudio T., Anne M. Whitney, Leonard W. Mayer, Roger Morey, Arnold Steigerwalt, Arijana Boras, Robin S. Weyant, and Tanja Popovi.** 2002. Sequencing of 16S rRNA Gene: A Rapid Tool for Identification of *Bacillus anthracis*. Emerg. Infect. Dis. **8**:1117-1123.

44. **Segonds, Christine, Thierry Heulin, Nicole Marty, and Gerard Chabanon.** 1999. Differentiation of Burkholderia Species by PCR-Restriction Fragment Length Polymorphism Analysis of the 16S rRNA Gene and Application to Cystic Fibrosis Isolates. J. Clin. Microbiol. **37**:2201-2208.

45. **Semprini, Lewis.** 1995. In situ bioremediation of chlorinated solvents. Environ. Health Perspectives. **103**:Supp.5.

46. **Sharma, Pramod K. and Perry L. McCarty.** 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenated tetrachloroethene to *cis*-1, 2-Dichloroethene. Appl. Environ. Microbiol. **62**:761-765.

47. **Shida, Osamu, Hiroaki Takagi, Kiyoshi Kadowaki and Kazuo Komagata**. 1996. Proposal for two New Genera, *Brevibacillus* gen.nov. and *Aneurinibacillus* gen. nov. Int. J. Syst. Bacteriol. **46**:939-946

48. **Speksnijder, Arrjen G.C.L., George A. Kowalchuk, Sander De Jong, Elizabeth Kline, John R. Stephen, and Hendrikus J. Laanbroek**. 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. Appl. Environ. Microbiol. **67**:469-472.

49. **Taylor, R.T.** 1993. In situ bioremediation of trichloroethylene-contaminated water by a resting-cell methanotrophic microbial filter. Hydrolog.Sci. Jour. **38**:323-342.

50. **Thompson, J., T. Gibson, D. Higgins.** Sequence Interpretation Tools: Multiple sequence alignment. http://clustalw.genome.ad.jp

51. **Vogel, Timothy M., Craig Criddle, and Perry L. McCarty.** 1987. Transformations of halogenated aliphatic compounds. Environ. Sci. Technol. **21**:722-736.

52. **Wackett, Lawrence P. and David T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54**:1703-1708.

53. **Wartenberg, D., D. Reyner, and C. Siegel.** Trichloroethylene and cancer: epidemiologic evidence. Environ. Health Perspect., Vol. 108 (Suppl 2), 2000, pp.161-176.

54. **Wilson, John T. and Barbara H. Wilson.** 1985. Biotransformation of trichloroethylene in soil. Appl. Environ. Microbiol. **49**:242-243.

55. **Yeager, Chris M., Peter J. Bottomley, and Daniel J. Arp.** 2001. Cytotoxicity Associated with Trichloroethylene Oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. **67**:2107-21185.

56. **Zhou, Jizhong, Mary Ann Bruns, and James M. Tiedje.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62**:316-322.

Chapter IV

Determination of different patterns of aromatic substrate use between clinical and environmental *Burkholderia cepacia* complex (Bcc) strains.

Introduction

Burkholderia cepacia, first described in 1950 as the cause of soft rot in onions, has since been recognized as an opportunistic human pathogen that causes disease in compromised patients and especially among cystic fibrosis (CF) patients (Bevivino, et al., 2001). This is especially important because of the patient-to-patient spread of the organism due to respiratory infections and the fact that this organism is resistant to many anti-microbial agents (Govan et al., 1996, Bevivino, et al., 2001), which limits many therapeutic options (LiPuma, 1999). The clinical outcome in *B. cepacia*-infected CF patients is variable and ranges from a fatal pneumonia, "the cepacia syndrome", that has caused epidemics as well as small disease clusters in CF patients to an unmodified respiratory status (Pitt et al., 1996, Segonds et al., 1997 and 1999). However, the Bcc is generally nonpathogenic for healthy humans (LiPuma et al., 1999).

The Bcc consists of nine specific genomovars, now described as species: *B. cepacia (genomovar I), B. multivorans (II), B. cenocepacia (III), B. stabilis (IV), B. vietnamiensis (V), B. dolosa (VI), B. ambifaria (VII), B. anthina (VIII), and B. pyrrocinia (IX)* (Coenye, et al., 2001). *B. cenocepacia* and *B. multivorans* constitute the majority of isolates from CF patients (LiPuma et al, 2001). Bcc strains are also of great interest in agriculture and biotechnology because of their potential as bio-control agents for root disease, N_2-fixing ability, and as bioremediation agents. This is important because of some Bcc's ability as a rhizosphere colonizing, plant-growth

promoting agent of several economic crops which subsequently increases crop yield (Coenye, et al 2001, Bowers and Parke, 1993). The Bcc is also important because some can degrade hydrocarbons and co-oxidize TCE and thus aid with the bioremediation of contaminated soil and water (Folsom et al., 1990, Holmes et al., 1998, and Bevivino et al, 2001). Whether or not there are distinguishing differences between Bcc strains isolated from the environment and those isolated from clinical patients is unclear (Govan et al., 1996, and Bevivino et al., 2001). What is clear, however, is that *B. cenocepacia* and *B. multivorans* constitute the majority of isolates from CF patients (LiPuma et al, 2001), while *B. cepacia, B. cenocepacia* and *B. ambifaria* are the most widespread in the rhizosphere of maize (Fiore et al., 2001). More specifically, it has been stated that the observation that clinical strains lack the ability to act as phytopathogens, and environmental isolates are unlikely to be responsible for human infections, may be because the strains have not been fully analyzed (Bevivino, et al 2002). Additionally, Coenye has noted that the taxonomic complexity of *B. cepacia*-like organisms and the lack of widespread and generally accepted identification schemes have hindered sound studies that could establish the roles played by and the pathogenic significance of the different *B. cepacia*-like organisms (Coenye et al., 2001).

Because members of the Bcc are thought to be potential agents for bioremediation and because they have not been well analyzed for this application, I sought to explore the relationship between clinical and environmental strains. Since Burkholderia have many aromatic oxygenases and the soil and lung are very different in their supply of these substrates, I evaluated whether there were any differences in

species and habitat source of strains using a variety of aromatic compounds. For example, aromatic compounds involved in lignin metabolism are a major carbon resource in soil, but not in the lung.

Lignin Biosynthesis and Associated Aromatic Compounds

Lignin and lignans are the major metabolic products of phenylpropanoid metabolism in vascular plants (Lewis et al, 1998). A complex aromatic polymer, lignin comprises about 25% of the land-based biomass on Earth, and the recycling of this and other plant-derived aromatic compounds is vital for maintaining the Earth's carbon cycle (Diaz, et al., 2001). These phenolic substances account for ~30-40% of all the organic carbon. **Phenol** is an industrial product although it is also produced naturally. It is a colorless aromatic alcohol to which individuals may be exposed to through breathing contaminated air or through skin contact in the workplace. It is considered to be very toxic to humans through oral exposure. The primary use of phenol is in the production of phenolic resins, used in plywood construction and automotive industries (Agency for Toxic Substances and Disease Registry (ATSDR), 1989).

The biosynthesis of lignin, which originates from carbohydrates that are formed from atmospheric carbon dioxide by photosynthesis, is usually described as: ***CO_2 →Carbohydrates →Phenylpropanoid amino acids→Cinnamic acid derivatives→Cinnamyl alcohol derivatives→lignins*** (Freudenberg et al., 1968). The first major step in lignin biosynthesis is the formation of phenylpropanoid amino acids (C_6-C_3) such as **phenylalanine,** an essential amino acid. Upon conversion of phenylalanine to lignin is the removal of ammonia by action of L-phenylalanine ammonia-lyase to a number of cinnamic acid derivatives which upon reduction give

corresponding cinnamyl alcohol derivatives from which lignin is formed (Weiss et al., 1980). The ring- substituted cinnamic acids are distributed as **p-coumeric and ferulic acids**, which are precursors of lignin since they have the required phenylpropane carbon structure [C_6-C_3 units](Schubert, 1965). Additionally, syringen is produced via the coniferyl-alcohol pathway that ultimately gives **syringic acid,** which along with **ferulic acid** is an intermediate product of the fungal degradation of lignin (Schubert, 1965).

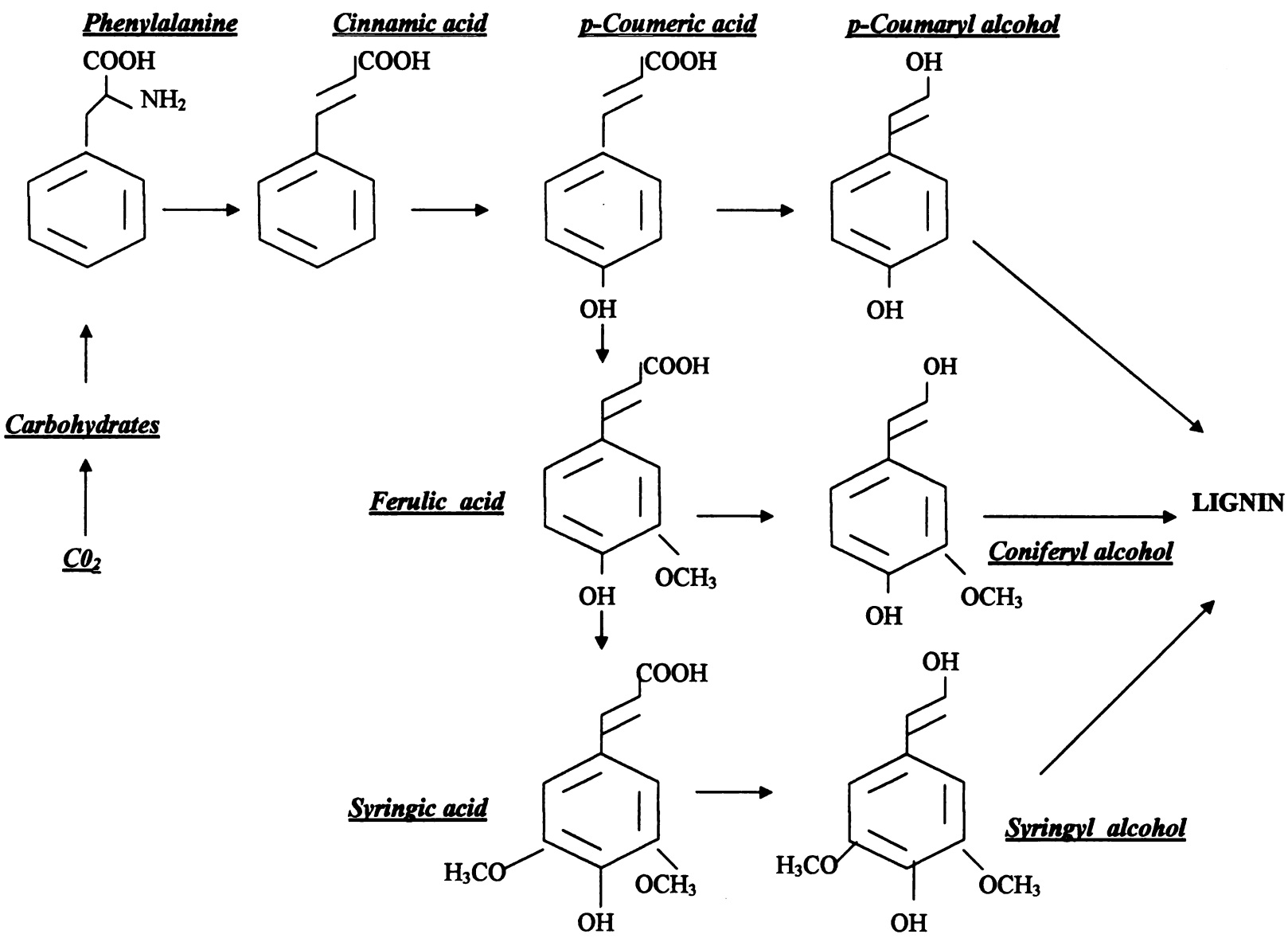
Microorganisms are almost entirely responsible for the degradation of such chemicals. In recent years there has been considerable interest in exploring the diversity and extent of microorganisms' ability to degrade or detoxify the increasing amounts of aromatic compounds that enter the environment as by-products of many industrial processes (Diaz, et al., 2001, Harayama, S, and K. N. Timmis, 1992, and Pieper, D. H., and W. Reineke. 2000). In higher plants, **salicylic acid**, a colorless crystalline organic carboxylic acid from willow bark, is formed by the hydroxylation of **benzoic acid**, the simplest aromatic carboxylic acid that may be obtained from resins, notably gum benzoin. **3-Chlorobenzoic acid**, which is also used in this experiment, is a product of bacterial transformations of polychlorinated biphenyls that is used as a model for study of the evolution of chloroaromatic degradative pathways (Fulthorpe et al, 1996). **Catechol,** a dihydric phenol that accounts for a small percent of lignin in wood, is the central intermediate in microbial catabolism of aromatic compounds (Crawford, 1981). We chose this range of aromatic compounds to examine the range of aromatic degrading abilities of different environmental and clinical strains of the Bcc.

Additionally because phenol and its derivatives are some of the major hazardous compounds in industrial wastewater their biodegradation has attracted great attention.

In this study, selected isolates that grow on phenol were screened for phenol hydroxylase genes using PCR primers that target conserved regions of the alpha subunit of the multicomponent phenol hydroxylase gene family (Futamata et. al., 2001, Ayala-del-Rio, 2002). Rapid methods for specifically detecting and quantifying toluene-degrading bacteria in the environment would aid the evaluation of site suitability for the implementation of toluene and phenol-stimulated TCE bioremediation.

Hence the objective of this study was to determine if there are different patterns of aromatic substrate use between clinical and environmental Bcc strains.

Figure 4.1. Simple and modified version (from Lewis et al 1998) of lignin biosynthesis involving aromatics used in this experiment.


Phenylalanine
Cinnamic acid
p-Coumeric acid
p-Coumaryl alcohol
COOH
NH2
OH
Carbohydrates
C02
Ferulic acid
OCH3
Coniferyl alcohol
LIGNIN
Syringic acid
H3CO
Syringyl alcohol

Materials and Methods

Strains used. Clinical Bcc strains isolated from patients and from the environment (no specific description of the origin for several strains) were provided by Dr. John LiPuma, Department of Pediatrics, University of Michigan, Ann Arbor, Michigan. Also strains isolated from rhizospheres of corn from a tall grass Iowa prairie were obtained from Dr. Alban Ramette, Michigan State University, East Lasing, Michigan. The strains were already identified as to which genomovar they belonged (Table 4.1).

Chemicals used Benzoic acid, catechol, 3-chlorobenzoic acid, *p*-coumeric acid, ferulic acid, phenylalanine, and syringic acid were all purchased from Sigma-Aldrich (St.Louis, MO). Salicylic acid was obtained from Matheson Coleman and Bell (Cincinnati, OH); phenol from Fisher Scientific Company (Fair Lawn, NJ); and toluene from Mallinckrodt Chemical Company (Paris, KY).

Table 4.1. Strains of the Bcc, the genomovar to which they belong, from where the strains were isolated, and the people who supplied them. Strains Corn 4, 5, 6, & 7 did not have a high similarity to the known genomovars when analyzed with restriction enzyme *HaeIII* as determined by Dr. Alban Ramette, Michigan State University.

Environmental Strains	Genomovar	Origin	Source
IO80-1	VII	Iowa Prairie	Dr. Alban Ramette
IO80-2	I	Iowa Prairie	Dr. Alban Ramette
IO80-3	VII	Iowa Prairie	Dr. Alban Ramette
IO80-4	I	Iowa Prairie	Dr. Alban Ramette
IO80-10	VII	Iowa Prairie	Dr. Alban Ramette
IO80-13	I	Iowa Prairie	Dr. Alban Ramette
IO80-21	I	Iowa Prairie	Dr. Alban Ramette
Corn 1	I	Corn Rhizosphere	Dr. Alban Ramette
Corn 2	I	Corn Rhizosphere	Dr. Alban Ramette
Corn 3	I	Corn Rhizosphere	Dr. Alban Ramette
Corn 4	B	Corn Rhizosphere	Dr. Alban Ramette
Corn 5	C	Corn Rhizosphere	Dr. Alban Ramette
Corn 6	D	Corn Rhizosphere	Dr. Alban Ramette
Corn 7	D	Corn Rhizosphere	Dr. Alban Ramette
HI2557	I	-	Dr.John LiPuma
PC783	I	onion	Dr.John LiPuma
HI2140	I	-	Dr.John LiPuma
HI2229	II	Soil enriched w/anthranilate	Dr.John LiPuma
ES1500	III	-	Dr.John LiPuma
HI2485	III	-	Dr.John LiPuma
HI2468	VII	-	Dr.John LiPuma
HI2474	VII	Corn roots	Dr.John LiPuma
ES0034	VII	-	Dr.John LiPuma
ES0609	VII	-	Dr.John LiPuma
ES0193	VII	-	Dr.John LiPuma
HI2725	VIII	Panama Palm Plant	Dr.John LiPuma
BC11	IX	Water	Dr.John LiPuma
HI2710	IX	-	Dr.John LiPuma
ES0196	IX	-	Dr.John LiPuma
ES0219	IX	-	Dr.John LiPuma
ES0164	IX	-	Dr.John LiPuma

Preparation for aromatic growth analysis. After growth on Plate Count Agar (PCA), each strain was inoculated with 2 ml Basal Salt Medium (BSM) into triplicate 20 ml bottles. Basal Salt Media consisted of 40 ml/L Na/KPO_4 buffer (MMO); 5 ml/L $MgSO_4$; 5 ml/L $CaCl_2$; 5 ml/L $FeSO_4$; 5 ml/L $NaMoO_4$; 1 ml/L Metal 44 (MMO); 10 ml/L 10% $(NH_4)_2$ SO_4. These three bottles of each inoculated strain were then spiked separately with 10 ppm of benzoic acid, catechol, 3-chlorobenzoic acid, *p*-coumeric acid, ferulic acid, phenol, phenylalanine, salicylic acid, syringic acid, and toluene. All bottles inoculated were sealed with Teflon stoppers capped with an aluminum top, placed on a shaker and incubated for 1 week at 25°C before analysis by visible turbidity except for toluene where optical density (Varian CaryWinUV) was measured.

Analysis of environmental vs. clinical strains. Data on substrates for each set of strains was analyzed via molecular statistical programs Systat 8.0 and Molecular Evolutionary Genetics Analysis Version, 2.1(MEGA) to construct dendrograms of the strains to and determine similarities or differences between them.

Determination of phenol hydroxylase genes. All Bcc strains that grew on PCA were first lysed and then PCR amplified with primers for the multi-component phenol hydroxylases, PheUf and PheUr (Futamata et al. 2001). PCR products were detected by electrophoresis gel and products that were amplified were purified and sequenced by the Genomics Technology Support Facility (GTSF) at Michigan State University. Sequences were aligned using BLAST 2.0 (**B**asic **L**ocal **A**lignment **S**earch **T**ool) against known phenol hydroxylases in the GenBank database.

Table 4.2. Growth of environmental Bcc strains and *Burkholderia vietnamiensis* G4 (positive control) on toluene and the total percentage of toluene degraded. Data are the means of triplicate samples.

Environmental Strains	Growth on Day 1 (OD_{600})	Growth on Day 5 (OD_{600})	% Toluene Degraded
IO80-1	0.04	0.04	59.7
IO80-2	0.06	0.06	35.9
IO80-3	0.07	0.08	54.0
IO80-4	0.05	0.05	54.5
IO80-10	0.04	0.05	74.5
IO80-13	0.06	0.06	57.1
IO80-21	0.06	0.07	15.5
Corn 1	0.02	0.03	13.8
Corn 2	0.04	0.08	69.1
Corn 3	0.05	0.05	23.4
Corn 4	0.06	0.06	21.7
Corn 5	0.10	0.19	40.8
Corn 6	0.03	0.03	82.2
Corn 7	0.07	0.12	57.4
IHI2557	0.01	0.01	0
IPC783	0.01	0.01	0
IHI2140	0	0.01	0
IIHI2229	0	0.01	0
IIIES1500	0.01	0.01	16.7
IIIHI2485	0.01	0.01	34.2
VIIHI2468	0	0.01	0
VIIHI2474	0.04	0.04	63.6
VIIES0034	0.07	0.08	28.9
VIIES0609	0	0.01	15.0
VIIES0193	0.03	0.03	32.6
VIIIHI2725	0.03	0.04	0
IXBC11	0.02	0.05	0
IXHI2710	0.02	0.02	0
IXES0196	0.01	0.01	22.9
IXES0219	0.01	0.01	28.1
IXES0164	0.02	0.02	12.9

Table 4.3. Growth of clinical Bcc strains on toluene and the total percentage of toluene degraded. Data are the means of triplicate samples.

Clinical Bcc strains	Growth on Day 1 (OD_{600})	Growth on Day 5 (OD_{600})	% Toluene Degraded
IHI2284C	0.02	0.02	0
IIHI2132C	0.02	0.03	0
IIHI2240C	0.02	0.03	50.0
IIIHI2711C	0.02	0.14	100.0
IIIHI3240C	0.02	0.03	62.8
IIIHI3248C	0.01	0.02	44.8
IVHI2210C	0.02	0.04	0
IVAU0244C	0.01	0.04	0
VPC259C	0.02	0.03	8.07
VAU1344C	0.03	0.03	30.0
VHI2238C	0.02	0.04	58.7
VIAU0645C	0.02	0.05	19.4
VIAU0158C	0.03	0.04	33.1
VIHI2238C	0.02	0.04	29.8
VIIAU0212C	0.02	0.03	39.9
VIIIAU1293C	0.01	0.02	44.2

Growth of clinical compared to environmental strains on aromatic compounds.

More environmental strains than clinical grew on all substrates except *p*-coumeric acid (environmental 39%, clinical 44%), syringic acid (environmental 23%, clinical 31%), and toluene (environmental 74%, clinical 75%) (Tables 4.2, 4.3, 4.4 and Fig. 4.2). I compared the prairie and corn isolates to see whether host plants affected their aromatic substrate range. The corn isolates grew better than the prairie ones on all aromatics except toluene, where all strains grew, catechol, where about one-half of each set grew, and syringic acid, where none of the strains grew. When all substrates are grouped together, the differences in growth between the environmental and clinical are clearly seen (Figure 4.3). To better observe these differences, dendrograms were constructed to cluster strains that had similar aromatic degrading ability.

Table 4.4. Growth of environmental Bcc strains on different aromatic compounds measured in triplicate. (+) represents at least two out of the three replicates exhibiting turbidity. (-) represents one or none of the isolates being turbid.

Strain	*Benzoic acid*	*p-coumeric acid*	*Ferulic acid*	*Phenol*	*Phenylalanine*	*Salicylic acid*	*Toluene*	*Catechol*	*3-chlorobenzoic acid*	*Syringic acid*
IO80-1	-	+	-	+	+	+	59.7	+	-	-
IO80-2	-	-	-	-	-	+	35.9	+	-	-
IO80-3	-	-	-	-	+	+	54.0	-	-	-
IO80-4	-	-	+	-	-	+	54.5	-	-	-
IO80-10	+	-	+	-	+	-	74.8	-	-	-
IO80-13	-	-	+	-	-	-	57.1	+	-	-
IO80-21	-	-	+	-	-	-	15.5	+	+	-
CORN-1	+	-	+	+	+	+	13.8	-	+	-
CORN-2	+	-	-	-	+	+	69.1	-	-	-
CORN-3	+	+	-	+	+	-	23.4	+	-	-
CORN-4	+	-	+	-	+	+	21.7	+	-	-
CORN-5	+	-	+	-	+	-	40.8	+	-	-
CORN-6	+	-	+	+	+	-	82.2	+	-	-
CORN-7	+	+	+	+	+	+	57.4	-	-	-
IHI2557	+	+	-	+	-	+	-	-	-	-
IPC783	+	+	-	+	-	+	-	+	+	+
IHI2140	+	+	-	+	-	+	-	-	-	-
IIHI2229	-	-	-	-	-	-	-	-	+	-
IIIES1500	-	-	-	-	-	-	16.7	-	+	+
IIIHI2485	+	+	-	+	+	-	34.2	-	+	+
VIIHI2468	+	-	-	-	+	+	-	+	+	+
VIIHI2474	+	-	-	-	-	+	63.6	-	-	+
VIIES0034	+	-	+	+	+	+	28.9	-	+	+
VIIES0609	+	-	-	-	-	+	15.0	-	+	+
VIIES0193	+	+	+	+	+	+	32.9	-	+	-
VIIIHI2725	+	-	-	+	-	+	-	-	-	-
IXBC11	+	+	+	+	-	-	-	-	-	-
IXHI2710	+	+	-	+	-	-	-	-	-	-
IXES0196	+	+	+	+	+	+	22.9	-	+	-
IXES0219	+	+	+	+	+	+	28.1	-	-	-
IXES0164	+	-	+	+	+	+	12.9	-	-	-

Table 4.5. Growth of clinical Bcc strains (isolated from patients), on different aromatic compounds measured in triplicates (+) represents at least two out of the three replicates exhibiting turbidity. (-) represents one or none of the isolates being turbid.

Strain	Benzoic Acid	p-coumeric Acid	Ferulic Acid	Phenol	Phenylalanine	Salicylic Acid	Toluene	Catechol	3-Chloro benzoic acid	Syringic acid
IHI2284C	+	+	-	+	-	-	-	-	+	+
IIHI2132C	+	+	-	-	-	+	-	-	-	-
IIHI2240C	+	+	-	+	-	-	50.0	-	+	+
IIIHI2711C	+	-	-	-	-	-	100	-	-	-
IIIHI3240C	-	-	-	-	-	-	62.8	-	+	+
IIIHI3248C	-	-	+	-	+	-	44.8	-	-	-
IVHI2210C	-	-	-	+	-	-	-	-	-	-
IVAU0244C	+	-	-	-	-	-	-	-	-	-
VPC259C	-	+	+	-	-	-	8.10	-	-	-
VAU1344C	+	+	+	-	-	-	30.0	-	-	-
VHI2238C	+	+	+	-	-	-	58.7	-	+	-
VIAU0645C	+	+	+	-	-	-	19.4	-	-	-
VIAU0158C	+	-	-	-	-	-	33.1	-	-	-
VIHI2238C	+	-	-	-	-	-	29.8	-	-	+
VIIAU0212C	+	-	-	-	+	+	39.9	-	-	+
VIIIAU1293C	+	-	-	+	-	-	44.2	-	-	-

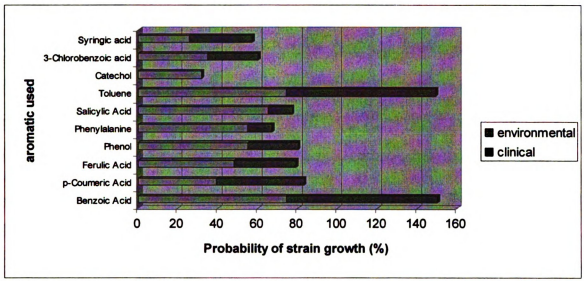


Figure 4.2. Probability of environmental/clinical strain growth on a variety of aromatics.

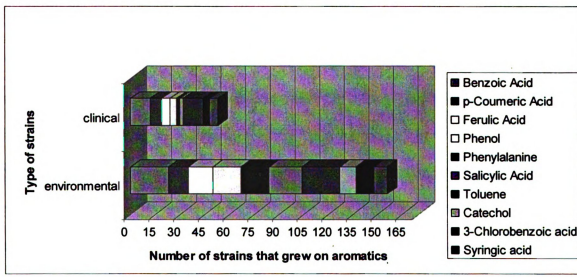


Figure 4.3. Growth of clinical vs. environmental strains of the Bcc on a variety of aromatics.

The strains are divided into eight clusters of aromatic substrates used (Fig. 4.4). The first cluster, A, is strictly environmental strains, including those from both the prairie and corn. The strains in this cluster grew on practically all aromatic compounds with only a few differences among them (Tables 4.4 and 4.5). Cluster B was similar to A except these strains did not grow on *p*-coumeric acid. Within this cluster is the prairie strain IO80-10 and the clinical strain IIIHI3248. These strains are included in this cluster probably because they are the only prairie and clinical strains that grew on both ferulic acid and phenylalanine. Cluster C (75% similarity to the previous clusters) includes prairie strains IO80-1 and Corn 3 and are two of the few strains that grew on *p*-coumeric acid and catechol, but not ferulic acid, which further shows why other prairie and corn strains were not clustered with them. The strains in cluster D only grew on three aromatics: phenylalanine, salicylic acid, and toluene, and thus had only a 72% similarity to other environmental strains.

Cluster E contained only clinical strains and had a 70% similarity to the other clusters because all these strains grew only on *p*-coumeric acid, ferulic acid and toluene and hardly anything else. A strictly prairie cluster (F) had only a 68% similarity to cluster E because these were the only prairie strains that did not grow on benzoic acid, phenylalanine or syringic acid, and also the only prairie strains that belonged to genomovar I. The large cluster, G, contains both clinical and environmental strains; the majority of these strains grew on benzoic acid and syringic acid and not ferulic acid whereas the strains in the previous cluster did not consume benzoic acid nor syringic acid.

Figure 4.4. Dendrogram showing both clinical and environmental strains with similar aromatic substrate use. X-axis represents the variable differences between each cluster. Symbols used represent the following:


Corn


Prairie


Patients w/Cystic Fibrosis


Patients w/Chronic Granulomatous Disease


Soil

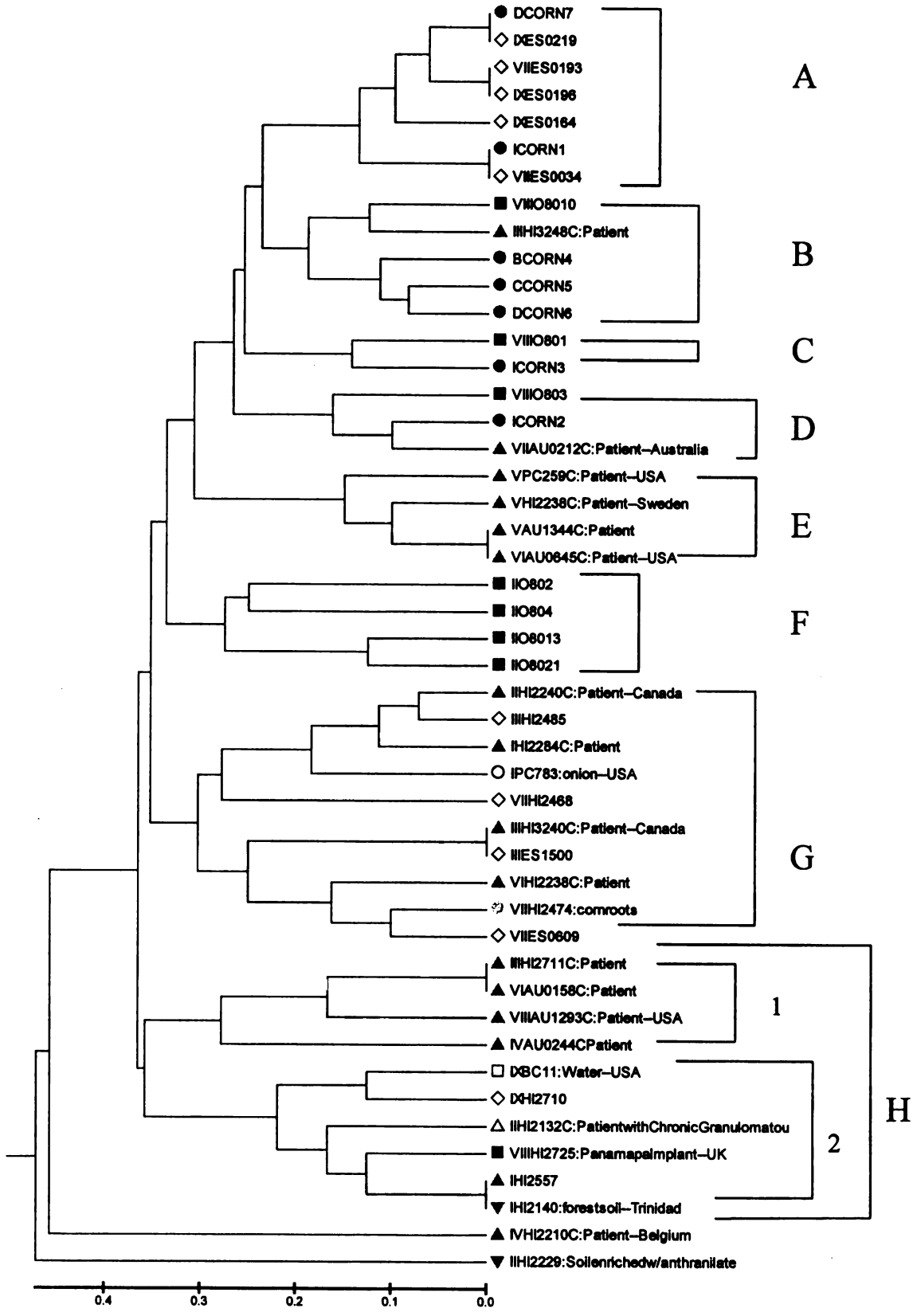

Panama Palm Plant


Onion


Water


Ubiquitous environmental strains

The eight major clusters of strains with similar substrate use are identified. Genomovars, when known, are indicated by the Roman numeral before the strain number.


DCORN7
DXES0219
VIIES0193
DXES0196
DXES0164
ICORN1
VIIES0034
VIIIO8010
IIIHI3248C:Patient
BCORN4
CCORN5
DCORN6
VIIIO801
ICORN3
VIIIO803
ICORN2
VIIAU0212C:Patient–Australia
VPC259C:Patient–USA
VHI2238C:Patient–Sweden
VAU1344C:Patient
VIAU0645C:Patient–USA
IIO802
IIO804
IIO8013
IIO8021
IIHI2240C:Patient–Canada
IIIHI2485
IHI2284C:Patient
IPC783:onion–USA
VIIHI2468
IIIHI3240C:Patient–Canada
IIIES1500
VIHI2238C:Patient
VIIHI2474:comroots
VIIES0609
IIIHI2711C:Patient
VIAU0158C:Patient
VIIIAU1293C:Patient–USA
IVAU0244CPatient
DXBC11:Water–USA
DXHI2710
IIHI2132C:PatientwithChronicGranulomatou
VIIIHI2725:Panamapelmplant–UK
IHI2557
IHI2140:forestsoil–Trinidad
IVHI2210C:Patient–Belgium
IIHI2229:Soilenrichedw/anthranilate
A
B
C
D
E
F
G
1
2
H
0.4
0.3
0.2
0.1
0.0

Cluster H had two sub-clusters, 1 and 2. Sub-cluster 1 had a 65% similarity to sub-cluster 2 and was limited in their degrading ability to consuming only benzoic acid and toluene, whereas sub-cluster 2, which had both environmental and clinical strains, consumed four aromatics: benzoic acid, *p*-coumeric acid, phenol, and salicylic acid. Thus, cluster H had only a 63% similarity to previous clusters. The final two strains did not cluster with any other because they each grew on only one aromatic each. The clinical strain (IVHI2210C) grew on phenol, and the environmental strain (IIH2229) on 3-chlorobenzoic acid. The results indicate that as a collective group, there are differences between environmental and clinical strains. However, the percentages that grew on toluene were similar as well as the percentage of strains that consumed 50% or more of the toluene (Tables 4.2 and 4.3), indicating that there is no significant difference on the growth of toluene between the clinical and environmental strains.

We examined the corn and prairie strains because we knew the exact origin of all of these environmental strains (Fig. 4.5). Cluster A consisted of four sub-clusters (1, 2, 3, 4) that had both corn and prairie strains. Sub-cluster 1 and 2 which had only 78% similarity are different from one another because although they grew on some of the same aromatics, benzoic acid and phenylalanine, for example, and they did not all share the property of utilizing salicylic acid and catechol (Fig. 4.4). These clusters also included strains that could not be placed into one of the nine known genomovars, based on *recA* RFLP-typing, i.e., **C** Corn 5, **D** Corn 6, **B** Corn 4, and **D** Corn 7. Sub-cluster 3, which consisted of IO80-1 and Corn 3, was not included in the previous cluster and had only a 75% similarity to the two previous sub-clusters because they did not consume ferulic acid. Sub-cluster (4) was also included in the larger cluster A, because

of these strains' growth on phenylalanine, toluene and salicylic acid. The prairie strains that were incorporated in cluster A did not group with cluster B because the strains in A had specific differences in that they grew on benzoic acid and phenylalanine and the strains in cluster B did not. Those strains included in cluster A also were members of genomovar VII, whereas the prairie strains in B were in genomovar I. These results reiterate the fact that even though strains are from the same location they are not siblings since they exhibit different growth phenotypes.

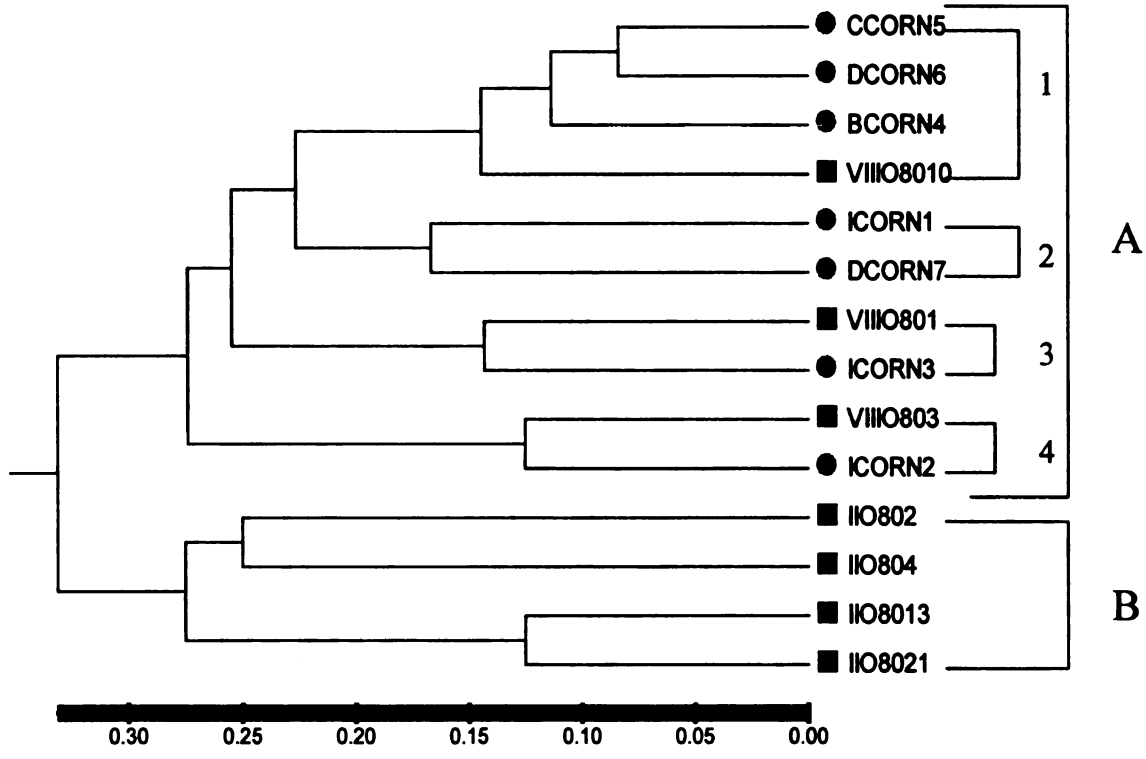


Figure 4.5. Dendrogram showing similar aromatic substrate use by strains from corn (●) and prairie (■) rhizospheres. X-axis represents the variable differences between each cluster.

Phenol hydroxylase analysis Of the 16 strains that grew on phenol only two produced amplified products using the phenol hydroxylase primers. Those strains were III HI2485 and I Corn 3 (Fig. 4.6). Only one of the two amplification products could be sequenced. When the amplicons from the corn strain was sequenced, its nucleotide amino acid sequence was 95% similar to a *Burkholderia cepacia* strain E1's gene for the alpha subunit of phenol hydroxylase.

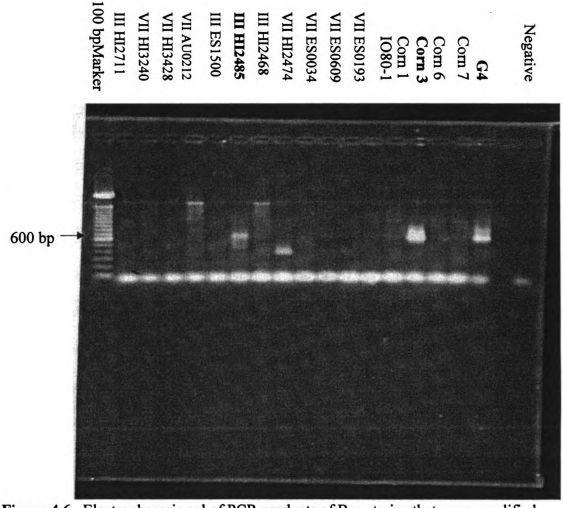


Figure 4.6. Electrophoresis gel of PCR products of Bcc strains that were amplified using phenol hydroxylase primers, PheUf and PheUr. Only IIIHI2485, Corn 3, and G4 (positive control) had bands at the expected 600bp site.

Discussion

Lignin is the second most abundant organic compound on Earth. It originates from the carbohydrates that are formed from atmospheric carbon dioxide by the process of photosynthesis. Lignins constitute a very widespread group of phenylpropanoid natural products found in plant parts including stems, rhizomes, roots, seeds, and oils (Lewis and Sarkanen, 1998). Lignification in the plant cell wall is initiated by the enzymatic formation of phenoxy radicals from cinnamyl alcohol precursors (Lewis and Sarkanen, 1998). Thus lignin biosynthesis is the charting of the macromolecule in the cell wall.

These phenolic substances account for ~30-40% of all organic carbon in vascular plants, of which the lignins are the predominant members. A few bacteria and some fungi are able to decompose lignin and to assimilate lignin degradation products as a carbon source eventually oxidizing it to CO_2 (Crawford, 1981). Lignins are relatively resistant to complete mineralization, with the greatest conversion to CO_2 occurring during the earlier stages of decomposition (Crawford, 1981). In this analysis, we studied the degradation of several aromatics including ones that are precursors for lignin biosynthesis to determine whether *Burkholderia cepacia* complex (Bcc) strains from clinical versus environmental sources had any differences in aromatic degrading abilities.

Overall, I found that the environmental strains did grow on more aromatic substrates than the clinical isolates. Within the environmental strains, the corn isolates grew on more substrates than the prairie isolates. The results from clustering indicated the relative growth or non-growth on the variety of aromatics has a profound effect on

how the strains are clustered together. There appeared to be ecological effects because even though some of the strains were taken from the same locations they are grouped with other strains from totally different areas. Importantly, most environmental and clinical strains clustered with strains from like habitats. Finally, there were differences between the environmental strains from the corn and prairie rhizospheres. The clear difference was in the prairie strains because all strains that were in a specific genomovar were clustered together signifying the disparity within one particular set of strains based on genomovar distinctions.

Contamination of the subsurface environment with chlorinated hydrocarbons, TCE for example, is a potentially serious threat to water sources. Laboratory studies have demonstrated that toluene and phenol-degrading bacteria co-metabolically transform these compounds to readily degradable oxygenated compounds. Implementation of phenol-stimulated TCE bioremediation would be aided by rapid methods for specifically detecting and quantifying groups of phenol-degrading bacteria in the environment. For this purpose, several strains of the *Burkholderia cepacia* complex were analyzed for the presence of genes for the largest subunit of multi-component phenol hydroxylases (LmPHs) that could predict the TCE degradation potential of bacterial populations (Futamata, 2001). I found that only 12% of the strains that grew on phenol produced an amplification product using the published phenol hydoxylase primers. As described by Futamata (2001), the primers used should amplify products at the 600 bp region when analyzed by electrophoresis gel. However, of the two research strains that produced bands, only Corn 3 produced a sequence similar to known phenol hydroxylase genes. The most likely explanation for this discrepancy is

that there is greater sequence diversity in phenol hydroxylase genes than recognized by the current primers.

In essence, the degradative properties of the strains from two sources were found to be quite different. Growth on the different aromatics such as those involved in lignin biosynthesis greatly affects how the strains clustered. While the strains can be separated, more work is needed to know whether one could safely distinguish a potential pathogen from a harmless strain by growth on aromatic substrates.

References

1. **Akin, DE, A. Sethuraman, WH Morrison III, SA Martin, and KL Eriksson.** 1993. Microbial Delignification with White Rot Fungi Improves Forage Digestibility. Appl. Environ. Microbiol. **59**:4274-4282.

2. **Agency for Toxic Substances and Disease Registry (ATSDR).** 1989. Toxicological profile for phenol. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

3. **Ayala-del-Rio, Hector.** 2002. Long-term effects of phenol and phenol plus trichloroethene application on microbial communities in aerobic sequencing batch reactors. Ph.D. Dissertation. Michigan State University, East Lansing, MI.

4. **Bevivino, Annamaria, Claudia Dalmastri, Silvia Tabacchioni, Luii Chiarini, Maria L. Belli, Sandre Piana, Alberto Materazzo, Peter Vandamme, and Graziana Manno.** 2002. *Burkholderia cepacia* Complex Bacteria from Clinical and Environmental Sources in Italy: Genomovar Status and Distribution of Traits Related to Virulence and Transmissibility. J. Clin. Microbiol. **40**:846-851.

5. **Bonnen, AM, LH Anton, and AB Orth.** 1994. Lignin-Degrading Enzymes of the Commercial Button Mushroom, *Agaricus bisporus*. Appl. Environ. Microbiol. **60**:960-965.

6. **Bowers, J., and J. Parke.** 1993. Epidemiology of *Pythium* damping-off and *Aphanomyces* root rot of peas after seed treatment with bacterial agents for biological control. Phytopathology **83:**1466-1473.

7. **Boyle, CD, BR Kropp, and ID Reid.** 1992. Solubilization and Mineralization of Lignin by White Rot Fungi. Appl. Environ. Microbiol. **58**:3217-3224.

8. **Camarero, S, GC Galletti, and AT Martinez.** 1994. Preferential degradation of phenolic lignin units by two white rot fungi. Appl. Environ. Microbiol. 60:4509-4516.

9. **Coenye, T., J. LiPuma, D.Henry, B. Hoste, K. Vandemeulebroucke, M. Gillis, D.P. Speert, and P. Vandamme.** 2001. *Burkholderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. Int. J. Syst. Evol. Microbiol. **51**:271-279.

10. **Crawford, Ronald L.** Lignin Biodegradation and Transformation. John Wiley & Sons. New York. 1981.

11. **Eduardo Díaz, Abel Ferrández, María A. Prieto, and José L. García.** 2001. Biodegradation of Aromatic Compounds by *Escherichia coli.* Microbiology and Molecular Biology Reviews. **65**:523-569.

12. **Duhalt-Vazquez, R., DWS Westlake, and PM Fedorak.** 1994. Lignin Peroxidase Oxidation of Aromatic Compounds in Systems Containing Organic Solvents. Appl. Environ. Microbiol. **60**:1548-1549.

13. **Fiore, A., S. Laevens, A. Bevivino, C. Dalmastri, S.Tabacchioni, P. Vandamme, and L. Chiarini.** 2001. *Burkholderia cepacia* complex: distribution of genomovars among isolates from the maize rhizosphere in Italy. Environ. Microbiol. **3**:137-143.

14. **Folsom, B.R., P.J. Chapman, and P.H. Pritchard.** 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia G4*: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56**:1279-1285.

15. **Freudenberg, Karl and Arthur Charles Neish.** Constitution and Biosynthesis of Lignin. Springer-Verlag. New York. 1968.

16. **Fulthorpe, Roberta R., Albert N. Rhodes, and James M. Tiedje.** 1996. Pristine Soils Mineralize 3-chlorobenzoate and 2,4-Dichlorophenoxyacetate via Different Microbial Populations. Appl. Environ. Microbiol. **62**:1159-1166.

17. **Futamata H., Shigeaki Harayama, and Kazuya Watanabe.** 2001. Group-Specific Monitoring of Phenol Hydroxylase Genes for a Functional Assessment of Phenol-Stimulated Trichloroethylene Bioremediation. Appl. Environ. Microbiol. **67:** 4671-4677

18. **Gold, M.H., L. Akileswaran, H. Wariishi, Y. Mino, and T.M. Loehr.** Spectral Characterization of Mn-Peroxidase, an Extracellular heme Enzyme from Phanerochaete chrysosporium. Etienne Odier ed. Lignin Enzymatic and Microbial Degradation. INRA publications. Paris. 1987, p.113-118.

19. **Gold, M.H., and M. Alic.** 1993. Molecular biology of the lignin-degrading basidiomycete *Phannerochaete chrysosporium.* Microbiol. Rev. **57**:605-622.

20. **Gonzalez, JM, WB Whitman, RE Hodson, and MA Moran.** 1996. Identifying numerically abundant culturable bacteria from compled communities: an example from a lignin enrichment culture. Appl. Environ. Microbiol. **62**:4433-4440.

21. **Govan, J.R.W., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia.* Microbiological Review. **60**:539-574.

22. **Govan, J.R.W, J.E. Hughes, and P.Vandamme.** 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. J. Med. Microbiol. **45**:395-407.

23. **Gutierrez, A, P. Bocchini, GC Galletti, and AT Martinez.** 1996. Analysis of Lignin-Polysaccharide Complexes Formed During Grass Lignin Degradation by Cultures of Pleurotus Species. Appl. Environ. Microbiol. **62**:1928-1934.

24. **Harayama, S., and K. N. Timmis.** 1992. Aerobic biodegradation of aromatic hydrocarbons by bacteria, p. 99-156. *In* H. Sigel, and A. Sigel (ed.), Metal ions in biological systems. Marcel Dekker, Inc, New York, N.Y.

25. **Harvey, P.J., H.E. Shoemaker and J.M. Palmer**. Mechanism of ligninase catalysis. Etienne Odier ed. Lignin Enzymatic and Microbial Degradation. INRA publications. Paris. 1987, p.145-150.

26. **Holmes, A., J. Govan, and R. Goldstein.** Agriculture use *of Burkholderia (Pseudomonas) cepacia*: a threat to human health? Emerg. Infect. Dis. **4**:221-227.

27. **Jokela, J., J. Pellinen, and M. Salkinoja-Salonen**. Initial Steps in the pathway for bacterial degradation of two tetrameric lignin model compounds. Etienne Odier ed. Lignin Enzymatic and Microbial Degradation. INRA publications. Paris. 1987, p.45-51

28. **Kawai, S. KA Jensen, Jr, W. Bao, and KE Hammel**. 1995. New polymeric model substrates for the study of microbial ligninolysis. Appl. Environ. Microbiol. **61**:3407-3414.

29. **Kersten, P.J., B. Kalyanaraman, K.E. Hammel, and T.K. Kirk.** Horseradish peroxidase oxidizes 1, 2, 4, 5-tetramethoxybenzene by a cation radical mechanism. Etienne Odier ed. Lignin Enzymatic and Microbial Degradation. INRA publications. Paris. 1987, p.75-80.

30. **Kirk, T.K.** "Enzymatic Combustion": The degradation of lilgnin by white-rot fungi. Etienne Odier ed. Lignin Enzymatic and Microbial Degradation. INRA publications. Paris. 1987, p.45-50.

31. **Lapadatescu, Carmen, Christian Ginies, Jean-Luc Le Quere and Pascal Bonnarme**. 2000. Novel Scheme for Biosynthesis of Aryl Metabolites from L-Phenylalanine in the Fungus *Bjerkandera adusta*. Appl. Environ Microbiol. **66**:1517-1522.

32. **Li, L, XF Cheng, J. Leshkevich, T. Umezawa, SA Harding, and VL Chiang.** 2001. The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell. **7**:1567-86.

33. **LiPuma, John J., Betty Jo Dulaney, Jennifer D, McMenami, Paul W. Whitby, Terrence L. Stull, Tom Coenye, and Peter Vandamme.** 1999. Development of rRNA-based PCR assays for identification of *Burkholderia cepacia* complex isolates recovered from cystic fibrosis patients. J. Clin. Microbiol. **37**:3167-3170.

34. **LiPuma, John J., T. Spiker, L.H. Gill, P.W. Campbell III, L. Liu, and E. Mahenthiralingam.** 2001. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. Am. J. Respir. Crit. Care Med. **164**:92-96.

35. **Lewis, Norman G. and Simo Sarkanen ed.** Lignin and Lignan Biosynthesis. American Chemical Society. Washington, DC. 1998

36. **Mahenthiralingam, Eshwar, Jocelyn Bischof, Sean K. Byrne, Christopher Radomski, Julian E. Davies, Yossef AV-Gay, and Peter Vandamme.** 2000. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkhoderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* Genomovars I and III. J. Clin. Microbiol. **38**:3165-3173.

37. **Mahenthiralingam, Eshwar, Tom Coenye, Jacqueline W. Chung, David P. Speert, John R. W. Govan, Peter Taylor, and Peter Vandamme.** 2000. Diagnostically and Experimentally Useful Panel of Strains from the *Burkholderia cepacia* Complex. J. Clin. Microbiol. **38**:910-913.

38. **Mahenthiralingam, Eshwar, David A. Simpson, and David P. Speert.** 1997. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. J. Clin. Microbiol. **35**:808-816.

39. **Matsui, N., K. Fukushima, S. Yasuda, and N. Terashima.** 1996. On the behavior of monolignol glucosides in lignin biosynthesis. III. Synthesis of variously labeled coniferin and incorporation of the label into syringin in the shoot of *Magnolia kobus*. Holzforschung. **50**:408-412.

40. **Miki, K., V. Renganathan, M.B. Mayfield,. and M.H. Gold.** Aromatic Ring Cleavage and Halogenation Reactions catalysed by the Phanerochaete chrysosporium Lignin Peroxidase. Etienne Odier ed. Lignin Enzymatic and Microbial Degradation. INRA publications. Paris. 1987, p.69-74.

41. **Miller, Suzanne C.M., John J. LiPuma, and Jennifer L. Parke.** 2002. Culture-Based and Non-Growth-Dependent Detection of the *Burkholderia cepacia* Complex in Soil Environments. Appl. Environ. Microbiol. **68**:3750-3758.

42. **Munakata-Marr, Junko, Perry L.McCarty, Malcolm S. Shields, Michael Reagin, and Stephen C. Francesconi.** 1996. Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered *Burkholderia* (*Pseudomonas*) *cepacia* G4 and PR1. Environ. Sci. Technol. **30**:2045-2052.

43. **Orth, AB, DJ Royse, and M. Tien.** 1993. Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Appl. Environ. Microbiol. **59**:4017-4023.

44. **Pitt, T.L., M.E. Kaufman, P.S. Patel, L.C. A. Benge, S. Gaskin, and D.M. Livermore.** 1996. Type characterization and antibiotic susceptibility of *Burkholderia (Pseudomonas) cepacia* isolates from patients with cystic fibrosis in the United Kingstom and the Republic of Ireland. J. Med. Microbiol. **44**:203-210.

45. **Phenol.** World Health Organization, Geneva. 1994.

46. **Pieper, D. H., and W. Reineke.** 2000. Engineering bacteria for bioremediation. Curr. Opin. Biotechnol. **11**:262-270

47. **Rodriguez, A., A Carnicero, F. Perestelo, G de la Fuente, O Milstein, and MA Falcon.** 1994. Effect of *Penicillium chrysogenum* on Lignin Transformation. Appl. Environ. Microbiol. **60**:2971-2976.

48. **Samejima, M., S. Abe, N. Habu, N. Tatazasako, Y. Saburi, and T. Yoshimoto.** Biodegradation Pathway of the lign in model Compounds with β-aryl Ether Linkage by *Pseudomonas paucimobilis* TMY1009. Etienne Odier ed. Lignin Enzymatic and Microbial Degradation. INRA publications. Paris. 1987, p.93-98.

49. **Schubert, Walter J.** Lignin Biochemistry. Academic Press. New York. 1965.

50. **Segonds, Christine, G. Chabanon, E. Bingen, Y. Michel-Briand, and G. Couetdic.** 1997. Epidemiiologie de la colonisation par *Burkholderia cepacia* des patients atteints de mucoviscidose en France: approche bioclinique, p.23. In Program and Abstracts of the 31st January 1997 SFM Conference on *Pseudomonas* et Bacteries Apparentees. Societe Francaise de Microbiologie, Paris, France.

51. **Segonds, Christine, Thierry Heulin, Nicole Marty, and Gerard Chabanon.** 1999. Differentiation of Burkholderia Species by PCR-Restriction Fragment Length Polymorphism Analysis of the 16S rRNA Gene and Application to Cystic Fibrosis Isolates. J. Clin. Microbiol. **37**:2201-2208.

52. **Temp, Ulrike, Claudia Eggert, and Karl-Erik L. Eriksson.** 1998. A Small-Scale Method for Screening of Lignin-Degrading Microorganisms. Appl. Environ. Microbiol. **64**:1548-1549.

53. **Toluene.** World Health Organization, Geneva. 1985.

54. **Watanabe, K., S. Hino, K. Onodera, S. Kajie, and N. Takahashi.** 1996. Diversity in kinetics of bacterial phenol-oxygenating activity. J. Ferment. Bioeng. **81**:562-565.

55. **Watanabe, K, Maki Teramoto, Hiroyuki Futamata, and Shigeaki Harayama.** 1998. Molecular Detection, Isolation, and Physiological Characterization of Functionally Dominant Phenol-Degrading Bacteria in Activated Sludge. Appl Environ Microbiol. **64**:4396-4402.

56. **Weiss, Ulrich and J.Michael Edwards.** The Biosynthesis of Aromatic Compounds. John Wiley & Sons. New York. 1980.

57. **Yeager, Chris M., Peter J. Bottomley, and Daniel J. Arp.** 2001. Cytotoxicity Associated with Trichloroethylene Oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. **67**:2107-21185.