RHIZOSPHERE/SOIL MICROORGANISMS IN THE PHYTOREMEDIATION OF BIPHENYL AND DIOXANE

by

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Abstract

Biphenyl and dioxane biodegradation by poplar and willow rhizosphere microorganisms was studied in a phytoremediation test-plot contaminated with biphenyl and dioxane. A dioxane-degrading consortium enriched from the contaminated rhizosphere soil did not use dioxane as the sole source of carbon and energy, but did co-metabolize dioxane in the presence of tetrahydrofuran (THF). An isolate obtained on agar plates containing basal salts and glucose grew on glucose and co-metabolically degraded dioxane after THF degradation. The rate and extent of dioxane degradation by this particular isolate increased with increasing THF concentration. This isolate was subsequently identified as a *Flavobacterium* by 16S rDNA sequencing. This is the first report of a dioxane-degrading *Flavobacterium* which is phylogenetically distinct from any previously identified dioxane degrader.

Rhizosphere microorganisms in the phytoremediation test-plot were capable of degrading biphenyl in the presence of the terminal electron acceptors (TEAs) nitrate, sulfate or carbon dioxide. TEAs (sulfate and carbon dioxide), nutrients in basal salts medium (BSM) or fertilizer enhanced biphenyl degradation. Although root exudates appeared to enhance biphenyl degradation slightly, it was not statistically significant ($p > 0.10$). A fungus enriched and isolated from the rhizosphere soil, was found to degrade biphenyl under anaerobic conditions only. The fungus was identified by a primer pair ITS4 and ITS1F as *Pseudallescheria boydii*.

The microbial community in the rhizosphere of the poplar and willow trees was sampled from different soil locations in the test-plot over a 5-year period. The denaturing gradient gel electrophoresis (DGGE) results indicated that soil type had a significant impact on microbial community composition with a more diverse microbial population in native soil samples than in engineered soil samples. Although tree type had less influence on microbial diversity, diversity

did decrease with time in the engineered soil of the willow rhizosphere. There was no significant influence of soil depth on the microbial community. Three aerobic biphenyl-degrading consortia were enriched from different rhizosphere soils, and the major microorganisms found in the enriched consortia were identified by 16S DNA sequencing as being in the family of *Flavobacteriaceae, Alcaligenaceae,* and *Mycobacteriaceae*.

Direct polymerase chain reaction (DPCR) without prior DNA extraction and DGGE yielded the same results as PCR assays using extracted DNA in the analysis of microbial populations in dioxane- and biphenyl-degrading consortia. Therefore, the combination of DPCR and DGGE has the potential for the fast analysis of the microbial populations of environmental and clinical samples with limited diversity.

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Abbreviations

AOP: Advanced oxidation process

BSA: Bovine Serum Albumin

BSM: Basal Salts Medium

DGGE: Denaturant Gradient Gel Electrophoresis

DPCR: Direct Polymerase Chain Reaction

EDTA: Ethylene diaminetetraacetic acid

GC: Gas Chromatography

MS: Mass Spectrometry

PCB: polychlorinated biphenyl

PCR: Polymerase Chain Reaction

PVPP: Polyvinylpolypyrrolidone

TE: Tris-EDTA

TEA: Terminal Electron Acceptor

THF: Tetrahydrofuran

UM-BBD: Minnesota Biocatalysis/Biodegradation Database

Chapter 1

General Introduction

This research was initiated by a pilot phytoremediation project implemented by a local environmental consulting company, Malroz Engineering. Malroz Engineering had been contracted to remediate an active industrial site contaminated with three major organic pollutants: biphenyl, dioxane and ethylene glycol. Traditional "pump-and-treat" was initially used but was not cost efficient for a contaminant like biphenyl, which is poorly soluble in water, highly hydrophobic, and adsorbs to the soil matrix tightly. Since pump and treat would take a very long time and be expensive, Malroz Engineering wished to evaluate phytoremediation to replace "pump-and-treat" at this site.

Phytoremediation is considered to be a "green", cost-effective, and aesthetically pleasing technology that can be applied over the long-term. It has been shown to be effective in removing many organic contaminants from soil and hydroponic experiments, but most studies have been conducted at the lab scale under controlled conditions. At the industrial scale, the situation is more complex and might be affected by multiple factors. Factors such as site conditions and history of use, the type of plant, and the properties of the contaminants might affect the success of phytoremediation. To test the effectiveness of phytoremediation, rows of poplar (*Populus balsamifera*) and willow (*Salix nigra*) trees were planted in an alternating pattern along the path

of a contaminated groundwater plume using both native (a clay soil) and engineered (a soil mixture designed to improve exchange with the water table) soils.

Phytodegradation, the use of plants and associated microorganisms to degrade organic pollutants, is a very important mechanism in phytoremediation. Soil microorganisms can utilize a wide range of organic contaminants as a carbon and energy source and degrade them to carbon dioxide and water or less toxic metabolites. In the rhizosphere, the plant and soil microorganisms interact very closely. For example, plant root exudates containing amino acids and organic acids can stimulate microbial growth and biodegradation activity. For some contaminants, the enhancement of microbial degradation in the rhizosphere might be very important for a successful phytoremediation project.

In collaboration with Malroz's phytoremediation project, the objective of this thesis is to investigate the microbial degradation of biphenyl and dioxane by the rhizosphere microorganisms in the phytoremediation test-plot. Focus is given to biphenyl since dioxane is likely to be transpired by plants. This research should not only help understand the degradation of biphenyl and dioxane on the site but also provide potential pathway(s) for the enhancement of contaminant removal. The knowledge obtained can also be applied to the remediation of similar contaminants and site conditions.

The thesis is organized as follows:

Chapter 2. Literature review. Current literature was reviewed to aid experimental design.

Chapter 3. Biodegradation of dioxane. The objective of this section was to investigate whether the indigenous microorganisms could aerobically degrade dioxane, and whether dioxane could be used as the sole carbon and energy source. Since dioxane was co-metabolized with another primary carbon source, tetrahydrofuran (THF), a strategy to minimize the use of THF was investigated. A dioxane-degrading microorganism was subsequently isolated and identified for further studies.

Chapter 4. Anaerobic biphenyl degradation. First the potential of anaerobic biphenyl degradation by the rhizosphere microorganisms was investigated in the phytoremediation test-plot in the presence of various terminal electron acceptors (TEAs). Since anaerobic biphenyl biodegradation was possible, the effect of TEA, fertilizer and root exudates on anaerobic biphenyl degradation was studied to find ways to enhance *in situ* anaerobic biphenyl degradation. An anaerobic biphenyl-degrading microorganism was identified in the process.

Chapter 5. Microbial community in the rhizosphere. The microbial community in the rhizosphere soil was analyzed by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) to see whether the rhizosphere population changed over the course of the phytoremediation study, especially in regards to specific biphenyl degraders. The effect of soil type, tree species, and soil depth on microbial community was also studied.

Chapter 6. Direct PCR analysis of a mixed, enriched culture. A faster method using direct PCR without prior DNA extraction was developed to analyze a mixed microbial population.

Chapter 7. Conclusions. The main results are concluded and future work is recommended.

Chapter 8. Contributions. The contributions of the research are summarized.

Chapter 2

Literature Review

Over the past century, a number of pollutants have been released into the environment through mining, manufacturing, industrial petroleum refining, and urban activities. The major pollutants are often aromatic compounds, which may be toxic, carcinogenic and mutagenic (Mishra et al. 2001), and have to be remediated. Various physical, chemical, and biological methods have been used to remediate contaminated groundwater and soil. The choice of a remediation strategy depends on the nature of the contaminants, soil properties, site conditions, and the volume of material to be treated (Cunningham et al. 1995). One commonly used technology in groundwater remediation is "pump-and-treat", which extracts the contaminated groundwater by pumping it to the surface where it is usually treated by physical or chemical means prior to surface discharge or it is returned to the subsurface upstream of the contaminated plume. Pump-and-treat provides quick reductions in groundwater concentrations, and is used as a management tool to prevent contaminant migration. However, if the contaminant is water insoluble and/or sorbs strongly to the soil, the non-aqueous phase contaminant in the subsurface will act as a continuous reservoir and will re-establish a new equilibrium with the "cleaned" groundwater. Hence, cleanup of groundwater contaminated with hydrophobic, water insoluble organic chemicals by pump-and-treat is typically slow (Mackay and Cherry 1989), and could be very expensive in the long term.

In general, *in situ* remediation technologies are less expensive than *ex situ* technologies (Cunningham et al. 1995). Organic contaminants can be removed *in situ* by microorganisms (biodegradation) and/or plants (phytoremediation). Since they are ecologically suitable and socially acceptable, bioremediation and phytoremediation of contaminated soil, sediment, surface water and groundwater are among the most rapidly developing fields in environmental science.

2.1 Phytoremediation

Phytoremediation is the use of plants for the remediation of contaminated soils, sediments and groundwater. Toxic heavy metals and organic pollutants are the major targets for phytoremediation and many reviews on this subject are available (Cunningham et al. 1995, Salt et al. 1998, Alkorta and Garbisu 2001, Dietz and Schnoor 2001, Glick 2003, Karthikeyan and Kulakow 2003, Singh and Jain 2003, Suresh and Ravishankar 2004, Krämer 2005, Pilon-Smits 2005, Reichenauer and Germida 2008). Phytoremediation of organic contaminants has been generally focused on chlorinated solvents, explosives and petroleum hydrocarbons (Dua et al. 2002). Based on the removal mechanisms, phytoremediation is divided into phytoextraction, phytodegradation, phytovolatilization, and phytostabilization (Cunningham et al. 1995, Salt et al. 1998, Singh and Jain 2003). Phytostabilization refers to the sequestration of organics and metals by the plants, and does not remove contaminants from the site.

The plant itself and its root-associated microorganisms are the two key players in phytoremediation. Although a plant will seldom directly degrade organic chemicals, the major mechanisms by which it can remove an organic contaminant is by adsorption, uptake, followed by transformation (phytostabilization) or transpiration (phytoextraction). On the other hand, soil microorganisms can use a wide range of environmental organic contaminants as a carbon and energy source. Biodegradation by the indigenous rhizosphere microorganisms has been shown as a promising technique for organic contaminant removal. More importantly, the interaction between the plant and microorganisms in the rhizosphere (i.e. root associated) provide a special environment that might significantly enhance the removal of contaminants.

2.1.1 Sorption and uptake of organic pollutants by plants

Of the major removal mechanisms of organic contaminants by plants, sorption and uptake is the first and very crucial step, and the efficiency depends on the nature of the organics and the soil (Dietz and Schnoor 2001).

The sorption of organic contaminants to the plant root structures and cell walls is nonspecific, relatively reversible, and can be measured using standard sorption isotherms. The root concentration factor (RCF), defined as the ratio of organic chemicals sorbed on the root to that in a hydroponic solution, is related to the octanol-water partition coefficient (K_{ow}), a measure of the

hydrophobicity of the organics (Briggs et al. 1980, Burken and Schnoor 1998). Both Briggs et al. (1980) and Burken and Schnoor (1998) showed that the greater the hydrophobicity of the organic compound, the higher the tendency of sorption to the roots. This result is reasonable since hemicellulose in the cell wall and lipid bilayer of plant membranes are hydrophobic and tend to bind hydrophobic chemicals.

Direct uptake by plants via diffusion through cell walls and membranes is an efficient way to remove organics from a contaminated site. The rate of chemical uptake depends on the transpiration rate, the concentration of chemical in soil water, and the transpiration stream concentration factor (TSCF) which is defined as the concentration in the transpiration stream divided by the bulk solution concentration in contact with the root tissue. TSCF can vary from zero (no uptake) to 1.0 (uptake at the same concentration as the soil water concentration) and is affected by the physico-chemical properties of the chemicals, the plant itself and plant growth. TSCF was also correlated to log K_{ow} (Briggs et al. 1980, Burken and Schnoor 1998), and the highest TSCF was found at a moderate hydrophobicity. Moderately hydrophobic organics (log K_{ow} = 1.0-3.5) bind to the cell membrane but not too strongly to prevent effective transmembrane transportation by roots (Dietz and Schnoor 2001).

Thus the role of plants in the removal of organic contaminants could be very significant when the contaminant has a moderate hydrophobicity. After being taken up by trees, contaminants might be transpired to the atmosphere, or be transformed and sequestered in plant tissues. If a

contaminant has a high hydrophobicity, it would have a high tendency to adsorb to roots but cannot be taken up by the trees effectively. Therefore, in the rhizosphere those organics might have a higher concentration, and this could favor the degradation by microorganisms.

2.1.2 Degradation of organic contaminants by microorganisms

Microorganisms are widely used to reduce the concentration and toxicity of various chemical pollutants, such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalate esters, nitroaromatic compounds, industrial solvents, pesticides and metals (Dua et al. 2002). Microbial degradation of organic contaminants has a long history, and is still a hot topic of research and industrial practice. As early as the 1920s, researchers found that certain soil bacteria like *Pseudomonas* can degrade different organic compounds. The early research centered on isolating microorganisms capable of degrading specific pollutants, and elucidating metabolic pathways and their enzymatic regulation. The degradation pathway of aromatic compounds began to be known in 1960s (Gibson 1968). Many organic contaminants are now known to be degraded by microorganisms, even ones previously shown to be recalcitrant to microbial degradation. The degradation pathways of many xenobiotics can be archived from online databases such as the Minnesota Biocatalysis/Biodegradation Database (UM-BBD) (Gao et al. 2010).

Molecular biology-based methods have been developed to identify and isolate microorganisms with degradative potential and to identify their catabolic genes (Widada et al. 2002). The accumulation of knowledge about the biochemistry and genetics of the biodegradation process makes it possible to modify the catabolic genes and enzymes to make 'Super bugs' (Furukawa, 2003) to enhance the biodegradation of xenobiotic compounds. However, the environmental and societal risks of applying genetically engineered microorganisms at the industrial scale or in field trials must be carefully assessed.

In situ biodegradation can occur naturally without intervention (natural attenuation) or be engineered to enhance biodegradation. Air sparging or injection of oxygen-releasing compounds (such as hydrogen peroxide, or magnesium peroxide) can be used to increase dissolved oxygen concentrations in ground water to stimulate aerobic degradation. However, air sparging is expensive and may be difficult to apply at some site conditions, and high concentrations of hydrogen peroxide can inhibit microbial degradation (Farhadian et al. 2008). The enhancement of anaerobic biodegradation by TEA other than oxygen has been reviewed by Foght (2008). Anaerobic biodegradation can also play a more important role under *in situ* conditions since the redox potentials are often low in contaminated groundwater and hence already provide reducing conditions. Anaerobic biodegradation of various contaminants has been reviewed by a number of groups (Holliger and Zehnder 1996, Zwolinski et al. 2000, Widdel and Rabus 2001, Mogensen et al. 2003, Zhang and Bennett 2005, Foght 2008).

2.1.3 Degradation of organic contaminants in the rhizosphere

The soil environment surrounding and influenced by plant roots is referred to as the rhizosphere. In the rhizosphere, the plant itself can directly degrade contaminants by releasing a number of enzymes such as laccases, dehalogenases, nitroreductases, nitrilases, peroxidases, and plant cytochrome P450 enzymes (Dietz and Schnoor 2001, Morikawa and Erkin 2003). Various ammunition wastes, nitroaromatic compounds, 4-chlorobenzonitrile, hexachloroethane, and trichloroethylene have been reported to be degraded by plant-derived enzymes (Alkorta and Garbisu 2001, Dietz and Schnoor 2001, Singh and Jain 2003). However, the enhancement of microbial degradation by plants in the rhizosphere might play a more important role in organic contaminant degradation.

Communication between plants and microbes which are in close proximity in the rhizosphere confers very specific characteristics to the rhizosphere compared to the bulk soil (outside the rhizosphere). The interaction between plants and microorganisms in the rhizosphere and their role in biodegradation of organic pollutants was reviewed by Shaw and Burns (2003). During growth, plants release large amounts of root exudates containing amino acids and organic acids which can be used as carbon and energy sources by rhizosphere microorganisms for their growth and metabolic activities. Many plants also release structural analogs of pollutants in the rhizosphere. These analogs may serve as microbial growth substrates, but more importantly, they can act as inducers for the expression for enzymes which can degrade an otherwise recalcitrant compound

of similar structure. Some root exudate chemicals can attract distantly located bacteria and fungi to the rhizosphere. Higher microbial numbers and activities are thus commonly found in the rhizosphere.

Conversely, plant growth-promoting rhizobacteria can beneficially influence the plants. In addition to supplying nitrogen, some nitrogen-fixers secrete phytohormones such as auxins, cytokinins and gibberellins to promote plant growth. Some rhizobacteria are also able to control plant diseases caused by fungi or bacteria (Bloemberg and Lugtenberg, 2001, Persello-Cartieaux et al. 2003).

2.2 Degradation of dioxane

1,4-Dioxane is a cyclic ether (Fig 2-1), primarily used as a stabilizer for chlorinated solvent. It is also used as a solvent for numerous organic and inorganic substances. Large amounts of dioxane have been released into the environment, totaling 1.13 million pounds in the USA in 1992 (OPPT Chemical Fact Sheets-1,4-dioxane 1995).

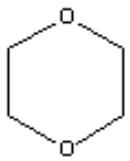

Figure 2-1 Chemical structure of dioxane

Dioxane is chemically stable, miscible with water, and has a low Henry's Law constant (Table 2-1), and natural attenuation of dioxane in contaminated groundwater is insignificant (Zenker et al. 2004, Mahendra and Alvarez-Cohen 2006). Various physical and chemical methods have been evaluated to treat dioxane-contaminated waters. Advanced oxidation processes (AOPs) using hydrogen peroxide, ozone, and UV photo-oxidation have been proven as suitable technologies for dioxane treatment (Zenker et al. 2003), but a major concern of AOPs is that the operating costs of these processes are normally high with additional costs incurred when used in combination with a "pump-and-treat" approach (Manendra and Alvarez-Cohen 2006).

Table 2-1 Properties of biphenyl and dioxane at 25 ℃

Chemical	Molecular Formula	Boiling Point (℃)	Melting Point (℃)	Henry's Law Constant atm m^3/mol	Log Kow	Water Solubility (mg/L)	Vapor Pressure mmHg
Biphenyl [a]	$C_{12}H_{10}$	254-255	69-71	3E-04	4.09	7.5	9.64E-03
Dioxane [b]	$C_4H_8O_2$	101.1	11.8	4.88E-06	-0.27	Miscible	30

[a]: OPPT Chemical Fact Sheets, 1,1'-Biphenyl, 1994
[b]: OPPT Chemical Fact Sheets, 1,4'-Dioxane, 1995

2.2.1 Uptake of dioxane by plants

Direct uptake and transpiration by plants might be an effective way to remove dioxane from a contaminated site. Hybrid poplar trees have been used to remediate dioxane in hydroponic and

soil experiments (Aitchison et al. 2000). The transpiration stream concentration factor (TSCF) was 0.72. When transpired to air, the half-life of dioxane was estimated to be 6.7-9.6 h as a result of photo-degradation (OPPT Chemical Fact Sheets-1,4-dioxane, US EPA 1995). The uptake of dioxane by a hybrid poplar tree is probably faster than microbial degradation (Kelley et al. 2001).

2.2.2 Degradation of dioxane by aerobic microorganisms

Dioxane has been reported to be recalcitrant to biodegradation, probably because it has a stable ether bond which requires 360 kJ/mol of energy for cleavage (Zenker et al. 2004). Biodegradation of dioxane using microorganisms is attractive because unlike transpiration by plants into air, it can be mineralized by microorganisms (Parales et al. 1994, Zenker et al. 2000). A potential dioxane degradation product was found to be 2-hydroxyethoxyacetic acid by a *Pseudonocardia sp*. strain ENV478 (Vainberg et al. 2006). Treatment with trickling filters (Zenker et al. 2004) resulted in dioxane concentrations as low as 2 to 10 ppb. Such concentrations are often below the regulated levels allowed in drinking water. To date, only a few aerobic microorganisms have been found to exhibit dioxane-degrading capabilities (Table 2-2).

Since dioxane is recalcitrant to biodegradation, using dioxane as the sole carbon and energy source in enrichment experiments is probably unsuccessful. THF, a structural analog of dioxane, has been used to enrich for dioxane degraders (Parales et al. 1994, Zenker et al. 2000 Vainberg et

al. 2006). In these cases, dioxane was co-metabolized in the presence of THF, except for a THF-degrading mutant *P. dioxaneivorans* CB1190 which was capable of using dioxane as the sole source of carbon (Parales et al. 1994). However, dioxane degradation has been reported by *Mycobacterium vaccae* JOB 5 (Burback and Perry 1993), the propanotroph SL-D (Fam et al. 2005), and *Methylosinus trichosporium* OB3b (Mahendra and Alvarez-Cohen 2006) without the presence of THF or other structural analogues. Mahendra and Alvarez-Cohen (2006) showed that methane monooxygenase could be a key enzyme in dioxane degradation.

Table 2-2 Aerobic dioxane degrading microorganisms

	Primary carbon source	Reference
Rhodococcus ruber 219	Dioxane	Bernhardt and Diekmann 1991, Bock et al. 1996
Pseudonocardia dioxaneivorans CB1190	Dioxane/THF	Parales et al. 1994, Mahendra and Alvarez-Cohen 2005
Cordyceps sinensis	Dioxane	Nakamiya et al. 2005
Pseudonocardia K1	THF	Kohlweyer et al. 2000
A mixed culture	THF	Zenker et al. 2000
Pseudonocardia sp. ENV478	THF	Vainberg et al. 2006
Mycobacterium vaccae JOB 5	Propane	Burback and Perry 1993
propanotroph SL-D	Propane	Fam et al. 2005
Methylosinus trichosporium OB3b	Methane	Mahendra and Alvarez-Cohen 2006

2.3 Degradation of biphenyl

Biphenyl is an aromatic hydrocarbon (Fig 2-2) having a high toxic rating to the environment. It has many industrial applications, such as being a dye carrier, as an intermediate of polychlorinated biphenyl (PCB), a fungicide and a heat transfer agent. In the past, a large amount of biphenyl was released into the environment, for example, 855,000 lbs of biphenyl was released in USA in 1992 (OPPT Chemical Fact Sheets-1,1'-biphenyl, 1994).

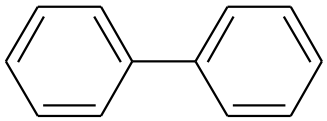

Figure 2-2 Chemical structure of biphenyl

2.3.1 Removal of biphenyl by plants

There are no reports on the phytoremediation of biphenyl in the published literature. Biphenyl has a low water solubility and is highly hydrophobic (log K_{ow} = 4.09) (Table 2-1). According to the relationship between hydrophobicity and uptake by plants (section 2.1.1), biphenyl is out of the range (log K_{ow} = 1.0-3.5) for effective uptake by roots (Dietz and Schnoor 2001). Phytoremediation has been reported as an effective way to remove PCB (Chekol et al. 2004, Zeeb et al. 2006, Mackova et al. 2009) where its removal was found mainly due to root uptake and

translocation to the shoots (Zeeb et al. 2006). Microbial counts and dehydrogenase activity in the rhizosphere was significantly increased in the presence of plants, and led to higher levels of PCB biodegradation (Chekol et al. 2004). The plant species was shown to be an important factor affecting PCB remediation with tobacco and horseradish displaying the best performance (Mackova et al. 2009). Some of this knowledge might apply to biphenyl since PCB degrading microorganisms commonly use biphenyl as the sole carbon and energy source.

2.3.2 Degradation of biphenyl by aerobic microorganisms

The aerobic biodegradation of biphenyl is reasonably easy. Several gram-negative bacteria were found to use biphenyl as the sole carbon and energy source in the early 1970s (Lunt and Evans 1970, Catelani et al. 1970). Higson (1992) reviewed the early development of microbial degradation of biphenyl. After growth on biphenyl, many bacteria can oxidize lowly chlorinated polychlorinated biphenyls (PCBs). PCBs are among the most widely identified organic contaminants in the environment and have been extensively researched. Therefore, many biphenyl degrading microorganisms have been identified in PCB studies. *Burkholderia xenovorans* LB400 and *Pseudomonas pseudoalcaligenes* KF707 are the two most extensively studied bacteria in biphenyl and PCB aerobic degradation. The metabolic pathway of biphenyl was reviewed by Pieper and Seeger (2008) (Fig 2-3).

The aerobic upper biphenyl pathway, i.e. the metabolism of biphenyl to benzoate and 2-hydroxypenta-2, 4-dienoate (Figure 2-3), was first proposed by Catelani et al. (1973). The gene and enzymes involved have been identified for *Pseudomonas sp*. LB400. Four enzymes, biphenyl-2, 3 dioxygenase (*Bph*A gene), 2, 3-dihydro-2, 3-dihydroxybiphenyl-2, 3 dehydrogenase (BphB), 2, 3-dihydroxybiphenyl-1, 2 dioxygenase (BphC) and 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate hydrolase (BphD) are involved in biphenyl degradation (Seeger et al. 1995). The benzoate and 2-hydroxypenta-2, 4-dienoate are further degraded to pyruvate through the benzoate and 4-chlorobiphenyl lower pathways before entering the TCA cycle.

Biphenyl is highly hydrophobic and poorly soluble in water. Biosurfactants containing both hydrophilic and hydrophobic groups may increase the availability of biphenyl in soil water. In the presence of a phytogenic surfactant, soya lecithin, the degradation of both biphenyl and PCBs was enhanced (Fava and Gioia 2001). Maltotriose esters might also increase solubility and thereby enhance the degradation of PCBs by *Burkholderia cepacia* LB400 (Ferrer et al. 2003).

2.3.3 Degradation of biphenyl by anaerobic microorganisms

Although the rate of anaerobic degradation is typically much slower than aerobic degradation, anaerobic biodegradation is likely to play a more important role than aerobic biodegradation *in situ*, since the redox potential in many subsurface environments is low. (Farhadian et al. 2008).

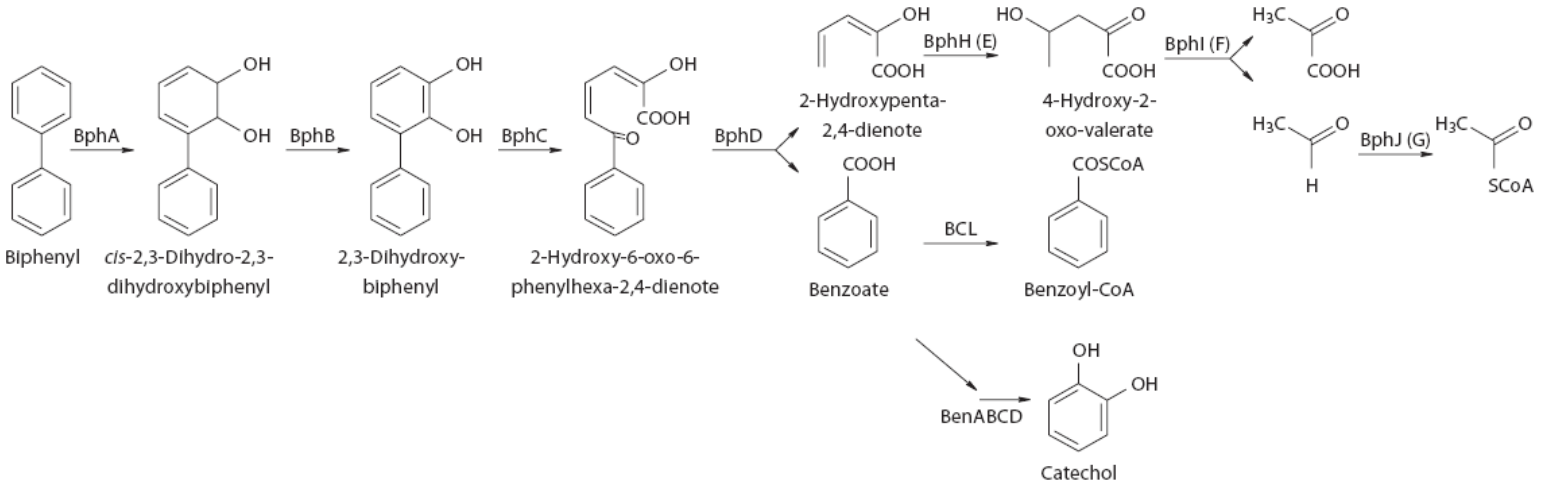

Figure 2-3 Aerobic biphenyl degradation pathway (Pieper and Seeger 2008)

BphA = Biphenyl 2,3-dioxygenase; BphB = cis -2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase; BphC = 2,3-dihydroxybiphenyl 1,2-dioxygenase; BphD = 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate (HOPDA) hydrolase; BphH(E) = 2-hydroxypenta-2,4-dienoate hydratase; BphI(F) =acylating acetaldehyde dehydrogenase; BphJ(G) = 4-hydroxy-2-oxovalerate aldolase; BCL = benzoate-CoA ligase; BenABCD = benzoate 1,2-dioxygenase and benzoate dihydrodiol dehydrogenase.

For some contaminants like tetrachloroethane and trinitrotoluene, the anaerobic process maybe the only solution to transform contaminants into harmless products (Holliger et al. 1997). Anaerobic PCB degradation has been extensively studied and normally leads to dechlorination but not ring opening. Biphenyl has been shown to be a product from the reductive dechlorination of PCB (Natarajan et al. 1996). Aerobic microorganisms can be used to degrade the low chlorinated PCBs resulting from the anaerobic process (Abramowicz 1994).

Biphenyl has been categorized as poorly anaerobically biodegraded by a quantitative structure-biodegradability relationship model (Yang et al. 2006). The anaerobic recalcitrance was probably associated with a lack of functional groups (Field 2002). So far very few studies have shown biphenyl degradation under anaerobic conditions (Table 2-3). Biphenyl was degraded under the conditions of nitrate reduction (Rockne and Strand 1998, 2001, Grishchenkov et al. 2002, Ambrosoli et al. 2005), sulfate reduction (Rockne and Strand 1998, 2001, Yang et al. 2008, Selesi and Meckenstock 2009), and methanogenesis (Sharak Genthner et al. 1997, Natarajan et al. 1999). In two studies, higher biphenyl degradation was seen when no TEA was added showing that biphenyl was degraded fermentatively (Ambrosoli et al. 2005, Yang et al. 2008). The anaerobic biphenyl degradation pathway is not very clear. Only one study showed that biphenyl-4-carboxylic acid was an intermediate under sulfate reducing conditions (Selesi and Meckenstock 2009). Adding acetate or glucose as a co-substrate might enhance biphenyl degradation but the effect was not significant when no nitrate was added (Ambrosoli et al. 2005). The effect of other nutrient factors on the biodegradation of biphenyl has not been previously reported.

Table 2-3 Anaerobic biphenyl degrading microorganisms

Inoculum	Culture condition	Reference
Creekbed sediment	Methanogenesis	Sharak Genthner et al. 1997
Paddy soils and river sediment	Sulfate-reducing & Fermentative	Yang et al. 2008
Sediment and enriched culture	Nitrate-reducing & Sulfate-reducing	Rockne and Strand 1998, 2001
Consortium ATCC 55616	Methanogenesis	Natarajan et al. 1999
Citrobacter freundii BS2211	Nitrate-reducing	Grishchenkov et al. 2002
Consortium	Nitrate-reducing Fermentative	Ambrosoli et al. 2005
Culture BiphS1	Sulfate-reducing	Selesi and Meckenstock 2009

2.4 Microbial community analysis

The ability to analyze the microbial community is fundamental to understanding the interaction between plants and microorganisms, and the role of microorganisms in the phytoremediation of organic contaminants. Since only 0.1 to 10% of bacteria can be cultivated using standard techniques, molecular biology techniques without the requirement for culturing have been applied often to analyze the microbial community structure (Torsvik et al. 1998, Theron and Cloete 2000). Spiegelman et al. (2005) surveyed the methodologies most frequently used for the identification and characterization of microbial consortia, and the advantages and disadvantages of various methodologies were assessed.

The combination of polymerase chain reaction (PCR) with denaturing gradient gel electrophoresis (DGGE) to analyze 16S rDNA is currently the most widely applied technology

for microbial community identification (Spiegelman et al. 2005). In DGGE analysis, a high GC content DNA fragment (GC clamp) is added to one end of the target 16S DNA during PCR. The amplified DNA is run on a gel with a denaturing agent concentration gradient from low to high under which double-stranded DNA denatures. Because the GC clamp is stable even at high denaturing concentration, the amplified DNA fragment will not totally separate and stops migrating where the 16S rDNA sequence is completely denatured. The DGGE technique is capable of separating DNA molecules with only one base pair difference (Myers et al. 1985). Since the separation is based on the melting property of DNA in a denaturing gel, and is not sequence specific, it is quite possible that DNA molecules with different sequences will have the same mobility and will not be separated on DGGE. Although Jackson et al. (2000) found that DGGE can always separate DNA differing by one base pair, DNA molecules with a difference of two base pairs can have identical migration. The DNA bands can be excised, extracted, and sequenced. By comparing the sequence information with publically available 16S rDNA databases, the microorganisms may be identified.

The extraction of DNA from environmental samples is the first and very important step for PCR-based microbial community analysis. The presence of bile salts, polysaccharides, heme, and humic acids in the soil samples can inhibit PCR reactions (Rådström et al. 2004), so DNA purification before PCR is often necessary.

2.4.1 Extraction of DNA from soil

The choice of DNA extraction method to use should be carefully considered with respect to recovery efficiency, obtaining a representative microbial population, and the purity and size of

the recovered DNA. DNA can be extracted directly from soil or from microorganisms that have been isolated from the soil matrix. Although DNA extracted from isolated microorganisms contains less soil organic materials, DNA extraction direct from soil normally yield greater quantities of DNA and the DNA from direct extraction is more representative of the soil microorganism (Roose-Amsaleg et al. 2001). Numerous protocols have been developed for the direct extraction of DNA from soil microbial communities and several commercial kits are also available for this purpose.

Different DNA extraction methods have been compared to obtain optimal soil DNA extraction and to quantitatively analyze the DGGE pattern. Yeates et al. (1998) compared bead beating, sonication, and enzymatic lysis methods. Bead beating caused mechanical disruption of the cells and was thought to result in effective lysis of all soil organisms (Yeates et al. 1998). Bead beating normally generates smaller-sized DNA than enzymatic methods. Bürgmann et al. (2001) investigated various factors on the DNA yield and fragment size in the bead beating protocol, and gave a guideline for optimizing DNA recovery. Kang and Mills (2006) investigated the effect of soil sample size on DNA extraction and microbial community structure identification. Soil samples between 0.1 and 1 g gave highly consistent DNA fingerprinting results. Since the DNA extraction efficiency may vary on a sample-by-sample basis, an external standard *Lambda* DNA (Mumy and Findlay 2004) and an internal standard (Petersen and Dahllöf 2005) can facilitate the determination of DNA recovery, and are important for the quantitative analysis of the microbial community.

2.4.2 DNA Purification

Co-extracted organic matter is the major source of inhibitors for PCR reactions. Humic material is derived originally from plant or microbial residues. Since humic acid has similar size and charge characteristics to DNA, it is the major contaminant in crude DNA. The presence of humic acid can be visualized by the brown colour of the DNA extract. The presence of humic-acid-like extracts inhibits DNA polymerase. A generic structure of humic acid is shown in Fig 2-4 (vanLoon and Duffy 2005).

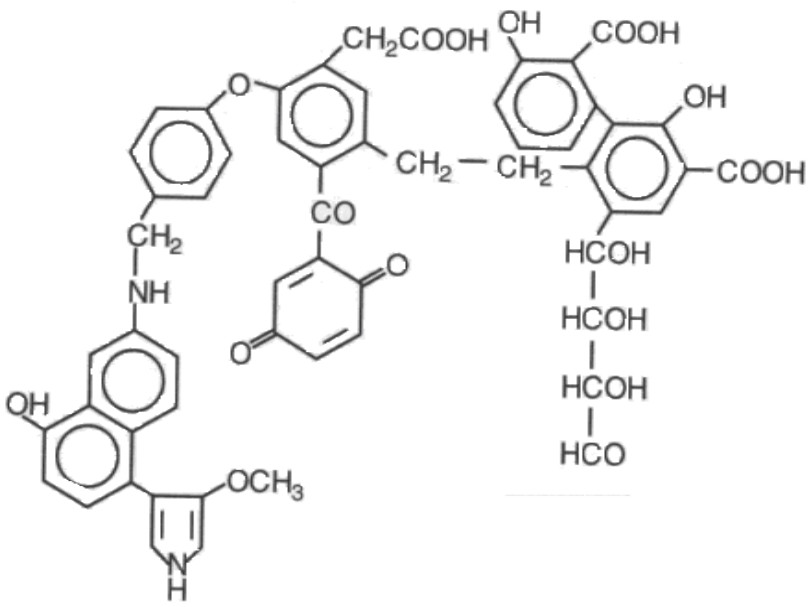

Figure 2-4 Representative chemical structure of soil humic acid (vanLoon and Duffy 2005)

Because of the similar physicochemical properties of humic acids to DNA, humic acids are difficult to separate from DNA. Several methods have been developed for DNA purification including cesium chloride (CsCl) density gradient ultracentrifugation, chromatography, electrophoresis, dialysis, and filtration (Roose-Amsaleg et al. 2001). Polyvinylpolypyrrolidone (PVPP) selectively binds to the phenol group in humic acids and has been successfully applied to a wide variety of soil types (Berthelet et al. 1996). PVPP spin columns was shown to have better purification efficiency than Sepharose B4 columns, and similar DNA purification efficiency and detection limit as the commercially available UltraClean kit (Mo Bio) (Trochimchuk et al. 2003).

2.4.3 Target DNA molecules for microbial community identification

In PCR amplification, the target genes have to be essential to cell function, be ubiquitous in all microbial populations, and possess conserved sequences for PCR primer design and have variable sequences to differentiate members of the microbial population. The 16S rRNA is the most commonly used target for microbial community analysis. Other target DNA sequences such as the gene spacer between those for 16S and 23S, chaperonin 60 (cpn60), the RNA polymerase ß-subunit (rpoB), and the DNA gyrase B protein (gyrB) coding for topoisomerase II (Spiegelman et al. 2005) have also been used.

2.5 Analysis of the microbial population in enriched culture

Culture-based detection methods like plating on agar are labour intensive, time consuming and might not work well with an enriched culture if two microorganisms have a similar cellular

morphology and Gram stain. Molecular based methods described in section 2-2 can be used for microbial population analysis, but they are designed for environmental samples containing many PCR inhibitors. In an enriched culture, the concentration of PCR inhibitors might be low enough for a more efficient analysis without DNA preparation prior to PCR.

PCR assays without DNA preparation, is known as Direct PCR (DPCR) or colony PCR. Since DPCR avoids the time-consuming and tedious DNA purification step, monitoring becomes faster, less costly and simpler. A smaller sample size may also be used since DNA loss during the extraction step is avoided. DPCR has been used to effectively amplify DNA contained in whole cells of blood samples (Mercier et al. 1990), solid murine liver tissue (Panaccio et al. 1993), fruitflies and blood flukes (Grevelding et al. 1996), bacteria (Fode-Vaughan et al. 2001, Fode-Vaughan et al. 2003, Benson et al. 2004, Klocke and Mundt 2004, Layton et al. 2006), and plant tissue (Yang et al. 2007). It is possible to use DPCR and DGGE for microbial community analysis in the enriched cultures.

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Chapter 3
Biodegradation of 1, 4-dioxane by a Flavobacterium

A manuscript of this chapter is in preparation for submission to Biodegradation (Springer, Netherlands)

3.1 Abstract

A dioxane-degrading consortium was enriched from soil obtained from a contaminated groundwater plume. The enriched consortium did not use dioxane as the sole source of carbon and energy but co-metabolized dioxane in the presence of tetrahydrofuran (THF). THF and dioxane concentrations up to 1000 ppm were degraded by the enriched consortium in about two weeks with a longer lag phase observable at 1000 ppm. Three isolates from the enriched consortium were then obtained on agar plates containing basal salts and glucose as the carbon source. Only one of the three isolates was capable of dioxane degradation. Further enrichment of this isolate in liquid media led to a pure culture that grew on glucose and co-metabolically degraded dioxane after THF degradation. The rate and extent of dioxane degradation of this isolate increased with increasing THF concentration. This isolate was subsequently identified as a *Flavobacterium* by 16S rDNA sequencing. Using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) analysis of microbial populations, *Flavobacterium* was determined to be the dominant species in the enriched consortium and was distinct from the two other isolates that did not degrade dioxane. This is the first report of a dioxane-degrading *Flavobacterium* which is phylogenetically distinct from any previously identified dioxane degrader.

3.2 Introduction

The cyclic ether 1,4-dioxane (dioxane) is used primarily as a stabilizer of chlorinated solvents and is considered a potential human carcinogen. In the past three decades, approximately 0.5 to 1.0 million pounds of dioxane were released in the United States each year (Toxic Release Inventory, 1988 - 2005), and it has been detected in groundwater samples (Zenker et al. 2000). The guidelines for maximum contaminant level (MCL) in drinking water ranged from 3 parts per billion (ppb) in California to 85 ppb in Michigan (Duncan et al. 2004). Dioxane is recalcitrant, chemically stable, miscible with water, and has a low Henry's Law constant. Such properties make natural attenuation of dioxane in contaminated groundwater insignificant (Zenker et al. 2004, Mahendra and Alvarez-Cohen, 2006) and physical separation difficult (Zenker et al. 2004).

Various physical and chemical methods have been evaluated to treat dioxane-contaminated waters. A combination of hydrogen peroxide and ferrous ion, or chlorine oxidation (NaOCl at 75 °C), can effectively oxidize dioxane (Klečka and Gonsior 1986). Beckett and Hua (2003) found that ultrasonic decomposition, at 358 kHz, removed 95% of the dioxane in 50 minutes with the addition of ferrous iron. Advanced oxidation processes (AOPs) using hydrogen peroxide, ozone, and UV photo-oxidation are proven technologies for dioxane treatment (Zenker et al. 2003), but the operating costs of these processes are normally high with additional costs incurred when used in combination with a "pump-and-treat" approach (Manendra and Alvarez-Cohen 2006).

Phytoremediation is also being assessed as another option for treating dioxane in groundwater. Hybrid poplar trees have been used to remediate dioxane in hydroponic and soil experiments (Aitchison et al. 2000). When transpired to air, the half-life of dioxane was estimated to be 6.7-9.6 h as a result of photo-degradation (OPPT Chemical Fact Sheets-1,4-dioxane 1995). Since

dioxane was not used by the trees as a growth substrate, transpiration is considered passive and nonspecific. However, the possibility of whether dioxane can be biodegraded in the tree rhizosphere remains an interesting question. Kelley et al. (2001) found that the combination of a dioxane degrader, *Amycolata sp*. CB1190, and hybrid poplar trees enhanced dioxane removal in soil. Poplar root extracts stimulated dioxane degradation, probably by increasing the growth and activity of *Amycolata sp*. CB1190.

Biodegradation of dioxane using microorganisms is an attractive remediation approach because dioxane can be mineralized if used as the sole source of carbon and energy (Parales et al. 1994) or as a co-metabolite (Zenker et al. 2000). However, dioxane has been reported to be recalcitrant to biodegradation. The inability to biodegrade is probably because it has a stable ether bond which requires 360 kJ/mol of energy for cleavage (Zenker et al. 2004). To date, only a few aerobic microorganisms have been found to exhibit dioxane-degrading capabilities. *Rhodococcus ruber* 219 (Bernhardt and Diekmann 1991, Bock et al. 1996), *Actinomycete* CB1190 (later identified as *Pseudonocardia dioxaneivorans* CB1190) (Parales et al. 1994, Mahendra and Alvarez-Cohen 2005) and a fungus, *Cordyceps sinensis* (Nakamiya et al. 2005) have been identified as micro-organisms capable of utilizing dioxane as a sole source of carbon and energy. *Mycobacterium vaccae* JOB 5 (Burback and Perry 1993), *Pseudonocardia* K1 (Kohlweyer et al. 2000), *Pseudonocardia sp*. ENV478 (Vainberg et al. 2006), and a mixed culture (Zenker et al. 2000) have been identified as microorganisms that degraded dioxane but were unable to grow on it. Treatment with trickling filters (Zenker et al. 2004) resulted in dioxane concentrations as low as 2 to 10 ppb. Such concentrations are often below the regulated levels allowed in drinking water.

CHAPTER 3. BIODEGRADATION OF 1, 4-DIOXANE BY A FLAVOBACTERIUM

Tetrahydrofuran (THF), a structural analog of dioxane, has been used to enrich dioxane degraders (Parales et al. 1994, Zenker et al. 2000, Vainberg et al. 2006). *R. ruber* 219 grew faster on THF than on dioxane, and *P. dioxaneivorans* CB1190 was thought to be a THF-degrading mutant capable of using dioxane as the sole source of carbon (Parales et al. 1994). The kinetics of the co-metabolic degradation of dioxane in the presence of THF by a mixed culture has also been studied (Zenker et al. 2002). However, since THF is also a significant environmental contaminant, its use should be minimized. Interestingly, there were reports of micro-organisms that did not require the presence of THF or other structural analogues to degrade dioxane. *Mycobacterium vaccae* JOB 5 (Burback and Perry 1993) enriched from propane, the propanotroph SL-D (Fam et al. 2005), and *Methylosinus trichosporium* OB3b (Mahendra and Alvarez-Cohen 2006) all degraded dioxane without using another dioxane-like carbon source. Mahendra and Alvarez-Cohen (2006) showed that methane monooxygenase could be a key enzyme in dioxane degradation. However, methane and propane are both of low water solubility, flammable gases, and growth would be low because of mass transfer problems.

The objective of this study was to isolate microorganism(s) from dioxane-contaminated soil capable of degrading dioxane as the sole source of carbon or co-metabolically in the presence of THF. Since the isolate obtained was co-metabolic in terms of dioxane degradation, a strategy to minimize THF usage in culture preparation and dioxane degradation was examined. The identity of this dioxane-degrading microorganism was revealed by denaturing gradient gel electrophoresis (DGGE) and 16S rDNA sequencing.

3.3 Materials and methods

3.3.1 Microcosm growth medium

Basal salts medium (BSM, modified from Cote and Gherna 1994) was modified and used in the enrichment and culturing experiments. Each liter of media contained 100 mL BSM stock solution and 100 mL trace element solution. BSM stock solution contained per liter: 32.4 g K_2HPO_4, 10 g NaH_2PO_4 H_2O, and 20 g NH_4Cl. The trace element solution contained per liter: 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4$ $7H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4$ $7H_2O$, 0.1 g $CoCl_2$ $6H_2O$, 0.132 g $CaCl_2$ $2H_2O$, 0.1 g $ZnSO_4$ $7H_2O$, 8.74 mg $CuSO_4$ $5H_2O$, 10.0 mg $AlK(SO_4)_2$ $12H_2O$, 10.0 mg H_3BO_3, 10 mg Na_2MoO_4 $2H_2O$, 27.1 mg $NiCl_2$ $6H_2O$, and 20.0 mg Na_2WO_4 $2H_2O$.

3.3.2 Enrichment and culturing conditions

The enrichment scheme for the reported dioxane degrader is presented in Fig 3-1 along with an outline of the experiments performed. To obtain an enriched consortium, soil from a groundwater plume contaminated with dioxane was added to 125 mL serum bottles containing 100 mL BSM and incubated at 25 $\pm$ 1 ^{o} C and 200 rpm on an Innova rotary shaker. THF or dioxane (80 to 320 ppm (v/v)) was added as the sole source of carbon and energy. After degradation was detected, enrichments were serially transferred to fresh BSM medium containing 100 ppm each of THF and dioxane. The enriched consortium was propagated under similar conditions in Erlenmeyer flasks with 50 mL BSM and capped with a rubber stopper in all experiments employing THF or dioxane.

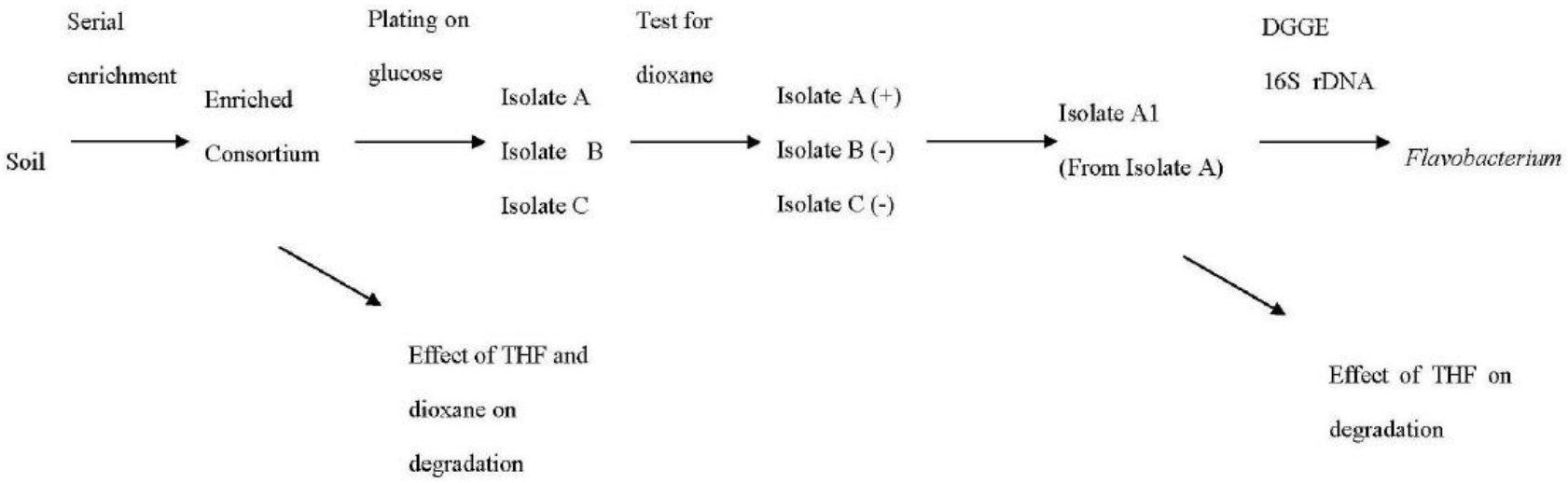

Figure 3-1 Enrichment and experimental outline for the dioxane-degrading isolate

3.3.3 Dioxane degradation with THF by the enriched consortium

To investigate if dioxane could be used as a sole source of carbon and energy by the enriched consortium culture, 5 mL of the enriched consortium was added to 45 mL BSM with 100 ppm dioxane with or without 100 ppm THF. The effect of substrate concentration was determined by adding 5 mL of enriched mixed culture to 45 mL BSM containing THF and dioxane, each at 100, 300, and 1000 ppm. Abiotic controls were identical to the biotic microcosms except without inocula.

3.3.4 Obtaining isolates from the enriched consortium culture and dioxane degradation

The enriched consortium culture was streaked on BSM agar plates containing THF (300 ppm), dioxane (300 ppm), or glucose (10g/L) as the sole source of carbon and energy. Three morphologically distinct colonies (designated Isolates A, B and C) found growing on the glucose plates were propagated separately for further studies. Each isolate was cultured in BSM containing 10 g/L glucose. After centrifugation at 7663 × *g* for 5 min in a Sorvall RC-5B centrifuge (Dupont), the resulting biomass was washed twice with sterile BSM to eliminate residual glucose, re-suspended in sterile BSM, and then used to inoculate BSM containing 100

ppm each of THF and dioxane. A dioxane-degrading culture from Isolate A was maintained on BSM-glucose (10 g/L) and designated Isolate A1.

3.3.5 Effect of THF concentration on dioxane degradation by Isolate A1

For these studies, the Isolate A1 (enriched from Isolate A) was grown in 2 × 50 mL BSM-glucose (10 g/L) for two days, collected by centrifugation, and washed as described above. The resulting pellet was resuspended in a final volume of 10 mL BSM. One millilitre of this final suspension was then added to BSM containing 100 ppm dioxane and different THF concentrations between 10 to 100 ppm to test the effects of THF concentration on dioxane degradation.

3.3.6 Microbial population analysis

The enriched dioxane-degrading consortium, the three glucose isolates, and two replicates of the Isolate A1 were used for the microbial population analysis. One millilitre of each type of culture was centrifuged at 8000 × *g* for 2 min in a bench top centrifuge (Eppendorf 5415C), washed twice with 0.5 mL deionized water, and resuspended in 50 μL deionized water for molecular characterization using polymerase chain reaction and denaturing gradient gel

electrophoresis (PCR-DGGE) (Fortin et al. 2004). Concentrated cells (1μL) were used as sources of PCR templates. The 16S rDNA fragments were amplified using a T1 thermocycler (Biometra, Goettingen, Germany), with an initial 10 cycles of 1 min denaturation at 94°C, 1 min annealing at 65°C stepping down 1 degree each cycle to 55°C, and 3 min extension at 72°C followed by 20 cycles of 1 min at 94°C, 1 min at 55 °C, and 3 min at 72°C. Each 50 μL PCR reaction contained 1 μL of concentrated cells, 8 μL of 1.25 mM dNTPs, 1 μL of each primer U341-GC2 and U758 (25 pmol) (Fortin et al. 2004), 4 μL 25 mM $MgCl_2$, 5 μL of 10X reaction buffer and 2.5 units of Taq polymerase (Fisher Scientific Ltd., Ottawa, Ontario). The amplified DNAs were separated using 8% (w/v), 0-80% denaturing gradient acrylamide gels in 1X TAE buffer. The gels were run using 80 V for 16 h at 60°C and stained for 10 min in 1X TAE containing SYBR Gold (Invitrogen, Burlington, Ontario).

For identification work, the 16S rDNA bands were excised and eluted with water at 4℃ overnight, precipitated with ethanol, re-amplified for 25 cycles of 1 min at 94℃, 1 min at 55 ℃ and 1 min at 72 ℃ using the U341 and 758 primers, and sequenced (Cortec, Kingston, Ontario). The 16S rDNA sequences were matched in GenBank using BLAST (Altschul et al. 1997) and the best matched sequences and those of previously reported dioxane or THF degraders between the U341 and U758 were retrieved. Phylogenetic and distance analysis were performed with MEGA

(4.0.2) and multiple sequence alignment was done with ClustalW which is integrated in MEGA (4.0.2) (Tamura et al. 2007). Bootstrap analysis was based on 1,000 replications.

3.3.7 Analytical methods

Dioxane and THF analysis

Various liquid culture samples were filtered through a 0.45 μm nitrocellulose filter (Millipore) and 1.5 μL of the filtrate with 200 ppm (v/v) 2-pentanol as the internal standard was injected into a Varian 3400 gas chromatograph equipped with a flame ionization detector and a 5 m guard column attached to a 30 m x 0.53 mm column (Restek stabilwax). The flow rates of helium, hydrogen and air were 4, 50, and 300 mL/min, respectively. The injector and detector temperature were held constant at 150 and 180°C, respectively, and the oven temperature profile was a 4 min hold at 60°C, followed by a ramp of 20°C/min ending at 150°C with a 1 min hold time.

Monooxygenase activity measurements

The monooxygenase activity of Isolate A1 was determined in triplicate using a colorimetric assay (Brusseau et al. 1990) which quantified naphthol formation from naphthalene using 1% o-dianisidine tetrazotized dye and glacial acetic acid at an absorbance of 530 nm and related to a standard curve (0-48.6 $\mu M\ l^{-1}$ 1-naphthol).

3.4 Results

3.4.1 Enrichment of a dioxane-degrading consortium culture

To enrich for dioxane-degrading microorganisms from soil contaminated with dioxane, nine microcosms were set up in BSM containing either dioxane or THF as the sole source of carbon and energy. After nine months, dioxane degradation was not observed in any of the microcosms, and only one showed THF disappearance. The THF-degrading microcosm was serially transferred to BSM containing 100 ppm THF and 100 ppm dioxane until a mixed culture with dioxane and THF degradation capability was obtained. A typical dioxane and THF degradation curve is shown in Fig 3-2. THF was completely degraded in about 5 days, and dioxane was degraded only when THF concentration reached a low level.

3.4.2 Co-metabolic degradation of dioxane by the enriched consortium

After the enriched consortium was obtained, the ability to degrade dioxane as the sole source of carbon and energy was tested. There was no significant loss of dioxane in the abiotic controls, and without THF, dioxane degradation was not observed in a three-week test period (Fig 3-3). However, in biotic experiments supplemented with THF, dioxane degradation occurred after THF

was degraded to low concentrations, with about 70% of the dioxane disappearing after 22 days (Fig 3-3).

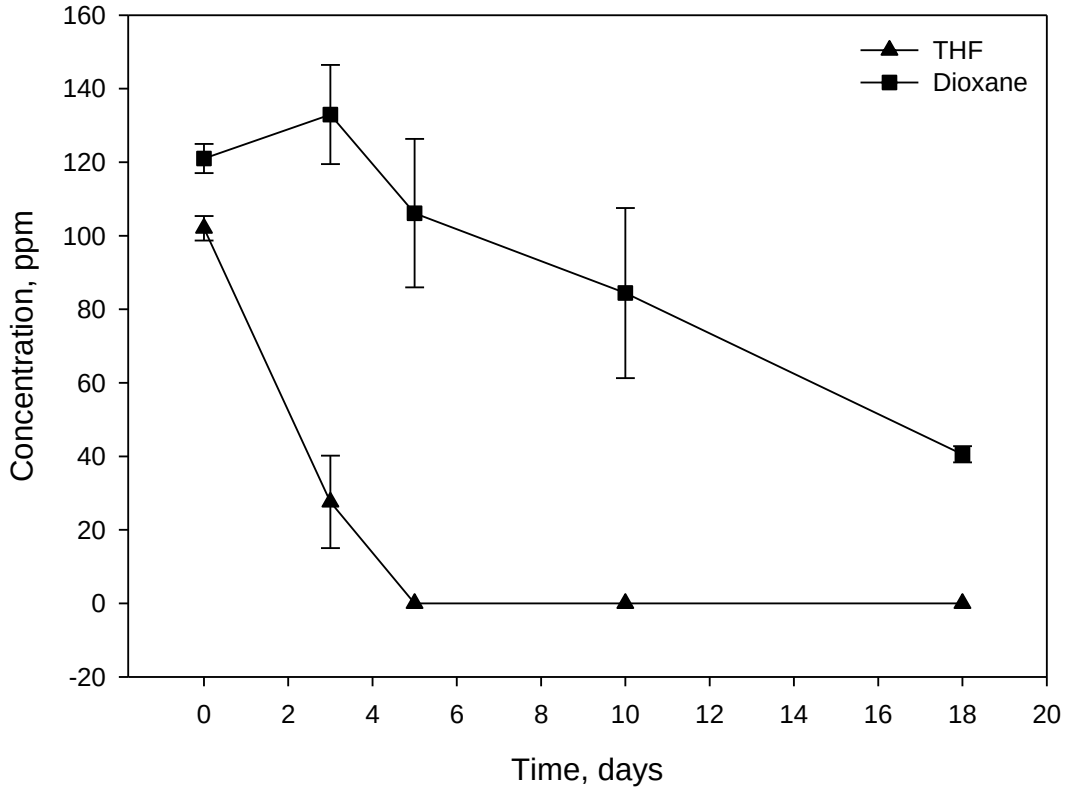

Figure 3-2 Dioxane (■) and THF (▲) degradation by the enriched consortium. The error bars represent the standard deviation of three independent replicates.

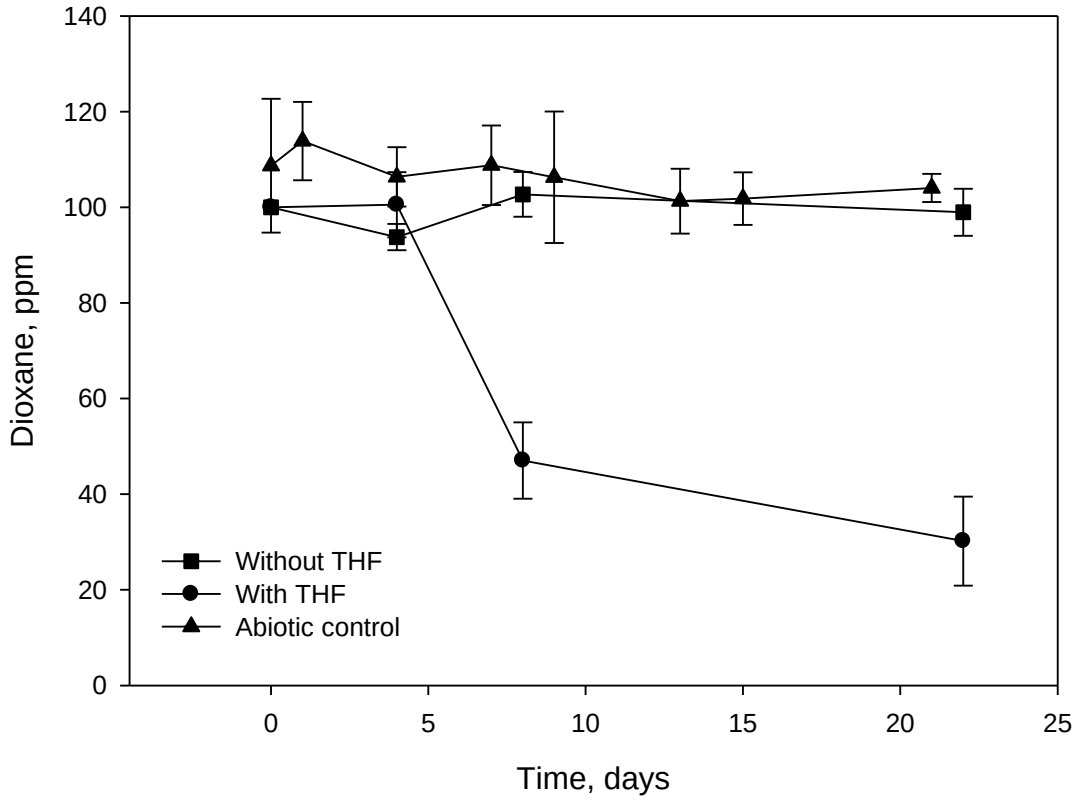

Figure 3-3 Dioxane degradation by the enriched consortium. Dioxane degradation was evaluated with (●) or without (■) THF and compared to the abiotic control (▲) with THF. Error bars represent standard deviation of three independent replicates.

3.4.3 Effect of THF and dioxane concentrations on the enriched consortium

To test whether THF and dioxane can be degraded at higher concentration than that in the enrichment experiments, the enriched consortium was grown on BSM containing THF and

dioxane each supplied at initial concentrations of 100, 300, and 1000 ppm. Dioxane and THF were degraded at all concentrations tested (Fig 3-4). The extent of dioxane degradation at all concentrations was similar with 90% dioxane degraded in about two weeks.

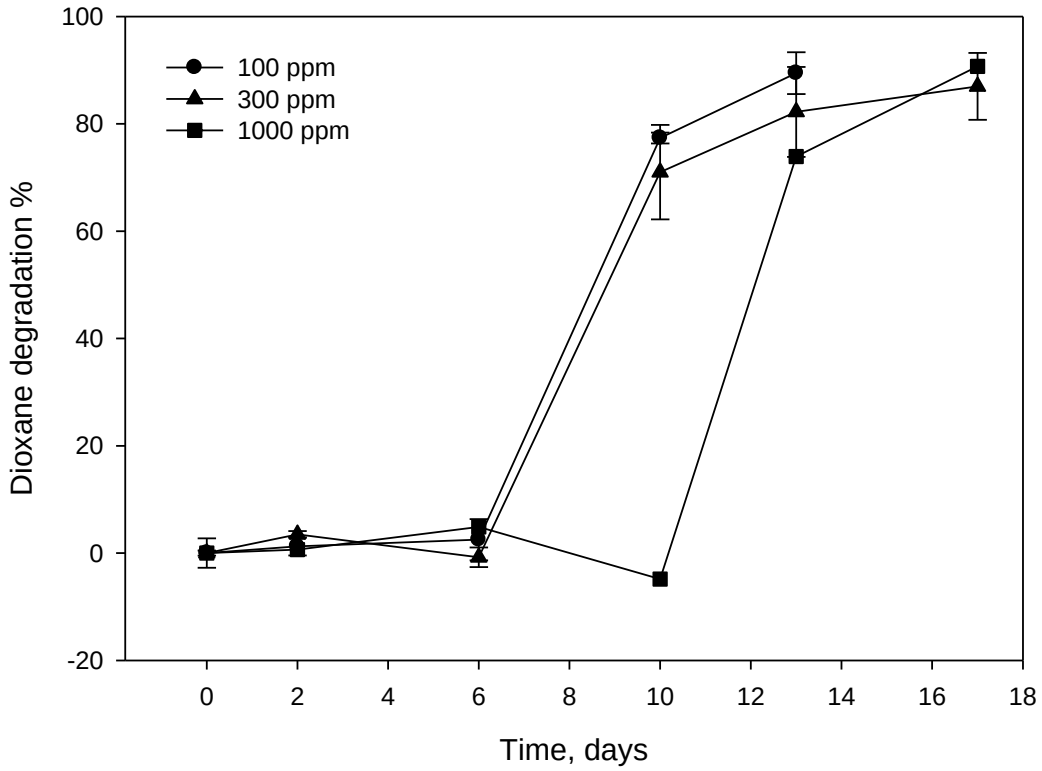

Figure 3-4 Dioxane degradation at different initial concentrations. Both THF and dioxane were each initially at 100 (●), 300 (▲), or 1000 (■) ppm. For 100 and 300 ppm, each point represents the average of two replicates, and the error bars represent the range of values obtained.

During the degradation phase, the maximum dioxane degradation rate increased with increasing initial dioxane concentration. These rates were calculated to be 12.8, 35.6, and 64.3 ppm per day at 100, 300, and 1000 ppm of initial dioxane concentration respectively. The lag phase at 1000 ppm seemed to be longer when compared to an initial concentration of 100 and 300 ppm indicating that a high concentration of THF and dioxane might inhibit the growth of microorganisms. A similar result was reported for *Pseudonocardia sp.* strain K1 (Kohlweyer et al. 2000).

3.4.4 Preparation and subsequent dioxane degradation assessment of isolates

To obtain isolates of the dioxane degraders, the enriched mixed culture was plated on BSM agar containing either dioxane, THF, or glucose as the sole source of carbon and energy. There was no observable growth on dioxane. Growth on THF was slow with colonies too small to handle. On the glucose plates, there were three distinct colony morphologies observed (designated Isolates A, B and C). Isolate A had light brown colonies which were smaller than Isolates B and C. All three were grown separately in BSM liquid medium containing 10 g/L glucose, centrifuged, washed with BSM, and then tested for dioxane-degrading capabilities. None of the three isolates degraded dioxane alone. In the presence of THF, only Isolate A degraded dioxane which decreased to below the detection limit (5 ppm) in about 20 days (Fig 3-

5). Isolate A also grew faster on glucose than on THF and dioxane and achieved a higher cell density. When grown on glucose, it appeared as a homogeneous suspension, but formed white clumps when grown on THF and dioxane. Similar observations were reported for *Pseudonorcardia* sp. strain ENV478 (Vainberg et al. 2006).

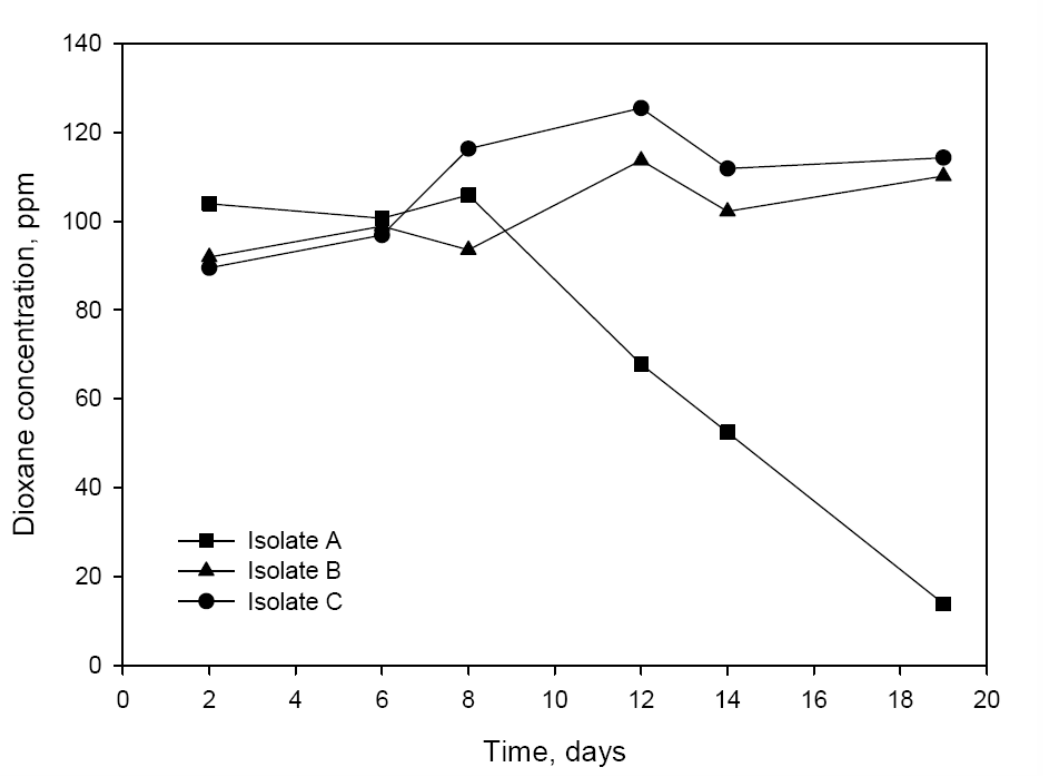

Figure 3-5 Dioxane degradation of three isolates obtained from the dioxane-degrading consortium on BSM-glucose agar plates. The three different colonies named Isolates A (■), B (▲), and C (●), were grown on BSM with 10 g/L glucose, and then transferred to fresh BSM containing about 100 ppm THF and 100 ppm dioxane.

After growing on THF and dioxane, Isolate A (2 mL) was transferred back to BSM-glucose (10 g/L) and maintained as a dioxane-degrading culture. The dioxane-degrading capability was re-tested in fresh BSM containing 100 ppm dioxane with or without THF. Again, this culture did not use dioxane as the sole source of carbon and energy, but co-metabolically degraded dioxane with THF. However, in a separate experiment in which the culture was pregrown on THF alone, once THF was exhausted and an aliquot of dioxane was added, 45% dioxane was degraded in 6 days but no more dioxane was degraded after. Dioxane degradation was very stable after 5 transfers between glucose and THF/dioxane and this enriched culture was named Isolate A1. It had no detectable naphthalene monooxygenase activity.

3.4.5 Microbial population analysis

The enriched consortium, Isolates A, B, and C from glucose plates, and two replicates of the Isolate A1 which was maintained in BSM-glucose medium were each analyzed using PCR and DGGE (Fig 3-6). Four major bands were seen in the enriched consortium (lane 5). The bands from Isolates B and C (lanes 4 and 6, respectively) were not major bands in the enriched consortium (lane 5).

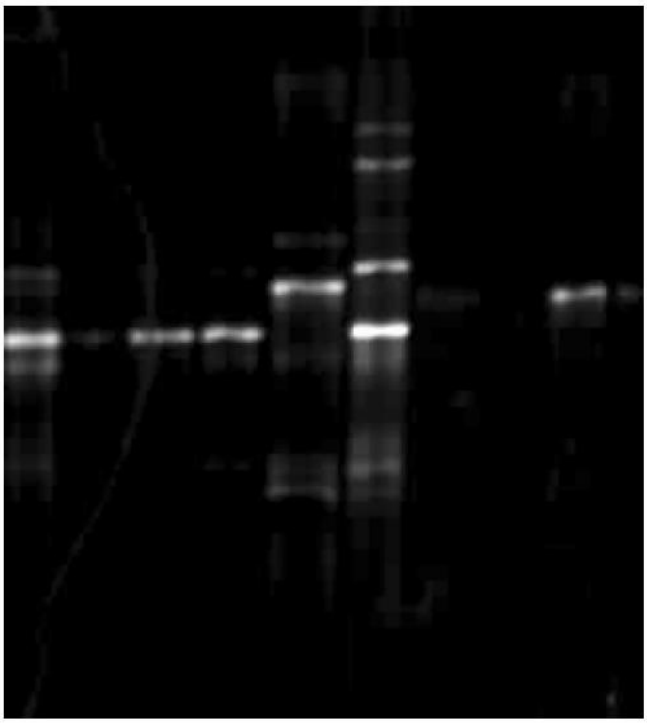

Figure 3-6 DGGE analysis of the microbial populations. The 16S rDNA of the enriched consortium, Isolates A, B, and C from agar plates, and two replicates of the pure dioxane-degrading culture (Isolate A1) were amplified using PCR and analyzed on DGGE. Lane 1: Isolate A, Lanes 2 and 3: Isolate A1, Lane 4: Isolate B, Lane 5: the enriched consortium, and Lane 6: Isolate C. Unmarked lanes were not loaded.

Neither of these isolates, B or C, degraded dioxane (Fig 3-5) and they were probably minor components of the enriched consortium but proliferated well on glucose. However, Isolate A (lane 1) degraded dioxane (Fig 3-5) and had a major band that matched the dominant band of the enriched consortium (compare lanes 1 and 5 in Fig 3-6). When an attempt was made to obtain a

pure culture from Isolate A by transferring it several times in dioxane and THF in BSM, Isolate A1was obtained. This had a single band which was clearly the dominant organism in the enriched culture (compare lanes 2 and 3 with 5). The two smaller bands were probably lost because those organisms did not use THF or dioxane as a source of carbon and energy. The results in Fig 3-6 were obtained when whole cells were used as the PCR template and had the same outcome as when extracted DNA was used (data not shown).

3.4.6 Identification of the dioxane degrader

The major 16S rDNA PCR product of the dioxane-degrading microorganism was excised from DGGE gels, eluted, re-amplified, and sequenced. It exhibited 99% homology with *Flavobacterium sp*. IP10, and *cf. Cytophaga sp.* MDA2507 using NCBI BLAST. *Flavobacterium* is the type genus in the family *Flavobacteriaceae* dealing with *Flavobacterium* and *Cytophage*-like bacteria (Bernardet and Bowman 2006) and its classification was described by Bernardet et al. (2002). Since *cf. Cytophaga sp.* is unclassified (NCBI taxonomy database) and has a high 16S rDNA similarity to *Flavobacterium*, the enriched dioxane degrader can be tentatively classified as a *Flavobacterium*. Further phylogenetic analysis showed that the dioxane degrader in this study is distinct from all previously reported dioxane and THF degraders (Fig 3-7).

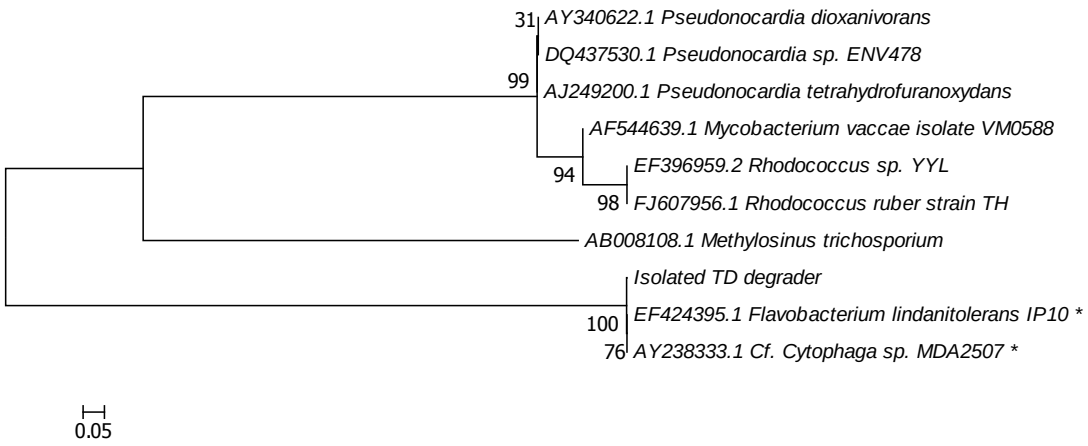

Figure 3-7 Dendrogram based on the polygenetic analysis of 16S rDNA. The result showed the relationship of the isolated dioxane degrader in this paper with previously reported dioxane and THF degraders. Since the 16S sequence of *Rhodococcus ruber* 219 and *Mycobacterium vaccae* JOB 5 are not available in GenBank, *Rhodococcus ruber* strain TH and *Mycobacterium vaccae* isolate VM0588 were used in the phylogenetic analysis. *Flavobacterium lindanitolerans* strain IP10 and Cf. *Cytophaga* sp. MDA2507 are not reported dioxane or THD degraders but were used as representative of the best BLAST hit in GenBank. The bootstrap values were based on 1000 resamplings.

3.4.7 Effect of THF concentration on dioxane degradation by the purified dioxane-degrader

To minimize using THF as a primary carbon source, an inoculum of the dioxane-degrading *Flavobacterium* was prepared on glucose and used to evaluate the effect of THF concentration on dioxane degradation. The rate and extent of dioxane degradation increased with increasing THF concentration (Fig 3-8).

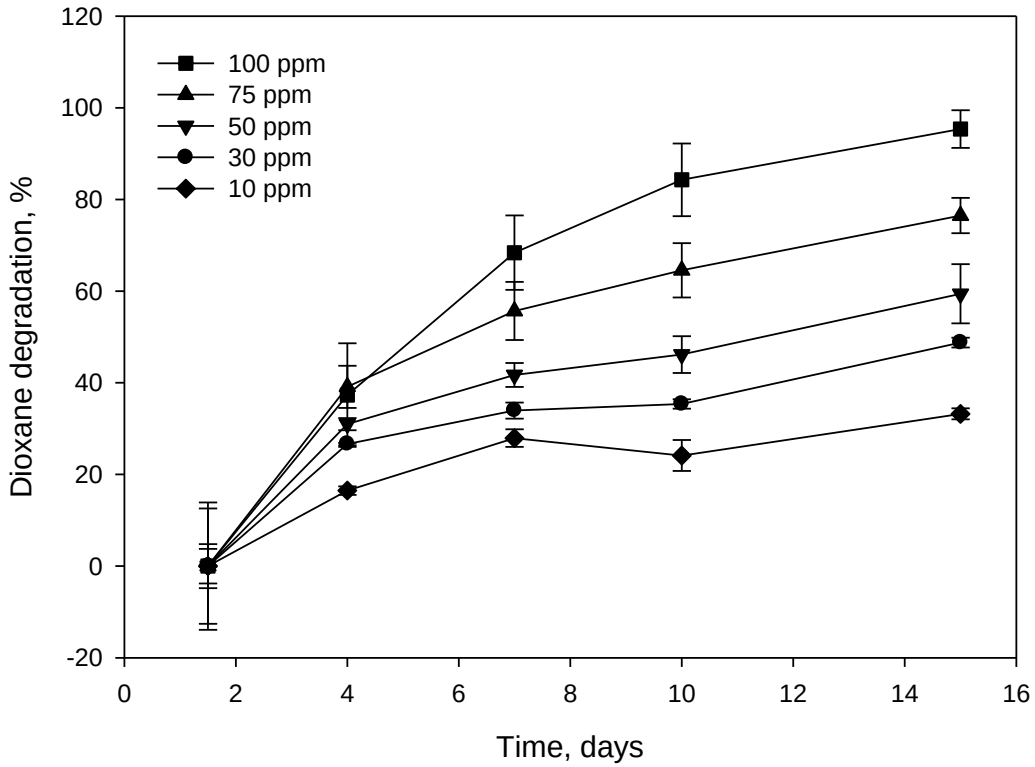

Figure 3-8 Effect of THF concentration on dioxane degradation. The Isolate A1 (a purified dioxane degrader from Isolate A) was cultured in BSM medium with 10 g/L glucose and then inoculated into fresh BSM medium containing 100 ppm dioxane and 10 (♦), 30 (●), 50 (▼), 75 (▲), or 100 (■) ppm of THF. Each point represents the average of two replicate cultures and the error bars represent the range of values obtained.

3.5 Discussion

Bioremediation is an attractive method to remove dioxane from the environment. However, few microorganisms have been reported to degrade it. In this study, a *Flavobacterium* was enriched from dioxane-contaminated soil. The enriched culture did not use dioxane as the sole source of carbon and energy, but co-metabolically degraded it in the presence of THF. To the authors' knowledge, biodegradation of dioxane by *Flavobacterium* has not been reported to date. *Flavobacterium* is taxonomically distinct from previously identified dioxane-degrading bacteria. *Pseudonocardia dioxanivorans* CB 1190, *Pseudonocardia* K1, and *Pseudonocardia sp*.ENV 478, are in the genus *Pseudonocardia*, and *Mycobacterium vaccae* JOB 5, and *Rhodococcus rubber* 219 are in the Suborder of *Corynebacterineae*, and are all in the Order of *Actinomycetales*. *Flavobacterium* is in a different Phylum. This distinction has also been substantiated by phylogenetic and distance analyses (Fig 3-7) (Yao et al. 2009).

Mahendra and Alvarez-Cohen (2006) have shown that microorganisms which express monooxygenase can degrade dioxane, and such a degradation pathway has been proposed by Vainberg et al. (2006). Based on bioinformatic approaches and the NCBI protein database, monooxygenases exist in *Flavobacterium* species. Although this information indicates that an oxygenase may be involved in dioxane degradation in *Flavobacterium*, naphthalene

monooxygenase activity was not detected in our culture using the method of Brusseau et al. (1990). Therefore, a different degradation mechanism may be utilized in *Flavobacterium*.

Plating microorganisms on nutrient rich agar is a standard technique to obtain or isolate contaminant degraders from environmental sources. However, in a similar dioxane enrichment experiment, attempts to isolate dioxane degraders on tryptic soy agar plates were unsuccessful (Zenker et al. 2000). The authors postulated a number of reasons for such a negative outcome: 1) their culture might have lost its degrading ability upon growth on rich media; or 2) the colonies were too small to be observed or handled because degraders grow slowly on nutrient plates; or 3) the syntrophic association with other microorganisms did not allow the degrader to form colonies. These difficulties were also observed early in our study. We were unable to isolate dioxane degraders when we initially transferred the enriched culture onto nutrient rich agar plates. To assess why it may be challenging to isolate dioxane degraders on nutrient plates, PCR and DGGE were used to analyze the enriched consortium and the isolates from the enrichment process. Isolates B and C did not degrade dioxane because they were different from the dioxane-degrading microorganism. Although *Flavobacterium* was the dominant organism in the enriched consortium, the non-dioxane degraders (Isolates B and C) grew more quickly on the glucose-supplemented agar plates than Isolate A (which was predominantly *Flavobacterium*), indicating that the major problem underlying the recovery of the dioxane degrader on nutrient rich agar was its slower

growth on glucose. This might be a common problem in the isolation of an axenic culture or a stable consortium with degradation capabilities since contaminant degraders are usually enriched from groundwater, soil or sludge, from a diverse range of microbes. The additional molecular techniques employed in this study proved to be useful assessment tools that complemented the traditional microbial isolation techniques used for establishing pure cultures of contaminant degraders. Whole cells as the PCR template has been previously successful with a pure culture (Fode-Vaughan et al. 2003) or when diversity is quite limited (Klocke and Mundt 2004) as in this case.

To biodegrade dioxane at industrial-scale levels, it would be crucial to possess the ability to prepare the degrading cultures in large quantities. Most dioxane-degrading cultures use either dioxane or THF as the source of carbon and energy. Since both chemicals are toxic and microbial growth rates in dioxane/THF are usually slow, culture maintenance and efficient production of large numbers of cells become a challenge. Maintaining a dioxane degrader on a carbon source structurally different from dioxane and keeping the ability to degrade dioxane was previously unsuccessful (Parales et al. 1994, Zenker et al. 2000). While the carbon sources of the propanotroph SL-D (Fam et al. 2005), and *M. trichosporium* OB3b (Mahendra and Alvarez-Cohen 2006) are less toxic, both propane and methane are flammable and must be treated with caution. The low solubility of propane and methane will result in mass transfer limitation and

lead to a low cell density. The *Flavobacterium* reported in this study was able to grow on glucose, and have a stable dioxane degradation capability after several transfers between glucose and THF/dioxane. The ability to grow on glucose without losing dioxane-degrading capabilities is highly advantageous. The use of glucose is more cost-effective and safer than THF or dioxane and is thus more beneficial in large-scale industrial applications. The *Flavobacterium* grows more quickly on glucose than on THF and dioxane, allowing faster production of high density cell cultures suitable for industrial-scale applications.

Since *Flavobacterium* can be grown and maintained on glucose without losing its dioxane-degrading capability, a bioremediation strategy can be developed for industrial-scale applications. Cultures can be grown first on glucose to obtain high cell densities before adding THF to stimulate dioxane degradation. After THF is totally degraded, the activated *Flavobacterium* can then be used in a dioxane bioremediation process such as for *ex situ* treatment of dioxane-contaminated groundwater in a bioreactor or for an *in situ* bioaugmentation treatment if a structural analog of dioxane is available in the groundwater.

3.6 Acknowledgements

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Chapter 4

Anaerobic biphenyl degradation by rhizosphere microorganisms

4.1 Abstract

Microorganisms present in the rhizosphere soil of poplar and willow trees from a phytoremediation test-plot were capable of degrading biphenyl in the presence of the terminal electron acceptors (TEAs) nitrate, sulfate or carbon dioxide. The TEAs tested were related to soil depth: nitrate for shallow soils where it is more likely to be used, carbon dioxide for the deeper soils, and sulfate for either depth. Nutrients in basal salts medium (BSM) or fertilizer was also found to enhance biphenyl degradation. Root exudates enhanced biphenyl degradation slightly but the enhancement was not statistically significant ($p > 0.10$). A fungus, *Pseudallescheria boydii*, was isolated from the enriched cultures and was found to degrade biphenyl under anaerobic conditions only. To the authors' knowledge, this is the first fungus found that exhibited anaerobic biphenyl degradation.

4.2 Introduction

Biphenyl has many industrial applications, such as a dye carrier, a fungicide, and a heat transfer agent (OPPT Chemical Fact Sheets-1,1'-biphenyl 1995). It is also an intermediate of polychlorinated biphenyl (PCB) production. As a result of industrial use, biphenyl is becoming a widely-distributed environmental contaminant. Aerobic degradation of biphenyl by microorganisms has been studied since the early 1970s (Lunt and Evans 1970, Catelani et al. 1970) and can be used as a strategy for surface remediation such as land-farming. However, in subsurface environments such as contaminated groundwater plumes, where conditions are generally anoxic or anaerobic and redox potentials are often low, anaerobic degradation is of particular interest because it may provide a potential mechanism for the natural attenuation of contaminants to occur. Such a mechanism could lead to the development of different strategies for *in situ* bioremediation. Anaerobic biodegradation is likely more important than aerobic biodegradation *in situ* (Farhadian et al. 2008), and maybe the best solution to transform contaminants like tetrachloroethane and trinitrotoluene into harmless products (Holliger et al. 1997). Since biphenyl is a potential product from the reductive dechlorination of PCB (Natarajan et al. 1996), knowledge of biphenyl's anaerobic degradation could also be important not only to biphenyl remediation in subsurface environments but also to PCB remediation.

There are a few reports on the anaerobic degradation of biphenyl by soil and sediment microorganisms and no reports related to its anaerobic degradation by rhizosphere microorganisms. Paddy soil and river sediment (Yang et al. 2008) and an anaerobically-enriched culture from a creosote-contaminated sediment (Sharak Genthner et al. 1997) have been shown to degrade biphenyl under anaerobic conditions. Biphenyl has also been degraded by *Citrobacter freundii* BS2211(Grishchenkov et al. 2002), and a mixed consortium (Ambrosoli et al. 2005) under nitrate-reducing conditions and through methanogenesis by a PCB-dechlorinating anaerobic microbial consortium ATCC 55616 (Natarajan et al. 1999). In a fluidized bed reactor under nitrate- and sulfate-reducing conditions, a culture enriched from a coal tar creosote-contaminated sediment was shown to degrade biphenyl (Rockne and Strand 1998, 2001). Another sulfate-reducing enrichment culture produced biphenyl-4-carboxylic acid as an intermediate (Selesi and Meckenstock 2009). However, anaerobic biphenyl degradation rates were generally 1-2 orders of magnitude slower than that observed for aerobic enrichments (Rockne and Strand 1998).

To determine whether *in situ* anaerobic biphenyl bioremediation is a strategy, it would be important to understand the effect of various nutrient factors on the biodegradation of biphenyl. Such studies have not been previously reported. We therefore investigated the effect of terminal electron acceptors (TEAs), root exudates, and fertilizer addition on anaerobic biphenyl

degradation by microorganisms found in rhizosphere soil of willow and poplar trees in a phytoremediation test-plot. An anaerobic biphenyl-degrading culture was subsequently enriched, characterized, and identified.

4.3 Materials and methods

4.3.1 Soil inocula

Rhizosphere soil samples obtained from a biphenyl contaminated industrial site were used as inocula in this study. Samples were from poplar or willow trees planted in either native (a clay soil) or engineered soil of the phytoremediation site (amended according to a proprietary formulation to improve exchange with the water table). All soil samples were taken from under the crown of the trees. Soils from different depths were acquired between the surface and 1 m below grade or to the water table at about 15 to 20 cm intervals. Samples were stored separately at -10 ℃ until use. Between each soil sampling, the auger was cleaned thoroughly with soapy water and rinsed extensively with distilled water.

4.3.2 Growth medium

Each liter of basal salts medium (BSM, modified from Cote and Gherna 1994) contained 100 mL BSM stock solution and 100 mL trace element solution. The BSM stock solution contained per liter: 32.4 g K_2HPO_4, 10 g $NaH_2PO_4 \cdot H_2O$, and 20 g NH_4Cl. The trace element solution contained per liter: 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.132 g $CaCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 8.74 mg $CuSO_4 \cdot 5H_2O$, 10.0 mg $AlK(SO_4)_2 \cdot 12H_2O$, 10.0 mg H_3BO_3, 10 mg $Na_2MoO_4 \cdot 2H_2O$, 27.1 mg $NiCl_2 \cdot 6H_2O$, and 20.0 mg $Na_2WO_4 \cdot 2H_2O$. All solutions were autoclaved at 121 ℃ for 20 minutes before use.

4.3.3 Anaerobic biphenyl-degrading enrichment cultures

Rhizosphere soil (20 g) samples derived from approximately 15 to 40 (shallow) or 65 to 80 (deep) cm below the surface were added to 100 mL BSM with 1 g/L biphenyl (the carbon and energy source) plus one of the following TEAs: 100 mol/L of NO_3^-, 100 mol/L of SO_4^{2-}, or 100 mol/L of CO_2 (added as $NaHCO_3$). Two replicates were set up for each culture condition. Separate abiotic controls were prepared by autoclaving once a day at 121 ℃ for 20 minutes for three consecutive days. All microcosms were sparged with nitrogen gas for 20 minutes according to Hungate (1969) and then incubated in an anaerobic glove box (Nexus One; Vacuum Atmospheres Company, Hawthorn, California) at 23 ± 1° C. After every 2 months, 20 mL of

culture were transferred to fresh medium containing the same TEA. Biphenyl disappearance was assessed beginning with the third transfer. Microcosms degrading biphenyl were selected and enriched further. Two such enrichment cultures with the best biphenyl degradation capability were selected for chemical analysis. Biphenyl and its degradation intermediates were analyzed by gas chromatography and mass spectrometry (GC/MS) (Testmark, Garson, Ontario) according to US EPA SW-864 method 8270.

4.3.4 Anaerobic biphenyl mineralization

In the mineralization experiments, soil from the willow rhizosphere previously shown to exhibit the best biphenyl degradation capability in the enrichment experiment was used as the inoculum. Typically, 20 g of the soil, 30 mL deionized water or BSM, 50 mg/L biphenyl were added to a 100 mL serum bottle with a 5-mL test tube containing 1 mL of 1 mol/L KOH to trap volatile ^{14}C compounds. Two replicates were set up for each culture condition. The initial amount of ^{14}C-biphenyl used was approximately 100,000 cpm. Microcosms were then capped with a butyl rubber stopper and an aluminum crimp. Radioactivity captured in the KOH was measured with a Beckman LS 6500 Scintillation Counter using the Optiphase "HiSafe 3" liquid scintillation cocktail (Wallac Scintillation Products, Turku, Finland). Abiotic controls were autoclaved once a day at 121 ℃ for 20 minutes for three consecutive days.

4.3.5 Effect of TEA, root exudates, and fertilizer on biphenyl mineralization

Microcosms were set up as described in the mineralization experiment. In the TEA experiment, each TEA (NO_3^-, SO_4^{2-} or CO_2 (as $NaHCO_3$)), was added to an initial concentration of 400 mM. Root exudates were prepared by Maureen Edwards, Biology, Queen's University. Exudates were prepared using willow or poplar trees grown hydroponically for two weeks in half strength MS medium (Murashige and Skoog 1962). The tree roots were washed twice in distilled water and transferred to distilled water for 1 week to obtain exudates. The exudates were filter sterilized with a 0.45 μm nitrocellulose membrane (Millipore) before use. Concentrated BSM stock solution (10X) was added to the poplar or willow root exudates to make the culture medium. To determine the effect of fertilizer concentration, the same fertilizer (CIL Tree and Hedge Feed) used to promote tree growth in the phytoremediation test-plot was added at increasing concentrations of 0.01, 0.05, 0.1, and 0.5 g/g soil in water alone or in BSM. In all experiments, the biphenyl mineralization was compared to control microcosms without TEA, root exudates, or fertilizer after about 40 days of incubation. Student's *t*-test was used to determine whether the effect of TEA, root exudates, and fertilizer were significant.

4.3.6 Isolation and growth of anaerobic biphenyl-degrading microorganisms on agar plates

Noble agar was used in the isolation experiment except where otherwise specified. All agar plates were inoculated and incubated in an anaerobic glove box. The enriched biphenyl culture (1 mL) exhibiting the best biphenyl-degrading capability was spread onto BSM agar plates (1.5 %) containing 0.05 g/L biphenyl. Sterile water (1 mL) was spread onto a separate plate as an abiotic control. Colonies were picked up with a sterile inoculation loop and spread on nutrient agar plates or noble agar plates made with 0.05 g/L biphenyl and sulfate or nitrate as TEA in distilled water, BSM, or BSM without trace elements. An aerobic control with biphenyl on noble agar plate was prepared in the glove box but incubated outside the glove box.

4.3.7 PCR and sequencing

The PCR protocol used was described previously in Fortin et al. (2004). The 16S rDNA fragments were amplified using a Px2 thermal cycler (Thermo Fisher, Ottawa, Ontario). The cycling scheme used was as follows: an initial 10 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C stepping down 1 degree each cycle to 40°C, and 3 min extension at 72°C followed by 20 cycles of 1 min at 94°C, 1 min at 40 °C, and 3 min at 72°C. Colonies were selected directly from an agar plate, and added directly to the PCR tube. Each 50 μL PCR reaction contained 8 μL of 1.25 mM dNTPs, 1 μL of 25 pmol ITS4 (TCCTCCGCTTATTGAT

ATGC) and ITS1F (CTTGGTCATTTAGAGGAAGTAA) primer, 4 μL 25 mM $MgCl_2$, 5 μL of 10X reaction buffer and 2.5 units of Taq polymerase (Fisher Scientific Ltd., Ottawa, Ontario). The amplified DNA fragments were separated using an agarose gel (1.4%), extracted, and concentrated by ethanol precipitation, and then sequenced (Cortec, Kingston, Ontario). The resulting DNA sequences were analyzed and matched to GenBank entries using BLAST (Altschul et al. 1997).

4.4 Results

4.4.1 Anaerobic enrichment cultures

To enrich for anaerobic biphenyl degraders, 48 microcosms with NO_3^-, SO_4^{2-} or CO_2 (added as $NaHCO_3$) as TEA were established with poplar or willow rhizosphere soils sampled from different depths (shallow- ~30cm or deep- ~70cm) of a phytoremediation test-plot. After two successive transfers, a qualitative assessment of the visual disappearance of biphenyl crystals indicated that anaerobic biphenyl degraders were widely distributed across the site in both native and engineered soils of both tree types (Table 4-1). There was no visual disappearance of the biphenyl crystals in the abiotic controls. Of the three TEAs provided, nitrate was clearly used by

rhizosphere microorganisms from the shallow depth, and carbon dioxide by the microorganisms residing in the deeper soil.

Table 4-1 Anaerobic biphenyl degradation in the presence of terminal electron acceptors

	TEA	NO_3^-		SO_4^{2-}		CO_2	
	Soil	Deep	Shallow	Deep	Shallow	Deep	Shallow
Poplar	Engineered	--	++	--	+	--	+
	Native	++	+++	+	+++	+++	+++
Willow	Engineered	--	+++	+++	+++	+++	--
	Native	--	+	+	+++	+++	--
Control	Sterile	--		--		--	

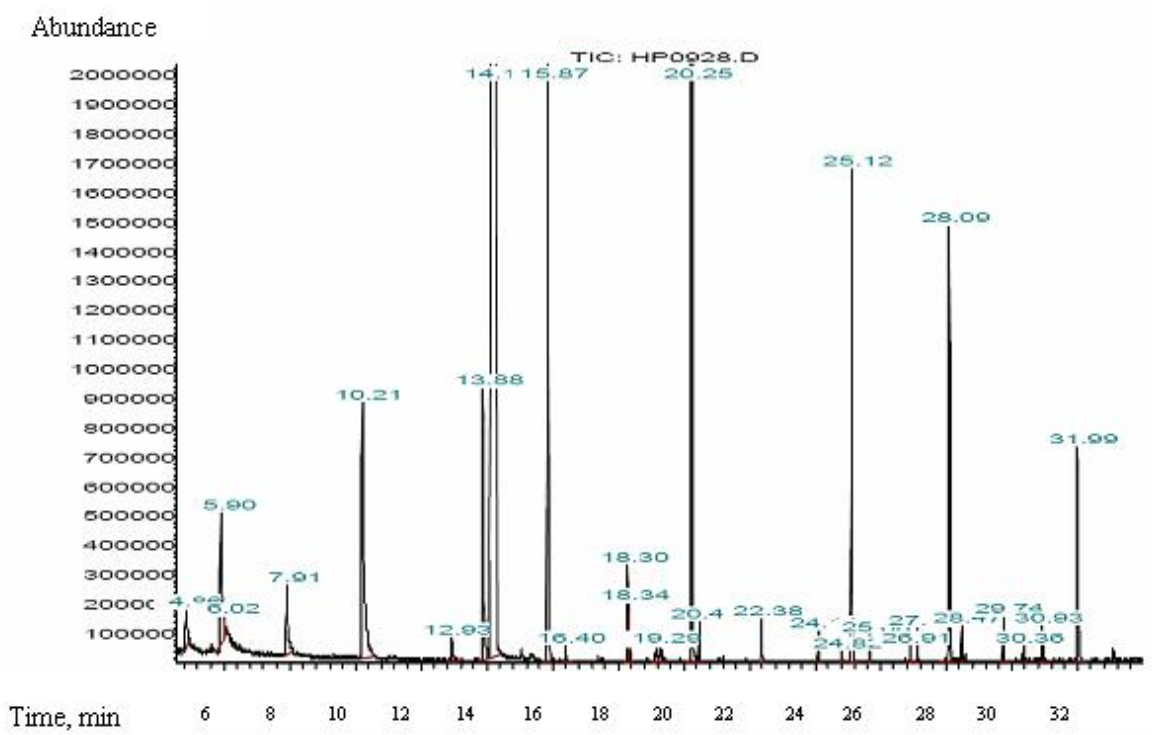
Anaerobic biphenyl degradation was assessed for the various enriched consortia listed. The origin and depth of the soil samples used to derive the enriched cultures are indicated in the corresponding rows and columns. Assessment was conducted after the third serial transfer and in the presence of the indicated terminal electron acceptors (TEAs). The level of degradation was based on visual assessment observed and categorized as follows: (--) 0-25%; (+) 25-50%; (++) 50-75%; and (+++) >75%.

After transfer to fresh medium with 1 g/L biphenyl and two months of incubation, GC/MS analyses were carried out for the nitrate- and sulfate-reducing microcosms exhibiting the highest level of biphenyl crystal disappearance. The biphenyl concentration was 0.65 ppm when sulfate was the TEA and 39.6 ppm when nitrate was the TEA. These concentrations represent biphenyl degradation levels of 99 and 96%, respectively. No significant accumulation of metabolic

intermediates was observed for both microcosms. Two metabolites, o-hydroxybiphenyl and p-hydroxybiphenyl, were detected under sulfate-reducing conditions (Fig 4-1). However, dihydroxybiphenyl, the first metabolite in the common aerobic biphenyl degradation pathway (Catelani et al. 1973) was not detected.

4.4.2 Mineralization of biphenyl by the rhizosphere soil

Since biphenyl adsorbs to soil organic matter and is poorly soluble in water, measuring biphenyl disappearance in soil samples can be very difficult. Therefore, $^{14}CO_2$ produced from uniformly-labelled ^{14}C-biphenyl mineralization was used in this study to monitor biphenyl degradation. Under similar grow conditions, a higher mineralization can indicate a higher biphenyl degradation. Instead of an enriched consortium, rhizosphere soil (willow in native soil) showing the best biphenyl degradation capability in the enrichment experiment was used as the inoculum to simulate an environment similar to the field site conditions. Higher biphenyl mineralization was achieved in BSM (3.6%) than in water (2.1%) over an 85 day period (Fig 4-2). In both cases, biphenyl mineralization levels were significantly ($p<0.05$) higher than the controls conducted with sterile soil.



Time	Chemicals	Concentration
5.9	1,4-dichlorobenzene-d4	Internal Standard
7.9	Nitrobenzene-d5	Surrogate Standard
10.2	Naphthalene-d8	Internal Standard
13.9	2-Fluorobiphenyl	Surrogate Standard
14.1	1,1'-biphenyl	0.6 mg/L
15.8	Acenaphthene-d10	Internal Standard
16.4	o-hydroxybiphenyl	6 ug/L
18.3	2,4,6-tribromophenol	Surrogate Standard
19.3	p-hydroxybiphenyl	12 ug/L
20.2	Phenanthrene-d10	Internal Standard
22.4	Di-n-butylphthalate	5 ug/L
25.1	p-terphenyl-d14	Surrogate Standard
28.1	Chrysene-d12	Internal Standard
32.0	Perylene-d12	Internal Standard

Figure 4-1 GC/MS analysis of the metabolites resulting from anaerobic biphenyl biodegradation. The culture for this analysis was enriched from rhizosphere soil of a willow tree planted in native soil at about 70 cm. The culture was grown on BSM with sulfate as TEA.

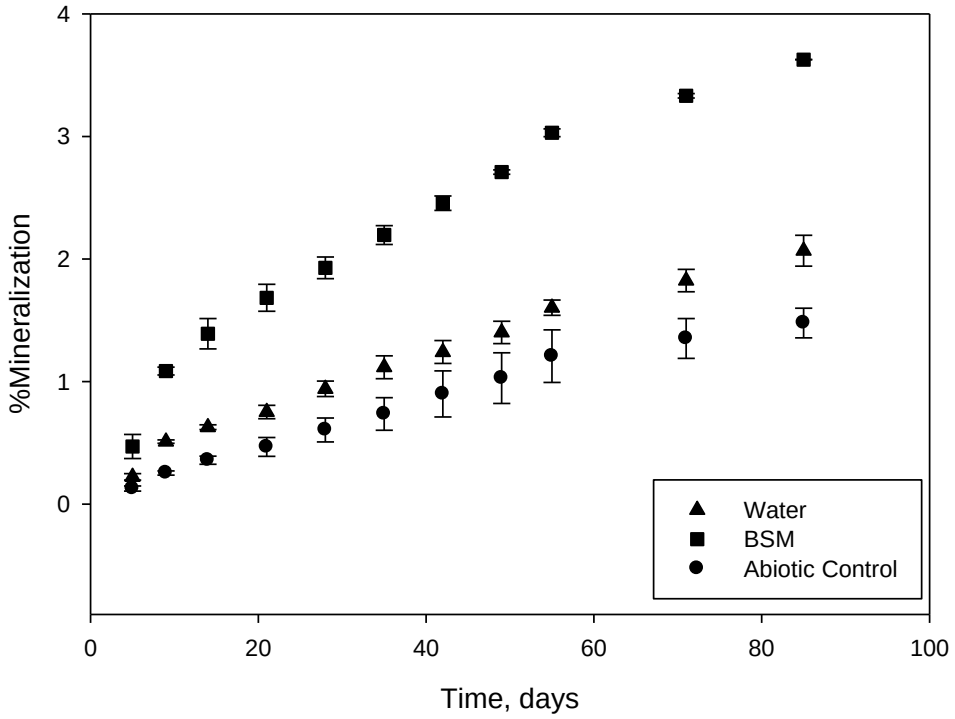


Figure 4-2 Biphenyl mineralization analysis using a native soil sample obtained from the rhizosphere of a willow tree as inoculums. The experiment was conducted with water or with BSM. For the abiotic control, the same soil inoculum was autoclaved 20 minutes at 121 ℃ once a day for three consecutive days. Each point represents the average of two replicates, and the error bars represent the range of values obtained.

4.4.3 Effect of TEA, root exudates, and fertilizer on biphenyl mineralization

Microcosms similar to those in the above mineralization experiment were set up to investigate the effect of TEA, root exudates and fertilizer on biphenyl mineralization. The presence of sulfate ($p<0.1$) and CO_2 ($p<0.05$) but not nitrate enhanced mineralization (Fig 4-3).

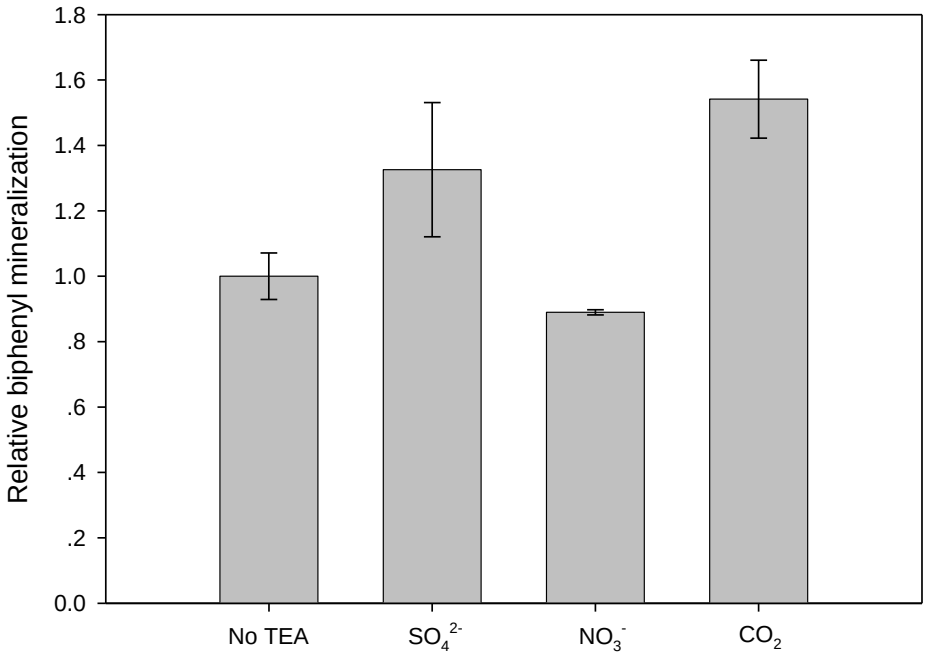


Figure 4-3 Effect of TEA on biphenyl mineralization in BSM after 40 days of incubation. The soil inocula were from native soil obtained from the rhizosphere of a willow tree. The concentration of each TEA used was 400 mM. Each microcosm was in duplicate and the error bars represent the range of values obtained..

A high level of mineralization was seen in one of the duplicate microcosms with poplar or willow root exudates, but when the values were averaged, the effect was not significant ($p>0.1$) (Fig 4-4). When the microcosms were prepared with distilled water instead of BSM, all fertilizer concentrations tested enhanced biphenyl mineralization (Fig 4-5). However, when prepared in BSM, the enhancement was only seen at the highest fertilizer concentration (0.5 g/g soil). Since

BSM also contains nitrogen and phosphate sources, the presence of low fertilizer concentrations was probably insufficient to result in a noticeable effect.

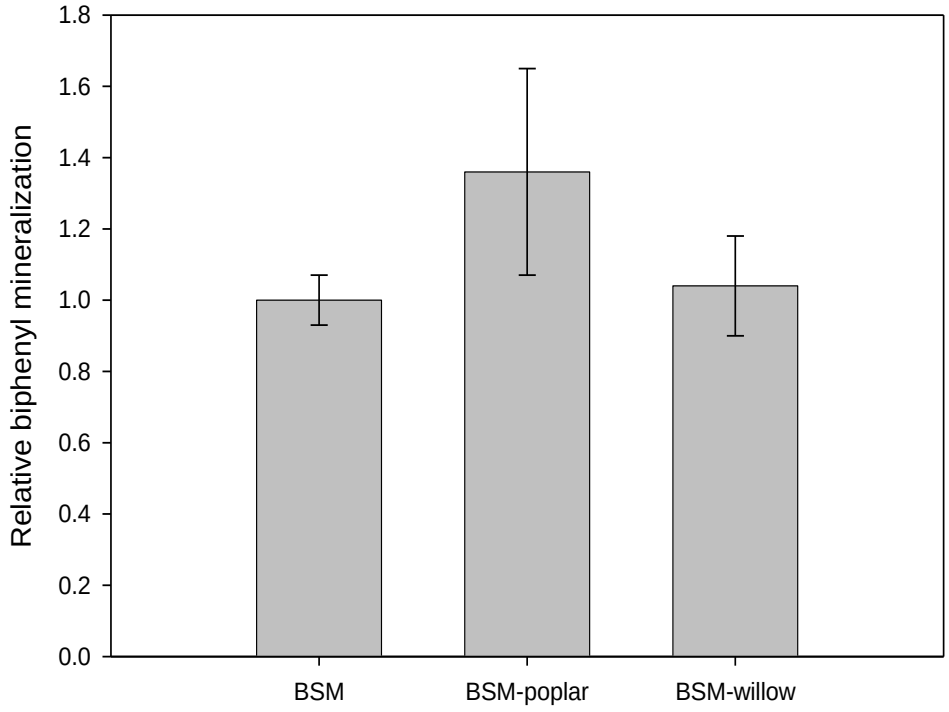


Figure 4-4 The effect of poplar and willow root exudates on biphenyl mineralization after 40 days of incubation. Microcosms were prepared in duplicate and the error bars represent the the range of values obtained.

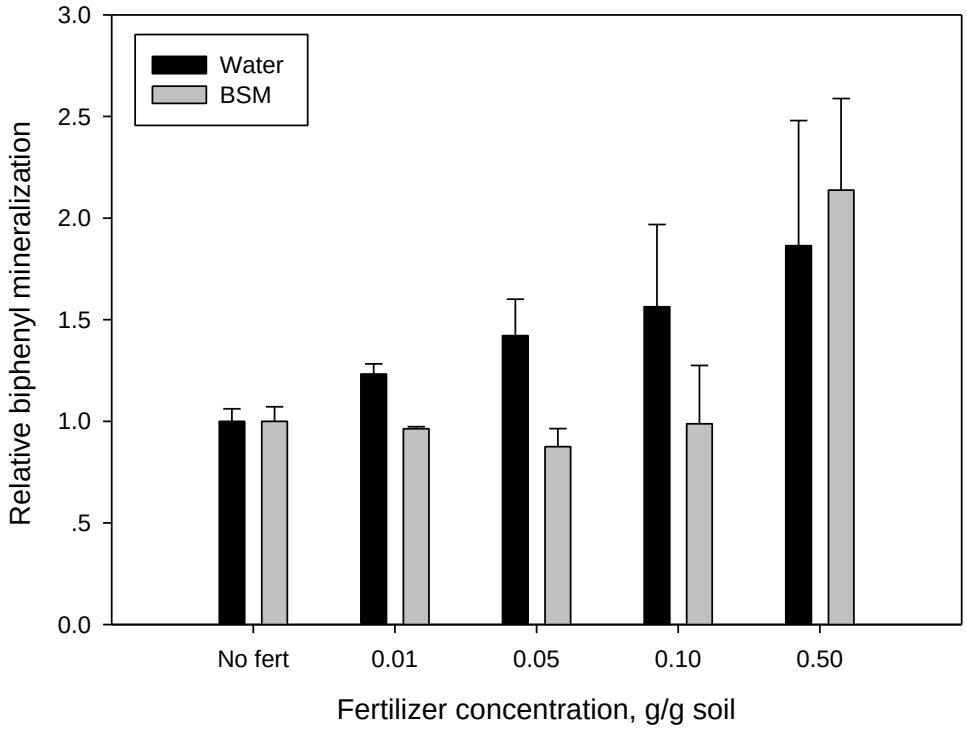


Figure 4-5 Effect of fertilizer concentration on biphenyl mineralization in water (light) or BSM (dark) after 40 days of incubation. Microcosms were prepared in duplicate and the error bars represent the range of values obtained.

4.4.4 Isolation and growth of anaerobic biphenyl-degrading microorganisms on agar plates

The enriched biphenyl-degrading culture under sulfate-reducing conditions was plated on BSM agar containing 50 mg/L biphenyl as the sole carbon and energy source and sulfate as TEA.

All colonies had the same appearance and the features of a filamentous fungus but no growth occurred on the abiotic controls where sterile water was used instead of the enriched culture. When the fungus was grown on different nutrient and TEA agar plates (Table 4-2), it did not grow under aerobic conditions or anaerobically on noble agar but it grew anaerobically on all plates containing biphenyl whether sulfate or nitrate was provided as TEA. The best growth was seen on nutrient agar. $Na_2MoO_4\ 2H_2O$ is reported to inhibit sulfate-reducing bacteria and was present in the trace elements solution. The fungus grew better on sulfate when the trace elements solution was omitted.

Table 4-2 Grown of the isolated fungus on agar plates with different nutrient and TEAs

Medium	Growth
Nutrient agar	+++++
Water + biphenyl	+++
Noble agar[a]	+/-
BSM + biphenyl	++
BSM (no trace[b]) + biphenyl	++
BSM + biphenyl + sulfate	++
BSM (no trace) + biphenyl + sulfate	+++
BSM + biphenyl + nitrate	++
BSM (no trace) + biphenyl + nitrate	++
Aerobic control, BSM + biphenyl	-

Noble agar was used to prepare all the plates except for the nutrient agar

[a] No added TEA or carbon source

[b] BSM was prepared without the trace elements solution.

4.4.5 Identification of the enriched fungus

The fungus appearing upon enrichment was identified using PCR and DNA sequencing. Fungal cells were taken directly from the BSM plates for PCR amplification. PCR amplification was carried out using the ITS4 and ITS1F primers. The colonies from nutrient agar and water or BSM agar with biphenyl as the sole carbon and energy source gave rise to 16S rDNA amplification products. No amplification was seen in the control without template DNA. The amplified 16S rDNA was sequenced and matched using BLAST. The best matches to GenBank entries were *Pseudallescheria boydii* and *Scedosporium apiospermum* at 96%. *Scedosporium apiospermum* is actually the asexual state of *Pseudallescheria boydii*.

4.5 Discussion

Biphenyl is difficult to degrade under anaerobic conditions. Most studies on the anaerobic degradation of PCBs show reductive dechlorination instead of ring opening, with biphenyl as an end product (Natarajan et al. 1996). In his review, Field (2002) concluded that anaerobic recalcitrance was associated with a lack of functional groups, and similar results have been illustrated by a quantitative structure-biodegradability relationship model (Yang et al. 2006). Consequently, biphenyl has been categorized anaerobically as poorly biodegraded. In this study, anaerobic biphenyl-degrading consortia were enriched from a phytoremediation test-plot

contaminated with biphenyl. When rhizosphere soil was used as inocula, about 3.6% of the biphenyl fed was mineralized in 85 days (Fig 4-2) while at least 96% disappearance was confirmed by GC/MS analysis in a separate experiment (Fig 4-1). The 3.6% mineralization is much lower than the levels achieved under aerobic conditions (81% in 24 days, Focht and Brunner, 1985). A small amount of radioactivity recovered as $^{14}CO_2$ (<1.3% in 45 days) was also seen in anaerobic biphenyl degradation study by Yang et al. (2008). A similar result was reported under aerobic conditions, that only a small amount of biphenyl (<1%) can be mineralized even when most of the biphenyl (>98%) was transformed (Moody et al. 2002). The complete disappearance but low mineralization of biphenyl might be due to the accumulation of metabolic intermediates in the microorganisms. Biphenyl mineralization in BSM was significantly higher than biphenyl in water alone (Fig 4-2) indicating that nutrients in BSM enhanced biphenyl mineralization.

In the enrichment experiments, greater biphenyl disappearance occurred in shallow soils when nitrate was provided as TEA, in deeper soils with CO_2, and with sulfate at both depths (Table 4-1). This is consistent with the results obtained in a mineralization experiment in which biphenyl mineralization with the deeper soil (70 cm below surface) was enhanced by the addition of sulfate and carbon dioxide but not of nitrate (Fig 4-3). Furthermore, without the sulfate-reducing inhibitor, $Na_2MoO_4\ 2H_2O$, there was better fungal growth on BSM biphenyl agar plates when sulfate was present indicating that the culture is capable of growing on biphenyl under

sulfate-reducing conditions. Yang et al. (2008) also found anaerobic growth on biphenyl without any added TEA and suggested that biphenyl degradation may have been fermentative. This could have been the reason for the good growth observed on only water and biphenyl agar plates.

Root exudates secreted by plants are well known to stimulate microbial growth and contaminant degradation. In this study, the effect of poplar and willow rhizosphere exudates on biphenyl mineralization was not statistically significant (Fig 4-4). Since the nutrients present in BSM were sufficient to mask the effects of low fertilizer concentration, it may be that any benefit from the exudates was masked by the BSM nutrients. Without BSM, low fertilizer concentrations did enhance biphenyl mineralization. Similar results by Bach et al. (2005) showed that slow release fertilizers enhanced anaerobic PAH degradation.

In this study, the biphenyl-degrading culture under nitrate- and sulfate-reducing conditions was tentatively identified as *Pseudallescheria boydii*. *P. boydii* has been shown to be involved in the degradation of aliphatic (April et al. 1998) and aromatic hydrocarbons (Claussen and Schmidt 1998), and has more recently been reported to degrade PCB aerobically, but was not able to grow on biphenyl or PCB as the sole carbon and energy source (Tigini et al. 2009). Although *P. boydii* in the current study was not able to grow aerobically, de Hoog et al. (1994) showed that all *P. boydii* strains in their study were able to grow anaerobically.

Two metabolites, o-hydroxybiphenyl and p-hydroxybiphenyl, were detected under sulfate-reducing conditions. In the aerobic biphenyl degradation pathway, dihydroxybiphenyl is the key

metabolic intermediate and mono-hydroxybiphenyl is not in the benzoate pathway. Less commonly, 4-hydroxybiphenyl, which has been reported as an intermediate in biphenyl degradation by *Mycobacterium sp.* PYR-1, can be formed by a P-450 monooxygenase in fungi with 4-hydroxybenzoic acid as the final degradation product (Moody et al. 2002). The presence of o-hydroxybiphenyl and p-hydroxybiphenyl suggests that the anaerobic biphenyl pathway utilized by *P. boydii* was different from the aerobic benzoate pathway, and 4-hydroxybenzoic acid was not detected in our cultures.

In conclusion, poplar and willow rhizosphere microorganisms were shown to anaerobically degrade biphenyl under nitrate-reducing, sulfate-reducing and methanogenic conditions. Although significant biphenyl disappearance (>98 %) can occur, mineralization to carbon dioxide was low and there was no significant accumulation of intermediates. Although key metabolites in the benzoate pathway, e.g., 4-hydroxybenzoic acid, were not detected, less common metabolites (o-hydroxybiphenyl and p-hydroxybiphenyl) were detected. This profile suggests that a different metabolic pathway is utilized. *P. boydii* was isolated from biphenyl enrichments under sulfate-reducing conditions. To the authors' knowledge, this is the first report of a fungus capable of degrading biphenyl anaerobically.

4.6 Acknowledgements

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Chapter 5

Microbial community analysis of willow and poplar rhizosphere

5.1 Abstract

The microbial community in the rhizosphere of poplar and willow trees of a biphenyl phytoremediation field trial was sampled over a 5-year period to assess changes in the microbial population. The different soil samples were derived from trees planted in native and engineered soils and at different depths. The denaturing gradient gel electrophoresis results indicate that soil type had a significant impact on microbial community composition. A more diverse microbial population was found in native soil samples than in engineered soil samples. Although tree type had less influence on microbial diversity, diversity did decrease with time in the engineered soil of the willow rhizosphere. In native soil, there was no significant influence of soil depth on the microbial community. Three biphenyl-degrading consortia, enriched from different rhizosphere soils, aerobically mineralized 8.5 to 16.4% of the fed biphenyl in about 50 days. The three major organisms found in the enriched consortia were identified by 16S DNA sequencing as being in the family of *Flavobacteriaceae*, *Alcaligenaceae*, and *Mycobacteriaceae*.

5.2 Introduction

The phytoremediation of organic contaminants has attracted increasing commercial and research interests as it is relatively inexpensive and has been recognized as a "green" and environmentally friendly process. The success of phytoremediation depends on the nature of the organic contaminants, soil properties, site conditions and the volume of material to be remediated (Cunningham et al. 1995). In terms of the removal mechanism, organic contaminants can be absorbed and taken up by plants, and then either stored in the harvestable parts of the plants or transpired into the atmosphere. Grasses and bushes have been used as model plants in a number of studies focused on the phytoremediation of organic compounds. Poplar and willow trees are attracting increasing attention as their long roots are able to reach contaminants deep in the soil and groundwater and the trees are capable of transpiring large amounts of water (200-800 gallons per day per tree). A few studies have reported on the use of poplar trees in the phytoremediation of contaminants including dioxane (Aitchison et al. 2000) and trichloroethylene (Gordon et al. 1998) and even fewer on the use of willow trees to phytoremediate contaminants including ethanol blended gasoline (Corseuil and Moreno 2001). Soil microorganisms can also utilize a wide range of organic contaminants as a carbon and energy source, and therefore can play an important role in phytoremediation.

In the field, the rhizosphere represents a unique environment in which soil microorganisms and plants must survive together in close proximity; therefore, interactions between plants and

soil microbes can play a major role in the degradation of contaminants during phytoremediation (Shaw and Burns 2003). Plants release large amounts of root exudates containing amino- and organic acids that promote microbial growth and metabolic activity. Rhizobacteria in turn can stimulate plant growth through their various metabolic activities (Bloemberg and Lugtenberg 2001). A number of studies have shown that plants can enhance the biodegradation of polycyclic aromatic hydrocarbons (PAHs) by rhizosphere microorganisms. For example, alfalfa enhanced the total number of microorganisms and the rate of PAH degradation (Muratova et al. 2003) while ryegrass and alfalfa increased the number of petroleum-degrading bacteria (Kirk et al. 2005). Poplar and pine tree rhizosphere communities were found to have a greater hydrocarbon degradation potential than those of bulk soil in the phytoremediation of PAH (Palmroth et al. 2007). The higher levels of petroleum hydrocarbon degradation found in the rhizosphere relative to bulk soil was due to higher numbers of contaminant-degrading genes in the root-associated bacteria (Siciliano et al. 2001, Siciliano et al. 2003). There were no detectable changes to the microbial population when assessed by denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA (Siciliano et al. 2003).

There are also examples of plant-independent effects such as that observed with diesel (Tesar et al. 2002), phenanthrene and pristane (Jones et al. 2004). These contaminants had been shown to have a more pronounced effect on the rhizosphere community than the plant variety, while diesel-degrading microorganisms were equally stimulated by the plants and the contaminant.

The influence of plants on rhizophere microorganisms is frequently species-specific. For the same contaminant, certain plant species have been shown to have more significant effects on rhizosphere microorganisms than others. For example, alfalfa was more effective in stimulating the rhizosphere microflora than reeds in the phytoremediation of PAH (Muratova et al. 2003). Similarly, DGGE studies showed that the greatest change in a bacterial community was caused by a mixture of ryegrass and alfalfa in petroleum degradation (Kirk et al. 2005). On the other hand, no correlation with plant species was found between PAH disappearance and the size of the PAH-degrading population in a greenhouse study (Olson et al. 2007). Although soil type was found to be the main factor affecting the microbial community in the phytoremediation of soil contaminated with hydrocarbons and heavy metals (Palmroth et al. 2007), Berg and Smalla (2009) concluded in a recent review that there was no general consensus on whether soil type or tree species was the key player. Differences in soil depth of up to 1 m have been reported to exhibit effects on microbial communities (Brad et al. 2008) as the redox potential may change with depth. Therefore, at this stage of our understanding, it is important to analyze and monitor the relationship between the rhizosphere and the plants in the field when developing phytoremediation strategies for particular contaminants.

Biphenyl is another organic contaminant that requires remediation because of its many industrial applications. Biphenyl is used in the manufacture of textiles, dyes and other chemicals such as high octane and aviation fuels. It is also a component of creosote which is widely used to

treat wood. In the US, 53 million pounds of biphenyl were produced in 1990, and 855,000 lbs were found to be released into the environment in 1992 (OPPT Chemical Fact Sheets-1,1'-biphenyl, 1994). The effect of biphenyl on human health is dependent on concentration, length and frequency of exposure. Short-term exposure to biphenyl fumes may result in nausea, vomiting, irritation of the eyes and respiratory tract, and bronchitis (OPPT Chemical Fact Sheets-1.1'-biphenyl, 1994). Over a long period of exposure, biphenyl may cause damage to the liver and central nervous system. Ingestion may cause damage to kidneys and blood, and reduce growth and life expectancy. Biphenyl is also known to be of high acute toxicity to aquatic life.

Although the phytoremediation of polychlorinated biphenyls has been studied (Chekol et al. 2004, Smith et al. 2007, Mackova et al. 2009), there are no published reports for biphenyl phytoremediation. Like many hydrocarbons, the absorption and uptake of biphenyl by plants is related to the hydrophobicity of the organic contaminant. Only moderately hydrophobic organics (log K_{ow} = 1.0 to 3.5) are effectively taken up by roots (Dietz and Schnoor 2001). Biphenyl has a low water solubility and is highly hydrophobic (log K_{ow} = 4.09). Therefore, direct uptake of biphenyl by the plants is not expected to be very effective. On the other hand, since the early 1970s, many enriched microbial cultures have been shown to use biphenyl as the sole carbon source (Lunt and Evans 1970, Catelani et al. 1970). The biphenyl degradation pathway and enzymes of microbes are now elucidated (Seeger et al. 1995). Therefore, it would be beneficial to study the combination of microbes and plants during the phytoremediation of biphenyl in the field.

There is no information in the literature on how the rhizosphere microbial community could be affected during the phytoremediation of biphenyl. Since the rhizosphere microorganisms may play a very important role in biphenyl removal, it is of interest to analyze the rhizosphere microbial population during a phytoremediation field trial. The objective of this study was thus focused on the relationship between tree type, soil type, soil depth, and the rhizosphere microorganisms of a phytoremediation field site for biphenyl to better understand the dynamics in play over a 5-year period. The diversity of biphenyl-degrading microbes of the rhizosphere microbial community was monitored over a 5-year period using PCR and DGGE and finally through enrichment cultures established using three different soil samples from the test-plot.

5.3 Materials and methods

5.3.1 Field site

A phytoremediation test-plot was established on an industry site (Kingston, Ontario) contaminated with biphenyl, dioxane, and ethylene glycol. Rows of poplar (*Populus balsamifera*) and willow (*Salix nigra*) trees were planted in an alternating pattern along the path of a contaminated groundwater plume. Trees were planted in native (clay soil) and engineered (a soil mixture designed to improve exchange with the water table) soils.

5.3.2 Soil samples

Soils were sampled from 2004 to 2008 either at the beginning and/or the end of the growing season where the contaminant concentration was highest in the groundwater. Samples were taken from poplar or willow trees in both native soil and engineered soil. At each sampling point, rhizosphere soils under the crown of the trees were augured up to 1 m below grade or to the water table at about 20 cm intervals. Bulk soils on the site located away from trees were used as control soil samples. The bulk soils contained lower contaminant concentrations based on chemical analysis of groundwater. Between each sample collection, the auger was cleaned thoroughly with soapy water and extensive rinsing in distilled water. Soil samples were stored at -10 ℃ until use. The rhizosphere soil samples taken at different times during phytoremediation, and representing different soil type (native and engineered), tree type (poplar and willow) and soil depth were chosen for microbial community analysis (Table 5-1).

5.3.3 Extraction of DNA from soil samples

DNA was extracted from soil samples as described by Fortin et al. (2004). Soil samples (10 g) were washed with three different buffers (20 mL each) (Buffer 1: 50 mM Tris-HCl, pH 8.3, 200 mM NaCl, 5 mM EDTA, 0.05% Triton X-100; Buffer 2: 50 mM Tris-HCl, pH 8.3, 200 mM NaCl,

Table 5-1 Soil samples used in the microbial community analysis

		Tree type	Soil type sampled	Year of sampling	Depth of sampling, cm
PN	1	Poplar	Native	2005	15~30
	2			2006	3~15
	3			2006	15~30
	4			2006	30~45
	5			2006	70~80
	6			2008	15~30
PE	1	Poplar	Engineered	2004	15~40
	2			2005	15~40
	3			2008	15~40
WE	1	Willow	Engineered	2005	15~40
	2			2006	15~40
	3			2008	15~40
WN	1	Willow	Native	2004	15~40
	2			2005	3~15
	3			2006	75~85
	4			2008	3~15
BK	1	Bulk	Native	2005	30~45
	2			2008	15~30

5 mM EDTA; and Buffer 3: 10 mM Tris-HCl, pH 8.3, 0.1 mM EDTA) to remove PCR inhibitor(s)and then resuspended in 9 mL of sterile, distilled water. Lysozyme (1 mL of 100 mg/mL in 250 mM Tris-HCl pH 8.0 at 37 ˚C with shaking at 295 rpm for 30 min, followed by

another 30 min at 37˚C in a water bath, and inverted every 10 min), proteinase K (100 µL of 20 mg/ml at 37˚C for 1 hr, and inverted every 10 min), and SDS (1000 µL of 20% (wt/v) at 85 ˚C for 30 min, and inverted every 10 min) were used stepwise to release total DNA from the washed soil samples. After centrifugation at 3,000 x g for 15 min at 23 ± 1 ℃, the supernatant was prepared for DNA precipitation with 7.5 M ammonium acetate (1/2 volume and on ice for 15 min). Total DNA was precipitated with cold 2-propanol (1 volume at -20 ˚C overnight). The recovered DNA was washed with ethanol (2 mL, 70% (v/v)) then redissolved with 200 µL Tris-EDTA (10 mM Tris-HCl, pH 8.0, 0.1 mM Na_2EDTA) at 23 ± 1 ℃ for 30 min and on ice for another 30 min. The DNA samples were incubated at 37˚C for 10 min and further purified using polyvinylpolypyrrolidone spin columns to remove humic acids before agarose gel electrophoresis (0.7% agarose gels).

5.3.4 PCR and DGGE analysis procedures

The PCR and DGGE protocol used were described previously in Fortin et al. (2004). The 16S rDNA fragments were amplified using a T1 thermocycler (Biometra). The cycling program employed was as follows: an initial 10 cycles of 1 min denaturation at 94°C, 1 min annealing at 65°C stepping down 1 degree each cycle to 55°C, and 3 min extension at 72°C followed by 20 cycles of 1 min at 94°C, 1 min at 55 °C, and 3 min at 72°C. Each 50 µL PCR reaction contained 1 µL of concentrated cells, 8 µL of 1.25 mM dNTPs, 1 µL of each primer U341-GC2 and U758 (25

pmol each) (Fortin et al., 2004), 4 μL 25 mM $MgCl_2$, 5 μL of 10X PCR reaction buffer and 2.5 units of Taq polymerase (Fisher Scientific Ltd., Ottawa, Ontario). The amplified DNAs were concentrated with ethanol. Concentrated samples containing approximately 400 ng DNA were resolved using 8% (w/v), 0-80% denaturing gradient acrylamide gels and 1X Tris-acetate-EDTA (TAE) buffer. The gels were ran using 80 V for 16 h at 60°C and stained for 10 min in 1X TAE containing SYBR Gold (Invitrogen, Burlington, Ontario). The 16S rDNA bands were excised and eluted with water at 4 ℃ overnight, precipitated with ethanol, re-amplified for 25 cycles of 1 min at 94 ℃, 1 min at 55 ℃ and 1 min at 72 ℃ using the U341 and 758 primers, and sequenced (Cortec, Kingston, Ontario). The resulting sequences were matched to GenBank entries using BLAST (Altschul et al. 1997).

5.3.5 Growth medium

Each liter of basal salts medium (BSM, modified from Cote and Gherna 1994) contained 100 mL BSM stock solution and 100 mL trace element solution. The BSM stock solution contained per liter: 32.4 g K_2HPO_4, 10 g NaH_2PO_4 H_2O, and 20 g NH_4Cl. The trace element solution contained per liter: 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4$ $7H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4$ $7H_2O$, 0.1 g $CoCl_2$ $6H_2O$, 0.132 g $CaCl_2$ $2H_2O$, 0.1 g $ZnSO_4$ $7H_2O$, 8.74 mg $CuSO_4$ $5H_2O$, 10.0 mg

$AlK(SO_4)_2 \cdot 12H_2O$, 10.0 mg H_3BO_3, 10 mg $Na_2MoO_4 \cdot 2H_2O$, 27.1 mg $NiCl_2 \cdot 6H_2O$, and 20.0 mg $Na_2WO_4 \cdot 2H_2O$.

5.3.6 Biphenyl-degrading enrichment cultures

Biphenyl-degrading cultures were enriched from the rhizosphere soil of poplar and willow trees under aerobic conditions. Ten grams of soil were added to 125 ml Erlenmeyer flasks containing 50 mL sterile BSM (121 °C for 20 min) and 2 g/L biphenyl as a carbon and energy source. The cultures were incubated at 23 ± 1 °C and 200 rpm using an Innova rotary shaker. In the abiotic control, 10 g Ottawa sand was used instead of rhizosphere soil. Since the water solubility of biphenyl is only 7.5 mg/L at 25 °C (Verschueren 1983), most of the added biphenyl crystals settled to the bottom of the flasks. If the biphenyl crystals disappeared, 10 mL were transferred to fresh BSM medium supplemented with 1 g/L biphenyl to obtain biphenyl-degrading consortia by serial transfer.

5.3.7 Biphenyl mineralization analysis

Each of the enriched biphenyl-degrading cultures (10 mL) was added to a 100 mL serum bottle containing 40 mL of fresh BSM medium supplemented with 1 g/L of biphenyl and ^{14}C-biphenyl. The initial radioactivity of the ^{14}C-biphenyl used was approximately 100,000 cpm. The

abiotic control was set up in the same way except that 10 mL sterile water was added. A 5-mL test tube containing 1 mL of 1 mol/L KOH was placed in each serum bottle to trap volatile ^{14}C compounds. The serum bottles were capped with a butyl rubber stopper and an aluminum crimp, and incubated without shaking at 23 ± 1 ℃. Radioactivity was measured with a Beckman LS 6500 Scintillation Counter using the Optiphase "HiSafe 3" liquid scintillation cocktail (Wallac Scintillation Products, Turku, Finland).

5.4 Results

5.4.1 Microbial population analysis of the various soil samples

Rhizosphere samples from various depths were obtained from poplar and willow trees planted in different soils at a biphenyl phytoremediation site (Table 5-1). Soils were sampled over a 5-year period, from 2004 to 2008 inclusive. The microbial communities in the various soil samples were then assessed for changes in diversity using DGGE. Although the microbial diversity profiles were different between samples from different trees and soil types, the profiles for most of the samples tested did not appear to change significantly within the same rhizosphere over the 5-year period. This was the case for the samples from the poplar or willow native soils (PN and WN), and the poplar engineered soil (PE) (Fig 5-1). However, diversity did decrease in

the willow engineered soil (WE) as one DNA band completely disappeared over the 5-year study period.

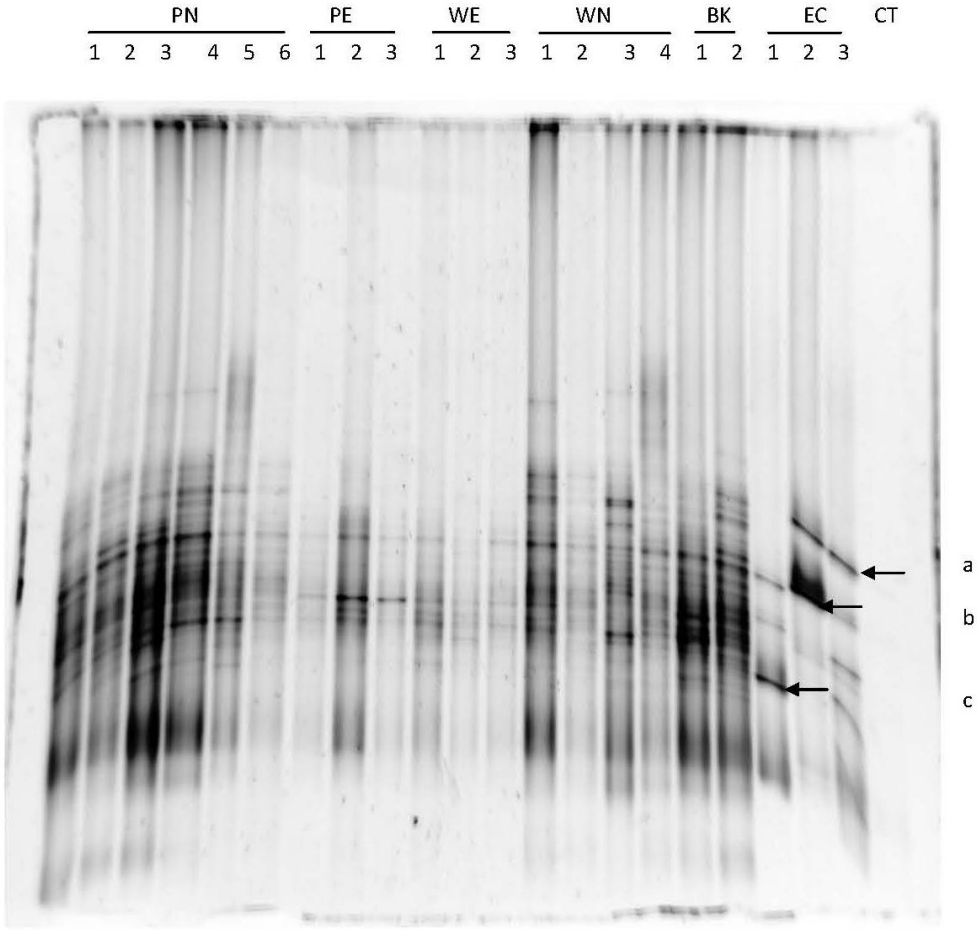

Figure 5-1 16S rDNA fingerprints of bacteria from a biphenyl phytoremediation site. Details concerning the different soil samples analyzed are listed in Table 5-1. With the exceptions of EC and CT, the codes used to identify the samples follow Table 5-1. EC 1 to 3 represent patterns derived from the three enriched cultures in Figs 5-2 and 5-3 with 1, 2 and 3 being poplar engineered, poplar native, and willow engineered, respectively. CT represents a PCR negative control.

Across the different samples, there is an observable trend that implicates soil type as an important factor affecting microbial diversity. The microbial community was more diverse in the native rhizosphere soil of either poplar (PN) or willow (WN) than the engineered soil (PE or NE). For most samples of the same soil type, the microbial community was very similar for both poplar and willow trees (compare PE and WE for engineered soil or PN and WN for native soil). Bulk soil (BK), where no trees had been planted, had a similar DGGE banding pattern to the native rhizosphere soil of both the poplar and willow trees (PN and WN). Soil depth in native soil did not affect the microbial diversity of the poplar rhizosphere in the 2006 (PN2~4) samples nor in the willow rhizosphere of 2004 to 2006 samples. However, in the latter case, the relative intensity of several bands at the same depth in 2004 and 2008 showed some differences. The effect of soil depth in engineered soils was not investigated.

The concentration of the contaminants in the impacted area did not have a significant effect as the microbial diversity was the same in the rhizosphere (PN and WN) where the concentration was high and in the native bulk soil (BK) where no contaminants was detected.

5.4.2 Enrichment of three aerobic biphenyl-degrading cultures

To determine whether aerobic biphenyl degraders are present on the site, serial enrichment experiments were set up to enrich for biphenyl degraders. The three rhizosphere soils used for the

enrichment cultures were from a poplar planted in the engineered soil (EC1), a poplar planted in native soil (EC2), and a willow planted in the engineered soil (EC3). All three samples were obtained in 2004. Aerobic biphenyl-degrading microorganisms were enriched from the three rhizosphere soil samples using 2 g/L of biphenyl as the primary carbon and energy source. After one week, the white biphenyl crystals disappeared from all flasks with rhizosphere soil but not from the abiotic sand control. After three serial transfers from the rhizosphere soil, there was little residual soil visible in the cultures with significant turbidity upon incubation.

Typical growth curves of the three transfer cultures are shown in Fig 5-2. The optical density at 600 nm increased quickly during the first four days after inoculation and displayed growth saturation thereafter. The concentration of biphenyl at the end of the one week incubation period was 0.4 mg/L, representing > 99.9% disappearance of the biphenyl fed into the cultures.

5.4.3 Biphenyl mineralization properties of the enriched cultures

The three resulting enriched cultures were analyzed directly for biphenyl-degrading properties using radioactive biphenyl usage. The amount of biphenyl mineralized by the three enriched cultures ranged from 8.5 to 16.4% in 50 days (Fig 5-3). Since only a small of amount of radioactivity (1.5%) was measured in the KOH trap of the un-inoculated control, mineralization in the biotic microcosms was due primarily to microbial activity. Since biphenyl completely

disappeared from the three enriched cultures in Fig 5-2, metabolic intermediates may be accumulating. The poplar rhizosphere consortium enriched from engineered soil exhibited the highest level of biphenyl mineralization. The willow rhizosphere consortium enriched from engineered soil displayed the lowest level of biphenyl mineralization.

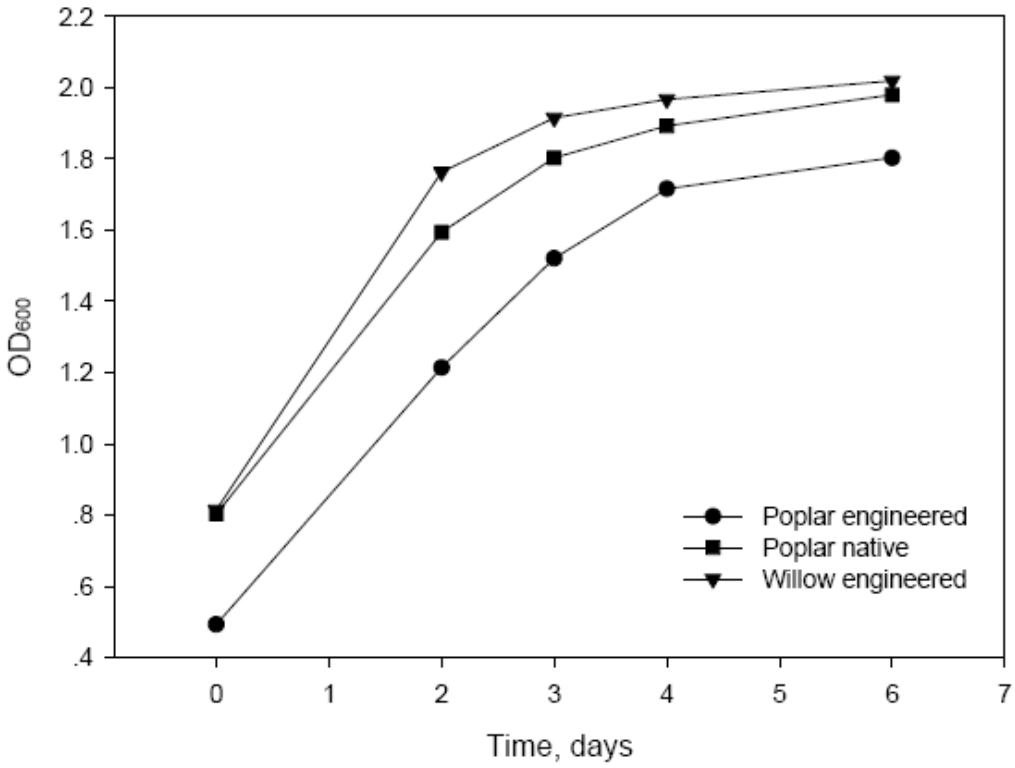

Figure 5-2 Aerobic growth on biphenyl by three consortia enriched from rhizosphere soils. The three separate rhizosphere soil samples were obtained from a biphenyl phytoremediation site where trees were planted either in native soil which had not been modified or in engineered soils which had been modified to a proprietary formulation. The three rhizosphere soils were from a poplar planted in the engineered soil (●), a poplar planted in native soil (■), and a willow rhizosphere planted in the engineered soil (▼). Shown are typical growth curves obtained after the third serial transfer at 23 ± 1 ℃.

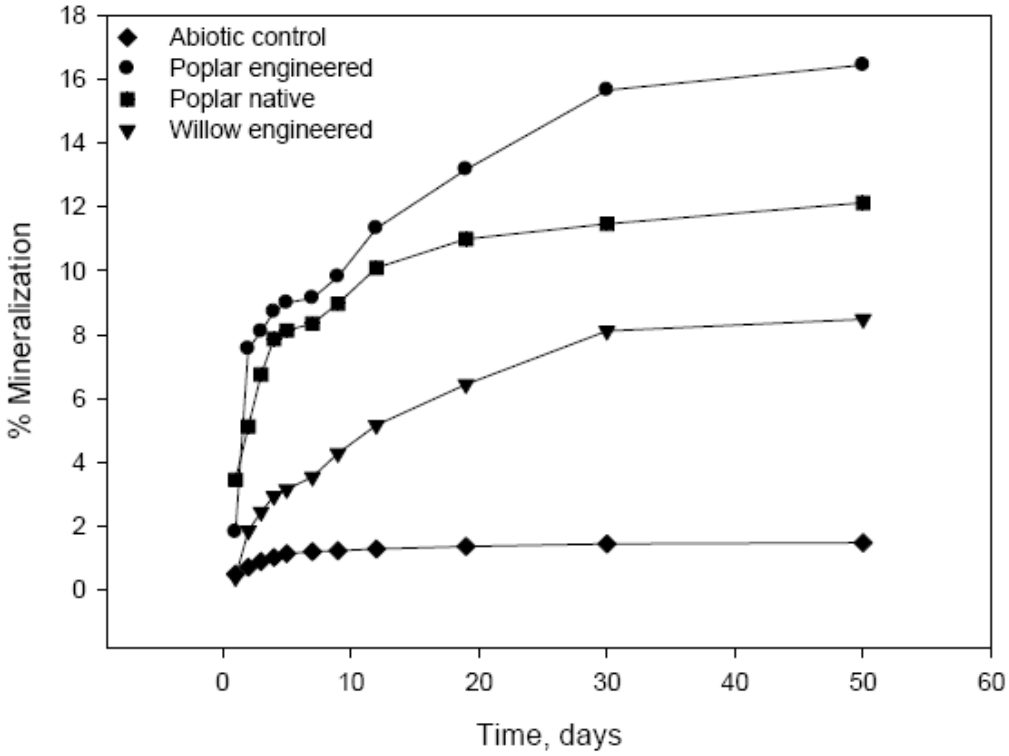

Figure 5-3 Aerobic mineralization of biphenyl by the enriched consortia shown in Fig 5-2. The three rhizosphere soils were from a poplar planted in the engineered soil (●), a poplar planted in native soil (■), and a willow rhizosphere planted in the engineered soil (▼). The abiotic control is shown for comparison (◆).

5.4.4 DGGE analysis of the enriched biphenyl-degrading cultures

The major DNA bands (labelled a, b, and c) in the three enriched consortia (EC1 to EC3) which degraded biphenyl were excised and tentatively identified. Band b was a major band universally present in all soil samples. Band a in EC2 (enriched from poplar native soil) and EC3 (enriched from willow engineered soil) were more abundant in native than the engineered soil.

Although band c was significantly present in the enrichment, it was not a major band in the soil microbial community. There was no significant change in intensity of these three bands over five years in any rhizosphere soil or the bulk soil (PN1, 3, 6; PE1-3; WE1-3; WN1, 2, 4; and BK1, 2) on the DGGE gels. When the DNA sequences of bands a, b and c were compared to GeneBank entries, the best matches were found to be *Flavobacterium*, *Alcaligenaceae*, and *Mycobacterium*, respectively (Table 5-2).

Table 5-2 Identification of the three major DNA bands of the enriched biphenyl-degrading cultures

Band	Representing microorganisms	Max identity %
a	*Flavobacteria* GASP-WC1W3_F09	99
b	*Alcaligenaceae* bacterium PB3-7B	100
c	*Mycobacterium sp*. GN-9188	99

5.5 Discussion

Phytoremediation is a complex process that could be influenced by a host of factors, from soil chemistry, physical characteristics, and pore water characteristics to microbial population dynamics. In this study, we set out to analyze the microbial rhizosphere community of a phytoremediation test-plot contaminated with biphenyl over a 5-year period to help better understand the phytoremediation process. Findings obtained from the field in turn should provide

insights for improving the remediation strategy. This particular analysis was focused specifically on the composition of the biphenyl-degrading microbial population over a 5-year period, and the effects of different soil types and different tree types on the microbial community. The results clearly show that the indigenous rhizosphere microorganisms were capable of aerobically degrading biphenyl. We were able to create three separate enriched consortia with biphenyl-degrading capabilities using soil samples from three different sites of the phytoremediation test-plot. The dominant DNA bands from the amplification of 16S rDNA in the DGGE gels matched sequences from organisms belonging to the family of *Flavobacteriaceae*, *Alcaligenaceae*, and *Mycobacteriaceae*. *Flavobacterium sp.* (Stucki and Alexander 1987). *Alcaligenes* (Dercová et al. 1995), and *Mycobacterium* Strain PYR-1 (Moody et al. 2002) have been reported previously to aerobically degrade biphenyl. Other microbes in the soil might also aerobically degrade biphenyl but these were likely the fastest growing ones on biphenyl.

Although microbial community profiles differed between tree type and soil type, the diversity of most samples from the same rhizosphere changed very little over the 5-year study period (Fig. 5-3). The concentration of biphenyl in the groundwater is likely too low (7.5 ppm) to contribute significantly to changes in microbial growth and diversity. In this test-plot, the concentration of another contaminant ethylene glycol is present up to 120,000 ppm in some areas (Carnegie 2006), and the ethylene glycol degrading population may be larger than the biphenyl degraders, but the

change of potential ethylene glycol degraders was not found by DGGE analysis. Furthermore, there was a great deal of other vegetation on the site that may have reduced any impact exudates from the poplar and willow roots have on microbial growth and diversity. Salles et al. (2004) had also found that plant species had less effect on a *Burkholderia* community than the history of land use. The only exception to this general lack of change observed in this study was a decrease in the microbial diversity of the willow engineered soil over the 5-year period. This willow tree might have promoted the growth of specific soil microbes and/or inhibited others. Since a change was not observed in the willow native soil, the rhizosphere microorganisms were likely affected by a combination of both soil type and tree type.

Soil type appears to exert a more significant impact on the diversity of the microbial community. A more diverse community was observed in the native soil samples than in the engineered soil samples for both poplar and willow trees (Fig 5-3). This is partly consistent with the results reported by Palmroth et al. (2007). They found that soil type was the main factor affecting microbial communities in the phytoremediation of soil contaminated with hydrocarbons and heavy metals. However, the effect of soil type and tree species appears to be very complex since a change in microbial diversity over time was seen only in the willow engineered soil in our study. Marschner et al. (2001) also found that rhizosphere bacterial communities can be affected by a complex interaction between soil type, plant species, and root zone location. Since different properties like hydraulic conductivity and pore water characteristics between the native and

engineered soils might also affect microbial community, the result of soil type shows the effect of a combination of various physical and chemical factors.

Differences in soil depth of 1 m (Brad et al. 2008) and even 1 cm (Beck et al. 2006) have been reported to affect the microbial community. However, in this study, soil depths between 3 and 80 cm (at about 15cm intervals) did not have a significant effect on the poplar rhizosphere microbial community in native soil. This result agrees with Shamir and Steinberger (2007) who showed no significant difference in the microbial biomass in the rhizosphere of *Tamarix aphylla* within a 50-cm soil depth. Even though the microbial community of the same rhizosphere was similar within a vertical distance of 80 cm, neighboring trees in different soil types (native vs engineered) did display different diversity patterns. Different diversity patterns were observed even though the trees were only 1 m apart. These patterns indicate that the microorganisms did not migrate freely, at least horizontally, in the soil matrix. This pattern may be related to the slow movement of groundwater flow in the native soil which is mainly composed of clay.

In summary, this study demonstrated that the indigenous rhizosphere microorganisms of poplar and willow trees were capable of aerobically degrading biphenyl and that microbial diversity in the field was affected by multiple factors. Soil type appeared to exert the most significant impact whereas tree type, soil depth, and location exerted the least.

5.6 Acknowledgements

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Chapter 6

Analysis of a mixed microbial population by direct PCR

6.1 Abstract

The effectiveness of direct polymerase chain reaction (DPCR) without prior DNA extraction was studied as a fast way to analyze microbial populations. Denaturing gradient gel electrophoresis (DGGE) analysis of dioxane- and biphenyl-degrading consortia in the early and late stages of enrichment showed that DPCR yielded the same results as PCR assays using extracted DNA. Therefore, the combination of DPCR and DGGE could be used for the fast analysis of the microbial populations of environmental and clinical samples with limited diversity.

6.2 Introduction

The ability to monitor microbial populations in environmental, food and clinical samples is crucial for the proper management of human health and safety. Detection or monitoring techniques should therefore be fast, sensitive, inexpensive, and applicable to small sample sizes. Culture-based detection methods such as colony/cellular morphology, Gram reactions, enzymatic and/or metabolic assays, are generally not sufficiently specific, are labour intensive and/or may take days or weeks to complete, especially when analyzing a large number of samples.

Monitoring techniques based on molecular approaches, such polymerase chain reaction (PCR), offer faster and more specific strategies for microbial detection. Of the many detection techniques, the combination of PCR with denaturing gradient gel electrophoresis (DGGE) is the most widely used method in microbial consortia and community analyses (Spiegelman et al. 2005). Traditionally, cells are lysed in order to purify high quality DNA templates prior to PCR. Cell lysis and DNA preparation require additional time to carry out and is an added expense to the monitoring process. Furthermore, PCR results could be biased by the DNA purification process since different microorganisms possess varying levels of resistance to enzymes or mechanical forces used to disrupt cells.

PCR assays can be carried out without prior DNA preparation in a technique known as direct PCR (DPCR). Since DPCR does not require the time-consuming and tedious DNA purification step, monitoring becomes faster, less costly and simpler. A smaller sample size may also be used since DNA loss during the extraction step is avoided. DPCR has been used to effectively amplify DNA contained in whole cells of blood samples (Mercier et al. 1990), solid murine liver tissue (Panaccio et al. 1993), fruitflies and blood flukes (Grevelding et al. 1996), bacteria (Fode-Vaughan et al. 2001, Fode-Vaughan et al. 2003, Benson et al. 2004, Klocke and Mundt 2004, Layton et al. 2006), and plant tissue (Yang et al. 2007). Previous reports indicate that DNA can be released effectively from *Esherichia coli* by boiling for 15 to 40 seconds (Holmes and Quigley 1981) or by incorporating in the PCR protocol a hot start step at around 95^{o}C for 5 to 10 minutes.

The DNA released from these intact bacterial cells was of sufficient quality to obtain amplified PCR products (Fode-Vaughan et al. 2001, Benson et al. 2004, Layton et al. 2006).

DPCR has been used successfully with bacteria to detect either a single species, for example, *E. coli* O157:H7 (Fode-Vaughan et al. 2003) or *Helicobacter pylori* (Benson et al. 2004), or a group of microorganisms such as *Bacteroides* (Layton et al. 2006) or methanotrophs and photosynthetic bacteria (Fode-Vaughan et al. 2001). Target organism(s) are identified by using DNA primers for species- or group-specific gene sequences. For example, primers for methane monooxygenase were used to detect methanotrophs (Fode-Vaughan et al. 2001), the Shiga toxin for *E. coli* O157:H7 (Fode-Vaughan et al. 2003) or subunit A of the *Helicobacter pylori* urease (Benson et al. 2004). However, 16S rDNA universal primers in DPCR have never been previously combined with DGGE for microbial community analysis. The only study in which 16S rDNA universal primers have been used in DPCR, only demonstrated the presence of bacteria using agarose gel electrophoresis. This study demonstrates the feasibility of using DPCR (without prior DNA extraction) with two universal 16S rDNA primers in combination with DGGE to characterize several enriched cultures and is compared to results with extracted DNA as the template.

6.3 Materials and methods

6.3.1 Composition of culturing medium

Each liter of basal salts medium (BSM, modified from Cote and Gherna 1994) contained 100 mL BSM stock solution and 100 mL trace element solution. The BSM stock solution contained per liter: 32.4 g K_2HPO_4, 10 g $NaH_2PO_4 H_2O$, and 20 g NH_4Cl. The trace element solution contained per liter: 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 7H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 7H_2O$, 0.1 g $CoCl_2 6H_2O$, 0.132 g $CaCl_2 2H_2O$, 0.1 g $ZnSO_4 7H_2O$, 8.74 mg $CuSO_4 5H_2O$, 10.0 mg $AlK(SO_4)_2 12H_2O$, 10.0 mg H_3BO_3, 10 mg $Na_2MoO_4 2H_2O$, 27.1 mg $NiCl_2 6H_2O$, and 20.0 mg $Na_2WO_4 2H_2O$.

6.3.2 Enrichment cultures

Early and late stage serially-enriched cultures containing dioxane- or biphenyl- degraders from contaminated soil were used in the microbial population analysis. To obtain an enriched consortium, soil from a contaminated groundwater plume was added to 125 mL serum bottles containing 100 mL basal salts medium (BSM) and incubated aerobically at 25 $\pm$ 1^{o}C and 200 rpm on an Innova rotary shaker. Tetrahydrofuran (THF, 100 ppm (v/v)) (a structural analog of dioxane) and dioxane (100 ppm (v/v)) was used as the only source(s) of carbon and energy to enrich for dioxane-degrading microbes. Similarly, biphenyl (1 g/L) was used to enrich biphenyl-degrading

microbes. Five serial transfers were performed in both cases. Each transfer was carried out after dioxane or biphenyl decreased to almost undetectable levels. At this point, a 10% volume was used to inoculate fresh medium. After five such transfers, the cultures were designated "early stage" enriched cultures. "Late stage" enriched cultures were obtained by plating the early stage cultures on BSM glucose (10g/L) agar plates and verifying which isolates were dioxane or biphenyl degraders in aqueous BSM medium with either both THF and dioxane or biphenyl alone.

6.3.3 Template preparation

In DPCR, concentrated intact cells were used as the template source in PCR amplifications. The enriched dioxane- or biphenyl-degrading culture (1 mL) was centrifuged at 8000 *xg* for 2 min in a bench top centrifuge (Eppendorf 5415C), washed twice with 0.5 mL deionized water, resuspended in 50 μL deionized water and then used as the source of DNA template. In conventional PCR, the total DNA in the pellet obtained after centrifugation and washing was extracted following the supplier's protocol of a bacterial DNA extraction kit (Omega Bio-Tek, Calgary, Alberta).

6.3.4 PCR amplification

The DNA contained within concentrated intact cells (1μL) or extracted DNA (1μL) was amplified with two universal 16S rDNA primers, U341-GC2 (GCGGGCGGGGCGGGGGC ACGGGGGGCGCGGCGGGCGGGGCGGGGGCCTACGGGAGGCAGCAG) and U758 (CTA CCAGGGTATCTAATCC) (Fortin et al. 2004) using a T1 thermocycler (Biometra, Kirkland, Quebec). The PCR tubes were preheat at 94°C for 3 min. After the first 10 cycles of 1 min at 94°C, 1 min annealing at 65°C stepping down 1 degree each cycle to 55°C, and 3 min extension at 72°C, there were 20 cycles of 1 min at 94°C, 1 min at 55 °C, and 3 min at 72°C. Each 50 μL PCR reaction contained 1 μL of extracted total DNA or 1 μL of concentrated cells, 8 μL of 1.25 mM dNTPs, 1 μL of each primer U341-GC2 and U758 (25 pmol each), 4 μL 25 mM $MgCl_2$, 5 μL of 10X reaction buffer and 2.5 units of Taq polymerase (Fisher Scientific Ltd., Canada). The amplification DNA products were separated on an 8% (w/v), 0-80% denaturing gradient acrylamide gel in 1X TAE buffer. The gel was run at 80 V for 16 h at 60°C and stained for 10 min in 1X TAE containing SYBR Gold (Invitrogen, Burlington, Ontario).

For identification, the 16S DNA bands were excised and eluted with water at 4 ℃ overnight, precipitated with ethanol, re-amplified for 25 cycles of 1 min at 94 ℃, 1 min at 55 ℃ and 1 min at 72 ℃ using the U341 and 758 primers, and sequenced (Cortec, Kingston, Ontario). The 16S DNA sequences were matched using BLAST (Altschul et al. 1997).

6.3.5 Analytical methods

Dioxane and THF analysis

Liquid culture samples were filtered through a 0.45 μm nitrocellulose filter (Millipore) before use. The filtrate (1.5 μL) was then mixed 200 ppm (v/v) 2-pentanol as the internal standard. The resulting mixture was then injected into a Varian 3400 gas chromatograph equipped with a flame ionization detector and a 5 m guard column attached to a 30 m x 0.53 mm column (Restek stabilwax). The flow rates of helium, hydrogen and air were 4, 50, and 300 mL/min, respectively. The injector and detector temperatures were held constant at 150 and 180°C, respectively, and the oven temperature profile was a 4 min hold at 60°C, followed by a ramp of 20°C/min ending at 150°C with a 1 min hold time.

Biphenyl analysis

Liquid culture samples were centrifuged at 8000 $\times g$ for 2 min in a bench-top centrifuge (Eppendorf 5415C). The supernatant was extracted with an equal volume of hexane and then supplemented with 5 ppm (v/v) of 2-fluorobiphenyl (the internal standard) before being analyzed by GC-MS (Varian 1200). GC parameters were: injector temperature 250 ℃, detector temperature 275°C, 1 μl injection, a split ratio of 10, and HP-1 column (60 m long x 0.32 mm i.d.). The oven temperature profile was: 90 ℃ for 1 min, 10 ℃/min to 150 ℃, 20 ℃/min to 275 ℃

and isothermal at 275 ℃ for 5 min. Mass range: full scan ion monitoring (m/z: 40-500), scan time was 0.5 second, with electron ionization at 70 V in a quadrupole mass analyzer.

6.4 Results

The four biphenyl-degrading consortia (designated as # 1-4) in this study were obtained from contaminated soil from four different sampling locations on the same site. The dioxane-degrading enrichment culture was established and was shown to co-metabolize dioxane using THF as the primary substrate. Intact cells or extracted DNA of the dioxane-degrading consortium and only biphenyl-degrading consortium 4 were used for the early-stage study (Fig 6-1). Consortium 4 displayed the highest level of biphenyl degradation. For the late-stage experiments, all five consortia were used in the assessment (Fig 6-2). All experiments were conducted with two 16S rDNA universal primers. When the resulting amplified products were compared by DGGE, the DNA band patterns were found to be similar between DPCR and PCR (Fig 6-1 and Fig 6-2). This was case for early- and late-stage enrichment cultures (Fig 6-1 and Fig6-2). In the late-stage experiments, fewer DNA bands were observed when compared to the corresponding early-stage patterns (Fig 6-1 vs. Fig 6-2). The dominant bands were now stronger than their counterparts in the early-stage profiles. It is likely that the microorganisms which were not involved in degradation were eliminated in the later-stage enrichment cultures.

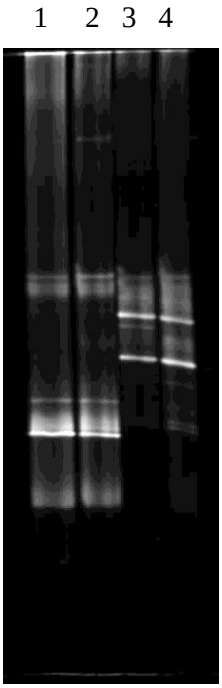

Figure 6-1 DGGE profiles for early stage enrichment cultures of biphenyl- (lanes 1 and 2) and dioxane- (lanes 3 and 4) degrading consortia. PCR amplifications were done directly with intact cells (DPCR, lanes 1 and 3) or with DNA extracted prior to PCR (lanes 2 and 4). Identification details for the DNA bands marked a, b, c and d are summarized in Table 6-2. Biphenyl-degrading consortium #4 was used in this experiment.

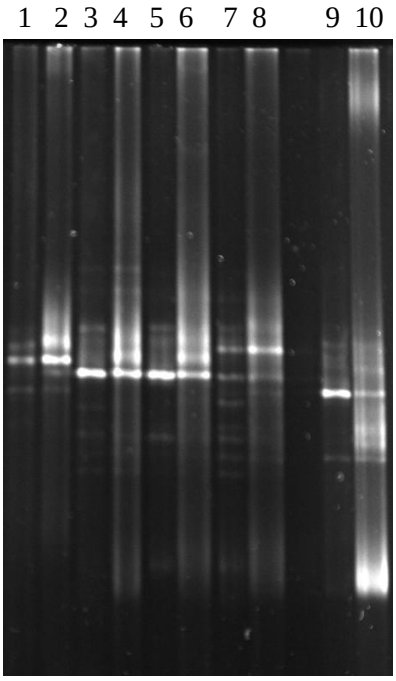

Figure 6-2 DGGE profiles for late stage enrichment cultures of dioxane- (lanes 1 and 2) and four biphenyl- (lanes 3 to 10) degrading consortia. The DPCR procedure was used in lanes 1, 3, 5 and 7. PCR amplifications using extracted DNA were used in lanes 2, 4, 6 and 8.

Dioxane-degrading consortium: DPCR (Lane 1); extracted DNA (Lane 2)
Biphenyl-degrading consortium #1: DPCR (Lane 3); extracted DNA (Lane 4)
Biphenyl-degrading consortium #2: DPCR (Lane 5); extracted DNA (Lane 6)
Biphenyl-degrading consortium #3: DPCR (Lane 7); extracted DNA (Lane 8)
Biphenyl-degrading consortium #4: DPCR (Lane 9); extracted DNA (Lane 10)

The changes in DNA patterns described above for early- and late-stage enrichment cultures appear to reflect what was happening at the chemical degradation level. The population shift from early to late stage enrichments is reflected in the increased rates of degradation by the late stage enrichment cultures (Table 6-1) for both biphenyl and dioxane degradation. The

degradation rates in Table 6-1 are averaged from duplicate experiments during the exponential disappearance of the target compound.

Table 6-1 Biphenyl- or dioxane-degradation properties of the enriched consortia

	Initial conc. (mg/L)	% Degradation	Degradation rate (mg/L/day)
Biphenyl #4			
Abiotic control	1000	0	0
Early stage enrichment	1000	99.9	142.9
Late stage enrichment	1000	99.9	200.0
Dioxane			
Abiotic control	100	1.4	0.07
Early stage enrichment	100	64.2	2.9
Late stage enrichment	100	95.0	5.0

For further identification purposes, the 16 S rDNA PCR products were excised from DGGE gels, eluted, re-amplified, and sequenced. The best matches for the two dominant bands in the early-stage enrichment cultures of biphenyl degraders were two species of *Burkholderia* (Table 6-2). From three independent series of enrichments of the same contaminated soil, two enriched for *Burkholderia sp.* CCBAU 11189 as the dominant organism in the late-stage enrichment cultures (Fig 6-2, biphenyl-degrading consortia #1 and #2). The third had both *Burkholderia* strains, but

mainly the *Burkholderia xenovorans* strain LB400. For the profiles developed from the early-stage enrichment culture of dioxane degraders, there were two DGGE bands. One had 99% homology with *Flavobacterium sp*. IP10 and the other was an uncultured bacterial isolate which disappeared in the late-stage enrichment steps. These findings were independent of whether the PCR template was extracted DNA or the DNA in the whole cells.

Table 6-2 Identification of the major DGGE bands of the early stage biphenyl-or dioxane-enrichment cultures used in Fig 6-1

Early stage enrichment	Best match	% Identity
Biphenyl consortium #4		
a	*Burkholderia xenovorans* strain LB400	100
b	*Burkholderia sp.* CCBAU 11189	94
Dioxane		
c	*Flavobacterium sp.* IP10	99
d	Uncultured bacterium	98

6.5 Discussion

This study was undertaken to determine whether 16S rDNA universal primers could be used in DPCR to characterize microbial populations of limited diversity without prior extraction of the community DNA. In previously reported microbial applications, DPCR was most often used with

specific primers to detect pathogens such as *E. coli* O157:H7 (Fode-Vaughan et al. 2003), *Helicobacter pylori* (Benson et al. 2004), and fecal *Bacteroides* (Layton et al. 2006) or a group of microorganisms which filled a particular ecological niche, i.e. methanotrophs and photosynthetic bacteria (Fode-Vaughan et al. 2001). To our knowledge, the use of universal primers such as 16S rDNA and DPCR was coupled only to agarose gel electrophoresis as an analytical tool (Fode-Vaughan et al. 2001). This combination can only confirm the presence of bacteria. Additional information about how many possible bacterial species are present and their relative concentrations cannot be resolved by standard agarose gels. DGGE separates the amplified DNA products by their melting points and can differentiate DNA sequences within one base pair difference (Myers et al. 1985). The number and intensity of DNA bands on DGGE gels can also give an estimate of the number of different microorganisms and their relative concentrations. So far, the PCR products analyzed by DGGE were derived using extracted DNA templates. To our knowledge, the combination of DPCR and DGGE has not been used to characterize mixed bacterial populations to date. In this study, DPCR gave the same results as with extracted DNA in the DGGE analysis of bacterial populations in several enriched cultures. This allowed us to eliminate the extra step of DNA preparation, and established DPCR as an effective and efficient method in the analysis of microbial populations of limited diversity.

The presence of bile salts, polysaccharides, heme, and humic acids can inhibit PCR reactions (Rådström et al. 2004) and can be problematic when studying or monitoring soil and groundwater

samples. Although DPCR was unsuccessful with undiluted groundwater samples (Fode-Vaughan et al. 2001, 2003), an appropriate dilution was able to relieve the inhibition and Layton et al. (2006) found that creek water from different watersheds did not inhibit DPCR. In the current study, PCR inhibition was not observed with the enriched cultures since potential inhibitors were likely removed during serial enrichment or the cell washing step in template preparation. Therefore, DPCR can be applied directly to the samples with low concentration of inhibitors. For samples with a high concentration of inhibitors, simple dilution (Fode-Vaughan et al. 2001) or washing (Fortinet al. 2004) might be enough for a successful DPCR.

Insufficient or preferential disruption of cells might bias results in microbial diversity, but the lysis should not be too rigorous to generate highly fragmented DNA (von Wintzingerode et al. 1997). DNA extraction by thermal lysis has been compared to other cell lysis protocols (Freschi et al. 2005, Leuko et al. 2008). For *Halococcus* which has rigid cell walls and is difficult lyse, thermal lysis was less effective in DNA release but badly sheared DNA was generated using a combination of bead beating, chemical lysis with lysozyme, and thermal shock (Leuko et al. 2008). In detecting *Salmonella*, thermal lysis achieved the similar results as using commercial kit and was thus recommended by Freschi et al. (2005). In our study, preheating at 94 ^{o}C for 3 min effectively released the DNA, and the microbial community was the same as obtained using a combination of enzymatic, chemical, and heating protocol. As *Escherichia coli* DNA was effectively released by boiling for only 15 to 40 s (Holmes and Quigley 1981), DPCR should

work for most cell types. For cells with rigid walls, a longer preheating time at 94^{o}C before PCR cycles should be applied.

In summary, it was demonstrated that DPCR can be successfully used in combination with DGGE to characterize enriched microbial cultures and obtain the same results as when extracted and purified DNA was used as the PCR template. DPCR clearly has potential in the analysis of microbial populations in samples of limited diversity.

6.6 Acknowledgements

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Chapter 7

Conclusions and Recommendations

7.1 Conclusions

1. The soil microorganisms at the contaminated site have the potential to degrade dioxane. A dioxane-degrading culture identified as a *Flavobacterium* by 16S rDNA sequencing was enriched from the contaminated soil. It co-metabolized dioxane in the presence of THF, and was able to grow on glucose, and maintain its dioxane degradation capability after several transfers between glucose and THF/dioxane.
2. The indigenous soil microorganisms present in the rhizosphere of poplar and willow trees in the phytoremediation test-plot were able to degrade biphenyl anaerobically. An anaerobic fungus, *Pseudallescheria boydii*, was isolated from the enriched culture and was unable to degrade biphenyl under aerobic conditions. Nutrients in BSM, fertilizer, TEAs (sulfate and carbon dioxide) significantly enhanced anaerobic biphenyl degradation. Root exudates might enhance biphenyl degradation, but the effect was not significant.
3. The microbial community in the rhizosphere of poplar and willow trees of a phytoremediation test-plot was investigated over a 5-year period. Soil type had a significant impact on the microbial community composition. A more diverse microbial population was found in native than in engineered soil. Tree type had less influence on microbial diversity,

but diversity did decrease with time in the engineered soil of the willow rhizosphere. Aerobic biphenyl degraders were found to be widely present in the contaminated site and the major organisms in enriched consortia were identified by 16S DNA sequencing as being in the family of *Flavobacteriaceae*, *Alcaligenaceae*, and *Mycobacteriaceae*.

4. Direct PCR without prior DNA extraction was shown to be a quick and effective way to analyze the microbial population in enriched cultures. DGGE analysis of dioxane- and biphenyl-degrading consortia in the early and late stages of enrichment showed that DPCR yielded the same results as PCR assays using extracted DNA.
5. The studies on microbial degradation of biphenyl and dioxane elucidated the presence of dioxane and biphenyl degraders and potential microbial degradation of dioxane and biphenyl at the industrial site. The enriched dioxane degrading *Flavobacterium* can be used for on ground *ex situ* dioxane treatment whenever "pump-and-treat" is necessary. Fertilizer and TEAs can be added to groundwater to enhance *in situ* biphenyl degradation.

7.2 Recommendations

1. The dioxane degradation pathway by the *Flavobacterium* isolate should also be determined as it will add to the limited pool of knowledge in regards to dioxane degradation. It is also useful

to demonstrate that degradation products are not toxic, or at least, are less toxic than dioxane. This may be evaluated using the Microtox toxicity assay which uses *Vibrio fisherii* as the test organism. Although glucose can be used for the growth of this *Flavobacterium* culture, a less toxic inducer than THF is needed to achieve dioxane degradation and such an inducer should be identified.

2. It is of interest to elucidate the pathway for the anaerobic degradation of biphenyl by the fungus, *Pseudallescheria boydii*, and to determine the range of PCB that it could degrade. Other factors to enhance biphenyl degradation such as the presence of surfactant should also be investigated. Biosurfactants have been shown to enhance the availability of the biphenyl to microorganisms and plants.
3. An interesting result obtained from the analysis of the rhizosphere microbial community is that diversity decreases with time in the engineered soil. It might be important to know the identity of the lost microorganisms and whether they are related to the degradation of other contaminants on the site.

Chapter 8

Contributions

1. Dioxane degradation

A dioxane-degrading culture enriched from dioxane-contaminated soil co-metabolized dioxane with THF, and was identified as a *Flavobacterium*. This is the first report of dioxane degradation by a *Flavobacterium* and it is phylogenetically distinct from the previously reported dioxane degrading microorganisms. The *Flavobacterium* was able to grow on glucose, and have a stable dioxane degradation capability after transfers between glucose and THF/dioxane. This ability is highly advantageous for the large-scale industrial applications, and was not reported in previous dioxane degradation studies. Adding *Flavobacterium* to the short list of the dioxane-degrading microorganisms will add to the knowledge of microbial dioxane degradation, and provide a new option for an industrial-scale for dioxane degradation. In addition to the traditional microbial isolation techniques, PCR and DGGE was shown to be a useful assessment tool for isolating pure cultures of contaminant degraders in this study. It will be especially useful for the enrichment of microorganisms capable of degrading environment contaminants recalcitrant to biodegradation. It might also be essential for the isolation of slow-growing microorganisms in a consortium.

2. Anaerobic biphenyl degradation

A fungus, *P. boydii*, enriched from a biphenyl-contaminated soil, anaerobically degraded biphenyl. To the author's knowledge, this is the first report of a fungus capable of degrading biphenyl anaerobically. Two less common metabolites (o-hydroxybiphenyl and p-hydroxybiphenyl) were detected indicating that the anaerobic biphenyl degradation pathway is different from the previously reported biphenyl degradation pathway. This study illustrated the potential of biphenyl degradation by a fungus and it will be of interest to see more research on this topic. In addition to previously reported TEA, the effect of BSM, fertilizer, and root exudates on anaerobic biphenyl degradation was investigated. Although not all the factors were statistically significant under the investigated conditions, enhancement of biphenyl degradation was seen. This provided useful information for applying anaerobic biphenyl biodegradation at the industrial scale.

3. Rhizosphere microbial community analysis

It is important to know how the rhizosphere microbial communities of poplar and willow trees are affected during the phytoremediation of organic contaminants. To author's knowledge, no such research has been reported for biphenyl. This thesis thus represents the first field analysis of microbial communities during biphenyl phytoremediation. The three aerobic biphenyl-degrading consortia obtained in this research can be potentially used to enhance the *in situ*

biphenyl degradation by bioaugmentation. Since they were enriched from the contaminated site, they might have a better probability of being compatible with the resident soil community of a phytoremediation site.

4. DPCR for the analysis of microbial populations

DPCR has been previously used to analyze a specific microbial species, but not for a mixed microbial population. In this study, DPCR yielded the same results as PCR assays using extracted DNA showing that DPCR without prior DNA extraction together with DGGE was an efficient method in microbial population analysis of an enriched consortium. The combination of DPCR and DGGE is fast, simple and less costly, and can be very useful in the analysis of microbial populations in samples of limited diversity.