ASSESSMENT OF AN ALASKAN WILLOW WITH POTENTIAL FOR POLYCHLORINATED BIPHENYL RHIZOREMEDIATION

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A

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Abstract

Polychlorinated Biphenyls (PCBs) are priority pollutants targeted for remediation efforts in many areas of the world. Rhizoremediation, the use of plants and their associated root microorganisms for the degradation of a pollutant, may be an inexpensive and effective approach to biodegrading PCBs and detoxifying contaminated soils *in situ*. Two Alaskan native tree species, *Salix alaxensis* (willow) or *Picea glauca* (white spruce), were assessed for the ability to stimulate microbial PCB degradation by measuring PCB loss, toxicity and microbial community shifts in soil microcosms following the addition of crushed roots. Incubation of polluted soils with root crushates of *S. alaxensis* led to significant losses (up to 30% in 180 days) of several PCB congeners, including many of the most toxic congeners, PCB 77, 105 and 169. Soil toxicity, measured using the Microtox assay, also decreased as a result of treatment with willow-roots. Treatment of soils with salicylate, a willow secondary compound hypothesized to promote aromatic pollutant biodegradation, inhibited PCB degradation, suggesting that other willow compounds are responsible for biostimulation. The disappearance of PCB congeners, detoxification of soil, and detection of microbes with PCB degrading abilities suggests that *S. alaxensis* is a promising plant candidate for rhizoremediation of PCBs.

Table of Contents

Page

List of Figures

Page

List of Tables

Page

Introduction

Polychlorinated biphenyls (PCBs) were widely used throughout the world for about four decades, up until worldwide production ceased in the mid-1980s. About 1.5 million tons of PCBs were produced worldwide and the majority of that is thought to have been released into the environment. PCBs are recalcitrant in the environment, subject to global transport and have potential to enter the food chain and adversely affect the health and development of humans and wildlife.

Due to these health risks, PCBs are now high priority targets for remediation efforts in many areas of the world. Rhizoremediation, the use of plants and associated root microorganisms for the degradation of a pollutant, is a possible alternative to traditional remediation methods for PCBs. Rhizoremediation relies on the roots of certain plant species to selectively foster the growth and activity of microbes with contaminant-degrading capabilities in the rhizosphere or the root zone. Plants release a wide variety of compounds from their roots that vary among plant species, with some being effective at promoting microbial degradation of PCBs. There is a need to identify effective plant species and to better understand the mechanisms of rhizoremediation in order to optimize this technology..

This thesis includes a review of PCB rhizoremediation studies including a discussion of the limitations of the current state of the field, and how those limitations may be addressed in future studies. Plant compounds may aid in stimulation of microbial degradation of PCBs in several ways, including providing a cometabolic substrate for

PCB degradation and/or increasing the bioavailability of PCBs in soils. The review addresses these possibilities as well as other approaches to rhizoremediation, such as genetic engineering.

Chapter two is an assessment of the ability of Alaskan tree species to promote PCB rhizoremediation. Crushed roots of two different plant species or chemical amendments were added to PCB contaminated soil microcosms, and PCB concentrations, soil toxicity and microbial community structure were analyzed following incubations of 180 days. The common Alaskan willow, *Salix alaxensis,* was found to promote the loss of several PCB congeners from polluted soils, including highly toxic congeners such as PCB-77, -105, and -169, while white spruce, *Picea glauca*, was not effective. Soil toxicity was also reduced as a result of treatment with willow roots. Addition of the willow secondary aromatic compound, salicylate, did not promote PCB degradation, suggesting that other chemical components of willow were responsible for its stimulatory effect. Microbial community analyses revealed that the various treatments resulted in shifts in community composition and/or structure. This finding supports the idea that willow species may be useful for PCB rhizoremediation.

The final chapter describes supplemental studies using unique methods to detect PCB degradation by microbes and to identify those microbes. This includes an attempt to detect enantioselective degradation of chiral PCB congeners PCB 95 and 149, which would be unique to biological transformation of the compounds, rather than losses to physical or chemical processes. Additionally, stable isotope probing (SIP), a molecular

technique that can help link metabolic function with taxonomic identity without the use of culturing, was explored in an attempt to indentify microbes involved in PCB degradation.

The authors who contributed to this work are designated in the footnotes for individual chapters. For all chapters, I, Heather Slater performed the laboratory work, data analysis and preparation of the thesis chapters. Dr. Mary Beth Leigh, co-author for all chapters, acted as primary advisor for the project, providing funding for materials, training for laboratory techniques including microbial methods from chapters two and three, and editing for all chapters. Dr. Todd Gouin, co-author for chapters two and three, provided the use of laboratory space and training for chemical extractions, instrumentation and data analysis of PCB congener concentrations for data included in chapters two and three. This project was supported by the National Science Foundation under Award 0626544, a graduate fellowship from Alaska Idea Network for Biomedical Research Excellence (INBRE) and the UAF Institute of Arctic Biology.

Chapter 1: Rhizoremediation of PCB contaminated soils: A review[1]

1.1 Abstract

Polychlorinated biphenyls (PCBs) are persistent organic pollutants of concern in many areas of the world. Remediation is often mandatory, yet conventional methods such as capping or incineration are quite costly. Rhizoremediation, the use of plants and their associated root microbes for the degradation of a pollutant, is a promising and cost-effective strategy for PCB remediation. This review will cover the background of PCB contamination, toxicity and remediation strategies, with a discussion of specific plants and plant compounds that may be effective for PCB rhizoremediation.

[1]Slater, H and Leigh, MB. University of Alaska Fairbanks. 2010. Rhizoremediation of PCB contaminated soil: A review. Prepared for submission to Environmental Science and Technology.

1.2 Introduction

Polychlorinated biphenyls (PCBs) were widely used throughout the world for about four decades, up until worldwide production ceased in the mid-1980s. In the United Sates, technical mixtures containing at least fifty congeners were sold and marketed by the Monsanto Corporation under the trade name "Aroclor." Similar commercial mixtures were sold in Japan, Italy, Germany, the Czech Republic and many other industrialized countries under different names. Many attractive qualities such as heat stability and a lack of chemical reactivity lead to the use of PCBs in many products from paint to electrical devices. About 1.5 million tons of PCBs were produced worldwide and the majority of that is thought to have been released into the environment. The same physical and chemical properties that made them popular in many industries contribute to our concern about the compounds today. PCBs are recalcitrant in the environment because they are not easily degraded by chemical or biological processes. They are also subject to global transport and have been detected in every part of the world, even where they were not used in industry. This is of concern because of their potential to enter the food chain and adversely affect the health and development of humans and wildlife.

One particularly disconcerting characteristic is that PCBs accumulate in fatty tissues of humans and other animals. PCBs have been found in the breast milk of women living in areas geographically distant from historically contaminated sites, and there is research to suggests that PCBs may be able to cross the placenta (Dekoning and Karmaus

2000). Several of the 209 possible congeners are highly toxic causing a variety of illnesses and even death. The most acutely toxic PCBs are dioxin-like in overall shape and are non-*ortho* substituted, such as PCB77 (3,3',4,4'). These acutely toxic PCBs bind the same aryl hydrocarbon receptor (Ahr) as dioxins (Van den Berg et al. 2006). In addition to acute toxicity, PCBs can also have chronic toxicity due to long term exposure. For example, PCBs are considered a probable human carcinogen, and are also implicated in several developmental disorders in children (Huisman et al. 1995) as well as liver dysfunction, chloracne and low birth weight.

Due to these health risks, PCBs are now high priority targets for remediation efforts in many areas of the world. Current remediation strategies include capping known polluted sites and excavation or incineration of contaminated soil. These approaches are not the most desirable because they are costly and often do not destroy the contaminants. Bioremediation with bacteria would be a more desirable solution due to affordability, decreased environmental impact and improved public opinion. However, PCBs are not easily biodegraded under normal conditions, as evidenced by their persistence in the environment.

Many studies have suggested that rhizoremediation, the use of plants and their associated root microorganisms to break down pollutants, is a possible approach for inexpensively and sustainably biostimulating PCB degradation (Gilbert and Crowley 1997; Leigh et al. 2002; Beranová et al. 2007). The basis for rhizoremediation is that certain plant roots may selectively foster the growth and activity of bacteria with specific

metabolic capabilities in the rhizosphere or root zone. Plants release a wide variety of compounds from their roots via exudation, and in particular during root turnover (mortality), that bacteria can use as carbon and energy sources. It has been hypothesized that some plant secondary compounds may promote the growth and activity of PCB degrading bacteria (Singer et al. 2003). PCBs are cometabolized through the biphenyl degradation pathway and there is evidence that salicylate and other secondary plant compounds can induce this pathway (Donnelly et al. 1994; Ohtsubo et al. 2000; Master and Mohn 2001). These compounds could promote the growth of pollutant degrading bacteria by increasing their numbers through selective enrichment and/or promoting expression of degradative genes (Master and Mohn 2001).

Bioremediation using microbes alone is subject to limitations including environmental conditions, genetic potential, and bioavailability of PCBs. Rhizoremediation strategies that target each of these limitations have been developed. This review will summarize research on rhizoremediation of PCB contaminated soils including a discussion of the role of plant secondary compounds, genetically engineered systems and surfactants as well as suggestions for the future of the field.

1.3 Microbial degradation and transformation of PCBs

PCBs are biodegraded aerobically through cometabolism using the genes in the biphenyl metabolism pathway. It is widely recognized that there is an upper pathway that takes the original molecule through several ring cleavage steps to a monoaromatic compound, either benzoate or chloro-benzoate. There is also a lower pathway, the β-

ketoadipate pathway, which funnels benzoate through catechol and produces products that can be used for carbon and energy in central metabolism. The upper pathway is depicted in Figure 1.1, adapted from Furukawa and Fujihara (2008).

The first step in the aerobic degradative pathway involves the biphenyl dioxygenase enzyme, which weakens the aromatic ring structure by introducing two hydroxyl groups to one of the rings. Biphenyl dioxygenases can operate through either a 2,3-attack or a 3,4-attack, and this capability varies among individual organisms (Field and Sierra-Alvarez 2008). This leads to different degradative microbes displaying different PCB congener specificities. In most cases, bacteria can degrade PCBs with one to five or six chloro substitutions (Bedard et al. 1986; Bedard 1990). PCBs with one or two chloro substitutions can sometimes be used as carbon sources, while PCBs with three to six substitutions are broken down through cometabolism, discussed below. PCBs with seven to nine chloro substitutions cannot be mineralized due to a lack of open sites on the rings for dioxygenase and other enzymes to attack. An anaerobic dehalogenation pathway for removal of chlorine atoms on highly substituted rings will be discussed later.

The majority of PCB congeners cannot be used as carbon and energy sources for microbes. They are instead broken down through cometabolism, therefore a primary substrate, such as biphenyl, is necessary for their degradation. Biphenyl, unlike PCBs, serves as a carbon and energy source for microbial growth and induces expression of biphenyl degradation genes (Ohtsubo et al. 2000). Enzymes in the biphenyl pathway may then attack PCBs and their metabolites, although they are not mineralized

completely to yield carbon and energy. Because biphenyl does not exist naturally in the environment, it has been postulated that plant aromatic compounds may be the natural substrate for the biphenyl degradation pathway (Gilbert and Crowley 1997). Aromatics of microbial origin might also be broken down by this pathway.

The aerobic degradation of aromatic rings by soil microbes proceeds by first preparing the aromatic ring for cleavage, cleaving the ring and finally degrading the resulting alkenes into intermediates that may then be funneled into primary metabolism. This general degradation scheme is found in many types of bacteria and fungi. The increased prevalence of microorganisms able to degrade aromatic compounds in the rhizosphere can be linked to the release of secondary metabolites from plant roots (Donnelly et al. 1994).

Aerobic aromatic ring cleavage requires molecular oxygen and is initiated by the addition of two hydroxyl groups to the ring structure by either the addition of one dioxygenase or two monooxygenase enzymes. After ring activation, the ring is cleaved. The *ortho*-cleavage pathway, also termed the β–ketoadipate pathway (Parales and Harwood 1992), is widely distributed among soil microorganisms and is often involved in the degradation of plant derived aromatic compounds such as lignin (Grbic-Galic 1985). After the aromatic ring has been broken, the resulting linear hydrocarbon is further degraded by enzymes and pathways that are ubiquitous in soils.

PCBs are not known to be mineralized under anaerobic conditions, however PCBs may be reductively dechlorinated in anaerobic environments. This dehalogenation

activity was first observed in aquatic sediments when lower chlorinated PCB congeners as well as congeners not present in the original contaminant mixture were detected and increased in concentration, which suggested that more highly chlorinated congeners were being transformed (Abramowicz 1995). As is the case with aerobic degradation, there is congener specificity associated with anaerobic transformation.

The extent and pattern of chlorination can greatly affect whether a PCB congener is aerobically degraded or not. In general, as the number of chlorine atoms on the ring increases, the ability for microbes to degrade the compounds decreases (Furukawa et al. 1978). This is likely due to steric hindrance of the biphenyl dioxygenase enzyme and a lack of open locations on the ring for dioxygenase attack. Many bacteria are able to use mono-chlorinated biphenyls as carbon sources and several di-chlorinated and tri-chlorinated biphenyls are quickly degraded by bacterial isolates, but even some di- and tri-chlorinated congeners can be difficult to degrade if the chloro-substitutions occur in both of the *ortho* positions of one ring, such as 2,6-chlorobiphenyl (Furukawa et al. 1978). As the number of chlorines increases to four, five or six, the ability of microbes to degrade the congeners varies widely between strains and between congeners (Rein et al. 2007). This depends on whether the organism has the 2,3-dioxygenase or the 3,4-dioxygenase enzyme. The aerobic degradation of PCB congeners with one to six chloro-substitutions, while possible, is often limited by the chlorination pattern with PCBs that are double *ortho* substituted being the most difficult for many microbes to degrade.

In anaerobic transformation, the chlorination pattern likewise impacts whether bacteria can act on the compounds or not. Chlorine atoms in the *ortho* position are the least likely to be removed, and this removal is often the slowest step in dechlorination (Field and Sierra-Alvarez 2008). After the discovery of the reductive dechlorination pathway, much research has focused on exploiting the bacterial ability to dechlorinate highly substituted congeners and degrade lower chlorinated congeners; some of these approaches will be reviewed later. Clearly the chlorination extent and position has a large impact on whether microbes are able to degrade PCBs successfully or not. PCBs that are chlorinated in the *ortho* position are usually the slowest to be dehalogenated and degraded because of the steric hindrance that this overall shape causes. There are some bacteria that have a different genetically encoded pathway that allows the degradation of congeners that are *ortho* substituted. Exploitation of certain microbes' unique abilities is one approach for treated PCB contaminated waste. It is possible to first dechlorinate PCB congeners and then degrade the molecules using sequential anaerobic and aerobic microbial processes.

Another limitation to PCB degradation in soils is expression of the *bph* pathway. The genetic pathway for PCB degradation, as discussed above, involves at least four enzymes that act on the PCB in succession. The activity of biphenyl dioxygenase (BPDO), the first enzyme in the pathway, is often considered the rate limiting step in the pathway. This is because the enzyme requires two adjacent positions on one ring to be open for attack which determines the congener specificity of the pathway, and this is the

initial step in the pathway which begins the cascade of enzyme activity involved in PCB degradation. Another enzyme in the pathway has been identified as a possible rate limiting step. The third enzyme in the pathway, dihydroxybiphenyl dioxygenase (DHBD), is another point where the pathway could be inhibited due to some metabolites of PCB degradation (Dai et al. 2002). More specifically, Dai and colleagues found that products of PCBs with *ortho* position chloro substitutions actually turned off the pathway at the third enzyme and that microbes growing on PCB congeners as a carbon source showed a decrease in the growth rate as a result of this inhibition. This finding has implications for bioremediation strategies because *ortho* chlorinated congeners are prevalent and ubiquitous. Furthermore, there is no efficient biological process to remove *ortho* chloro substitutions because of the steric hindrance that this chlorination pattern introduces to the molecule. There are many aspects of microbial physiology that may limit the degradation of PCBs in sediments and some are more easily addressed than others. Bioremediation strategies, such as rhizoremediation, address some of these limitations in soils.

1.4 The rhizosphere effect

Lorenz Hiltner was the first to describe the rhizosphere, in 1904, as the area of soil which is directly affected by the root system of a plant (Hartmann et al. 2008). In the rhizosphere, plants secrete a variety of inorganic and organic substrates, including secondary plant metabolites (SPMEs), from the root into the rhizosphere (Lynch and Whipps 1990). It is estimated that the roots receive 30-60% of photosynthetic carbon of

which 10-20% is secreted into the rhizosphere (Singer et al. 2003). This continual input of substrates to the rhizosphere results in elevated microbial growth within the rhizosphere of plants when compared to the bulk soil (Lynch and Whipps 1990; Singer et al. 2003).

Microbes in the rhizosphere are more abundant than in bulk soil for several reasons. Plant exudates in the rhizosphere include simple sugars, nitrogen containing compounds and more complex SPMEs. All of these compounds can support the growth of microbes, which is why it has been estimated that soil microbial populations associated with plant roots can be as high as 10^9 to 10^{12} per gram of soil (Narasimhan et al. 2003). Oxygen is more prevalent in the rhizosphere than in bulk soil, which contributes to the high numbers of aerobic bacteria associated with plant roots (Bertin et al. 2003).

The abundance of microbes in the rhizosphere may also be beneficial for the plant. Some rhizosphere bacteria can produce and secrete antimicrobial compounds that inhibit the growth of plant pathogens (Kang et al. 1998). These compounds can be aromatic and sometimes contain halogens (van Pée 1996).

Due to the diversity of plant and microbial compounds in the rhizosphere, the microbes that inhabit the area likewise may possess diverse metabolic capabilities. These include complex aromatic compound degradation, discussed above, but also the ability to degrade potential toxins such as antibiotics and halogenated compounds produced by both plants and microbes.

SPMEs are found in the plant rhizosphere, and can serve as novel carbon sources creating niches for certain bacteria. SPMEs are a diverse class of organic compounds, with 500,000 structures estimated and over 100,000 structures already described (Hadacek 2002). These compounds do not have a direct role in the growth or development of plant species (Taiz and Zeiger 2002), but function to maintain or increase the reproductive fitness of plants. Many act as deterrents against predation, such as herbivory, or to prevent infection by microorganisms (Hadacek 2002). Other roles include attracting pollinaters or seed-dispersing animals, providing structural support, or acting as agents of plant-plant communication (Taiz and Zeiger 2002). Many SPMEs resemble environmental pollutants such as pesticides, polyaromatic hydrocarbons (PAHs) and PCBs. Figure 1.2 shows some environmental contaminants and their plant compound analogs (Singer et al. 2003). The similarity between some pollutants and plant compounds lead to the idea that microbes that are able to degrade plant compounds may also be able to degrade pollutant analogs. The rhizosphere may be a reservoir for these bacteria and microbes with desirable metabolic abilities. Levels of SPMEs leached from roots into the rhizosphere have been seen to increase dramatically during fine root turnover events (Fletcher and Hegde 1995). Since SPMEs are often sequestered in the plant vacuole, cell lysis following root turnover may be a major mode for releasing biostimulatory SPMEs (Wink 1997). In this way, SPMEs in the rhizosphere may stimulate bacteria to breakdown pollutants, especially during root turnover.

SPMEs in the rhizosphere may lead to pollutant degradation through biostimulation of aromatic degrading bacteria. The presence of these compounds in the rhizosphere can provide a carbon source to microbes with the capacity to degrade them, which may result in increased growth and degradative activity in the rhizosphere community (Donnelly et al. 1994). Several studies have observed lower concentrations of pollutants in the rhizosphere when compared to bulk soil. For example, in an area with PAH contaminated sludge, concentrations in the root zone of mulberry trees were significantly lower than in bulk soil, even in PAHs considered to be more recalcitrant and difficult to degrade (Olson et al. 2001). Mulberry trees release aromatic compounds similar to PAHs which may promote PAH degradation in the rhizosphere (Olson et al. 2001).

1.5 SPMEs and PCB degradation

SPMEs in the rhizosphere may stimulate specific bacteria with unique metabolic abilities. PCB degrading bacteria have been detected in association with many plants using both culture dependent and culture independent techniques. The flavones morusin, morusinol, and kuwanon C, were found to accumulate in mulberry fine roots as they age and remain in roots as they died, becoming available to microorganisms during root decomposition. These compounds were shown to serve as growth substrates for some PCB degrading bacteria when provided as sole carbon sources (Leigh et al. 2002). Naturally vegetated PCB contaminated sites are one place to look for elevated levels of PCB degrading bacteria associated with certain plants. PCB degrading bacteria were detected in the root zones of pine, willow, birch and black locust trees at an actual

contaminated site, with significantly higher numbers associated with pine and willow, implicating these plant species as possible stimulators of PCB degrading bacteria (Demnerova et al. 2003; Leigh et al. 2006). These findings suggest that certain plant species may stimulate PCB degrading bacteria.

Donnelly and colleagues were the first to hypothesize that plant compounds may be the natural inducers for aromatic degradative pathways such as the biphenyl degradation pathway (Donnelly et al. 1994). Since that time, the search for plant compounds that can induce the *bph* pathway or biostimulate PCB degradation has led to many plants and plant compounds being investigated for PCB rhizoremediation. Phenolic compounds are one class of SPMEs that have been proven to support the growth of PCB degrading bacteria (Fletcher and Hegde 1995). Isolates in culture have also been shown to degrade PCBs while growing on secondary metabolites from spearmint such as carvone, p-cymene, limonene (Gilbert and Crowley 1997) and mulberry secondary compounds such as morusin, morusinol and kuwanon C (Leigh et al. 2002). Other studies suggest that plant compounds such as coumarin and naringin can stimulate PCB degradation more efficiently than biphenyl (Singer et al. 2003). These secondary compounds range from phenols to terpenoids and can have diverse chemical structures (Singer et al. 2003), as is demonstrated in Figure 1.3.

Litter from aboveground portions of plants may also impact the microbial community and produce an environment that favors PCB degrading bacteria. Orange peels, eucalyptus leaves, ivy leaves and pine needles are examples of plant litter that have

been reported to stimulate PCB degradation in soils (Hernandez et al. 1997; Dzantor and Woolston 2001; Dzantor et al. 2002). The compounds introduced to soil through plant litter represent many classes and a variety of structures of SPMEs as well as other common plant metabolites. These stimulatory effects could impact the rhizosphere as well as bulk soil, depending on how deeply into the soil the litter and its chemical components permeate.

Willow trees have recently received much attention as possible plants for PCB rhizoremediation. This is in part because willows produce salicylate, a phenol known to funnel into microbial degradation pathways of monoaromatics (Chen and Aitken 1999). Salicylate can also induce expression of *bphA*, the gene that codes for the first enzyme in microbial PCB degradation, widely considered to be the rate limiting step in degradation (Master and Mohn 2001). However in one study, salicylate was added to soils, but it did not lead to a significant increase in PCB degradation, whereas biphenyl did promote PCB degradation (Luo et al. 2008). A few studies have focused on whole willow plants, rather than individual secondary compounds, for PCB rhizoremediation. When willows were introduced to soils, the microbial community shifted towards bacteria including *Pseudomonas* and β-Proteobacteria which are taxonomic groups that include PCB degraders (de Carcer et al. 2007). This study focused on the microbial community and did not look at PCB concentrations to determine whether these increases were correlated with PCB degradation. A field study in a PCB-contaminated site found that significantly higher numbers of culturable biphenyl-utilizing bacteria were associated with the root

zone of willow (*Salix caprea*) in comparison to several other tree species (Leigh et al. 2006). In a recent pot study, the willow species *Salix caprea* led to about 15% more PCB degradation than in unplanted soil and more biphenyl utilizing bacteria were isolated from treated soil than untreated soil (Ionescu et al. 2009). These studies indicate that willows may be promising plants for PCB rhizoremediation, however few studies use culture independent techniques to study the microbial community while also investigating the extent of PCB degradation in soils.

SPMEs are the probable natural substrates for the PCB degradation pathway and many studies have shown that PCB degrading bacteria can utilize these compounds as primary carbon sources while degrading PCBs. A few plants, such as mulberry and willow, are the most promising candidates for PCB rhizoremediation, although PCB degrading bacteria are often detected in the root zones of many other plants (Leigh et al. 2006). These rhizoremediation strategies rely upon the presence of indigenous PCB degrading bacteria. PCB degrading bacteria can usually be isolated from contaminated soils; however they may not be present in sufficiently high numbers and/or may not be metabolically active. Plants may have the capacity to overcome these limitations. Another issue is whether the indigenous PCB degrading bacteria have sufficient enzymatic capabilities to degrade the variety of PCB congeners present. This has led researchers to genetically engineer bacteria for enhanced PCB remediation.

1.6 Engineered systems for PCB rhizoremediation

One possible approach to increasing PCB degradation rate in soils is to target the genetic potential of the bacteria at the polluted site. PCB degradation in soils is limited by several factors including the presence of certain degradative pathways and the activity or expression of certain genes and enzymes needed to degrade some PCB congeners. Genetic engineering of bacteria may address these limitations. The goal of genetically engineered microbes for this purpose is to alter the metabolic capabilities of a certain organism at the genetic level. This approach assumes that the main limitation in PCB degradation is the lack of the correct genes being present or being expressed at a sufficiently high level.

Burkholderia xenovorans LB400 is a well studied PCB degrader that exhibits broad congener specificities. The nucleotide sequence of the *bph* pathway in LB400 has been sequenced and this critical information has allowed much more research into the genetics of PCB degradation, including congener specificity (Erickson and Mondello 1992). The research surrounding LB400 has provided valuable information about genes and enzymes in the biphenyl pathway, including sequences, evolutionary relationships to genes in other pathways, crystal structures and enzymatic capabilities. This information has illuminated several targets for genetic engineering.

One of the central goals of genetically engineering microbes for this purpose is to insert a complete set of genes for biphenyl mineralization into a single organism, including the upper and lower pathways. This would enable an organism to degrade

PCBs and biphenyl to carbon dioxide, without accumulating other metabolic intermediates. Recently, one group designed *Cupriavidus necator* to grow on commercial mixtures of PCBs as sole carbon sources while avoiding the accumulation of benzoates and chloro-benzoates (Wittich and Wolff 2007). This approach can be implemented with current technology and there are several ways to transfer genes into a selected organism.

The easiest way to transfer genes is with the use of a plasmid that encodes the gene of interest. Some microbes easily take up plasmids and pass them between other organisms. In order to prevent the microbe from losing the plasmid, transposable elements were exploited so that the gene would transfer from a plasmid to the genome of a microbe and the gene would be a more permanent part of the organism's genome (Kleckner et al. 1977). Another technique that has proven successful is the direct implantation of a gene into a target organism's genome using a transposon shuttle system, which led to stable gene integration into the organism's genomic DNA (Wittich and Wolff 2007). Two disadvantages of these techniques are that they require genomic sequence data and gene presence may not result in gene expression under conditions found in a polluted site. A unique way to tackle these limitations is to ensure gene expression with promoters. One group used recombination to implant a promoter upstream of the *bph* operon which lead to high levels of gene expression in the absence of a substrate and even in the presence of carbon sources that would normally down-regulate the pathway (Ohtsubo et al. 2003). This technique bypassed the normal pathway

regulation where a protein BphS represses the expression of the *bph* operon under standard conditions and the pathway is turned on when a metabolic product of biphenyl metabolism is present (Ohtsubo et al. 2001). Recently the rhizosphere bacterium, *Pseudomonas fluorescens* F113 has been manipulated to overexpress the *bph* pathway using a nod box system, which led to greater degradation extent for some congeners than LB400 (Villacieros et al. 2005).

Once a genetically modified strain has been developed, based on knowledge of enzymatic function on a molecular level and what characteristics contribute to congener specificity, the challenge becomes implementing this knowledge effectively. A successful strategy for microbe delivery to the target soil is necessary, as well as the viability of a strain in the target soil.

One major issue with implementation of genetically modified PCB-degraders is whether the engineered strain will not only survive but also thrive in the target community. Thompson et al. (2005) suggested that to find a microbe that will survive in the presence of metals and other pollutants, we must go to the target soil and obtain a clear view of the microbial ecology unique to the site, since isolating organisms for engineering from the target site will give a better chance of microbial survival than if a strain were introduced to a foreign site. In addition to choosing a strain that already exists in the target environment, another consideration in strain selection is whether the organism colonizes the root zone of a useful plant. Plant-microbe pairing is key to microbe survival in the rhizosphere (Gilbertson et al. 2007) and rhizosphere bacteria

could be useful targets for genetically engineered bacteria (Villacieros et al. 2005). If an effective plant-microbe pair are found for certain sites, this strategy could be useful to introduce engineered organisms capable of degrading many classes of organic pollutants.

Plants can also be used as a delivery and support system for introduced microbes allowing *in situ* treatment of contaminated sites. This technique was successfully implemented in one case where the bacteria were altered to express green fluorescent protein (GFP) gene at all times. These bacteria were delivered to all areas of soil that the roots contacted, yet they had a low rate of survivability in the long term (Gilbertson et al. 2007). The use of GFP-tagged bacteria as a bioreporter system demonstrated that a root system is a promising way to introduce bacteria to target soil, but the plant-microbe pairing is important for microbe survival. Several plant species have been identified as good host plants for genetically engineered microbes for PCB degradation. These include sugar beets (Brazil et al. 1995) and willow trees (de Cárcer et al. 2007; Rein et al. 2007) due to high survivability of the introduced strain in both cases. In another case, engineered microbes were successfully added to the rhizosphere of a plant as the delivery system, yet degradation of the target pollutant did not increase accordingly (Plangklang and Reungsang 2008). This suggested that the genetic potential was not the limiting factor in degradation, and that other attempts at bioaugmentation should couple genetic engineering with another target such as cosubstrate addition, bioavailability of the target compound or nutrient concentration (Plangklang and Reungsang 2008). There are many challenges to overcome when implementing genetically engineered bacteria for this

purpose including survivability, ensuring the desired effect and delivery to the contaminated site.

Due to the limitations and challenges of engineered bacteria for PCB remediation, some researchers have begun to focus on other points within the system that may be manipulated. Plants may be another target for genetic engineering that could increase the rate and extent of PCB degradation in soils. Genetically engineered plants are often used in agriculture, and public opinion may be more favorable toward genetically engineered plants than microbes.

Plants have their own metabolic pathways that can act on PCBs through cytochrome P450, but plant transformation of PCBs is slow and incomplete, which led to plants being candidates for genetic engineering for PCB degradation (Mackova et al. 1997; Mackova et al. 2006; Macková et al. 2007). Recently, several plants including tobacco, alfalfa, horseradish, and nightshade, have had the bacterial *bph* degradation pathway inserted into the plants' genomes (Macková et al. 2007; Novakova et al. 2009). Genetically engineered plants transformed PCBs in the study, and microbes were able to complete the degradation and act on the PCB metabolites produced by plants (Macková et al. 2007), an occurrence observed in other studies involving plant transformation of PCBs (Francova et al. 2004). Furthermore, it was demonstrated that the plant cells were able to transform chlorobenzoic acid intermediates into more easily degraded hydroxylated forms and that bacteria were able to degrade these molecules (Macková et al. 2007). Plants may prove to be an easy target for genetic engineering for this purpose,

especially when microbial genes are inserted. For a complete review of this topic, see Sylvestre et al. (2009).

An additional strategy for PCB rhizoremediation could be to engineer plants for overexpression of genes for SPME production. Although not explored for this purpose, many plant SPME pathways have been engineered for the purpose of controlling pests and infections (Dixon et al. 1996; Siebert et al. 1996; Verpoorte et al. 1999). This hypothesis assumes that the necessary microbes are present for PCB degradation, but that the level of plant metabolites is not high enough in the wild type plant to stimulate PCB degradation. This option is yet to be investigated, but could prove useful in the future.

1.7 Rhizoremediation strategies addressing bioavailability

The low bioavailability of PCBs in soils contributes to their persistence in the environment. PCBs can adsorb to soil and other particles, making them difficult to degrade. Bioavailability is one of the major limiting factors in microbial degradation of PCBs, and a successful remediation strategy will address this limitation.

Plant growth may be a natural way of increasing PCB bioavailability in soils. In soil contaminated with the PCB mixture Aroclor 1242, plant growth led to degradation of the Aroclor to a point where it was no longer detectable and the authors suggest that the plants increased the bioavailability of the PCBs (Hernandez et al. 1997). Plants may release surfactants into the rhizosphere, increasing the bioavailability of pollutants. In this way, rhizoremediation may also be a strategy that addresses bioavailability of the pollutant.

Surfactant addition to contaminated soil is another way of increasing the bioavailability of the target pollutant. After the realization that certain compounds could change the bioavailability of PCBs in soils, many groups have tried to discover or create a surfactant to add to soils. Full scale field studies have shown that it is possible to successfully introduce a surfactant to contaminated soil *in situ* (Mulligan et al. 2001). Synthetic surfactants often last longer in soils because they are not easily degraded by bacteria, however they may also be toxic to soil microorganisms, preventing the degradation of pollutants (Fava and Gioia 1998; Mulligan et al. 2001). Biogenic surfactants, produced by plants or microbes, could be a better solution because they may be less toxic to microbes while increasing the bioavailability of PCBs and other pollutants. Surfactants produced by plants, such as soya lecithin and sorbitan trioleate, increase biological degradation of PCBs and do not exhibit toxic effects on soil microbes (Singer et al. 2000; Fava and Di Gioia 2001). Bacteria can also produce surfactants that are non-toxic to soil microbes and increase the bioavailability of PCBs (Van Dyke et al. 1993). Surfactants produced by plants appear to be the most promising approach to increasing the bioavailability of PCBs in soils because their production is easily engineered and they are often non-toxic to soil microbes.

Another strategy is the introduction of cyclodextrins (CDs). CDs act as surfactants in soils, with the advantage that they are non-toxic and slowly biodegradable (Fava and Ciccotosto 2002). Particularly promising is the observation that CDs effectively lead to biodegradation of PCBs in many soil types as well as historically

contaminated, aged soils (Fava and Ciccotosto 2002; Fava et al. 2003). Surfactants are a good option for increasing the bioavailability of PCBs in soils, but the appropriate surfactant will be non-toxic to soil microbes and also increase the degradation of PCBs *in situ.*

In addition to surfactants, there are several other approaches to increasing the degradation of PCBs. One of the easiest and simplest suggestions is to use earthworms to introduce oxygen, increase nutrient concentrations, and mix soil which increases the degradation of PCBs (Singer et al. 2001). Many soil conditions can support earthworms, and this bioturbation could be another way to increase PCB degradation.

Addressing the low bioavailability of PCBs in soils is essential for effective bioremediation of PCBs. Rhizoremediation of PCBs is promising because many of the most effective treatments to increase bioavailability are plant based. Since some plants are also capable of enhancing the growth and activity of PCB-degraders, this suggests that identification of the correct plant or mixture of plants could address multiple limitations to biodegradation, including bioavailability and cosubstrate availability.

1.8 Future Studies

In the future, rhizoremediation studies should continue to focus on the identification of plant species that promote PCB degradation in soils. Likely, there are many plants worldwide that could be good hosts for PCB-degrading bacteria in the rhizosphere. Temperature and soil conditions are unique to each area and native plants could be the best choice for rhizoremediation because they would be adapted to the

conditions at the contaminated site. This would require a different plant choice for different polluted areas, but the choice of plant could perhaps be narrowed to a widespread family or genus. For example, if local members of the genus *Salix* share the appropriate phenotypes and then that plant would be introduced to the contaminated area.

Another goal in the future is the identification of active plant compounds, in order to clarify the mechanism of rhizostimulation. Mixed results have been observed with the introduction of salicylate to induce *bph* gene expression or stimulate PCB degradation (Master and Mohn 2001; Luo et al. 2008), yet willow appear to be useful host plants for PCB rhizoremediation (de Carcer et al. 2007; Ionescu et al. 2009). In order to understand how plants are stimulating bacteria to degrade PCBs, more plant compounds produced by the roots of promising plants should be identified and their biostimulatory capabilities assessed.

Future studies involving the implementation of rhizoremediation of PCBs must also integrate culture-independent methods of studying the microbial community involved with degradation studies in soils. Several studies have investigated microbial communities using stable isotope probing and terminal restriction fragment length polymorphism, useful culture independent techniques, yet these studies have taken place at actual contaminated sites, and were not coupled with observations of PCB degradation in soil (Leigh et al. 2006; de Carcer et al. 2007). In some studies where PCB degradation in soil is the focus, culture dependent tools were used to study the microbial community (Ionescu et al. 2009). This could give an incomplete picture of the microbial community

involved in PCB degradation. A combination of the two approaches would be advantageous to understanding how a microbial community responds to plant amendments in contaminated soil as well as how effective a given plant could be for rhizoremediation. Soil toxicity is one aspect of PCB rhizoremediation that is rarely addressed. Many groups do not investigate whether soil toxicity has decreased due to lower concentrations of PCBs, or possibly increased due to the accumulation of toxic metabolites during the degradation process. The following chapter describes a study that addresses the microbial community using culture independent methods, determine the extent of PCB degradation, and assess soil toxicity after treatment to ensure that no metabolites that are more toxic than the parent compound have accumulated.

1.9 Conclusions

Plant compounds released into the rhizosphere can support the growth and activity of native PCB degrading bacteria. Plants may also release surfactants that can increase the bioavailability of PCBs and lead to degradation in soils. Rhizoremediation is a possible technique to treat PCB contaminated soils *in situ*, and more plant candidates must be tested for this purpose, especially how they affect microbial communities, PCB degradation, and soil toxicity. Long term and field scale studies are also needed in order to determine if the degradation observed in some lab studies is transferable to actual contaminated sites. While rhizoremediation of PCBs is a promising and affordable way to effectively clean up lower chlorinated PCBs at contaminated sites, higher chlorinated congeners, which are resistant to aerobic degradation, would need to undergo anaerobic

pre-treatment in order to remove chloro substitutions and make the PCB vulnerable to aerobic degradation.

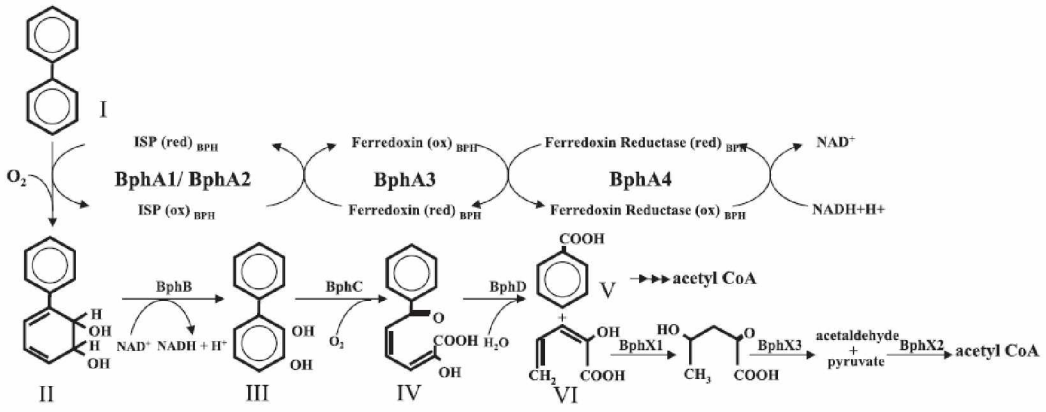

Figure 1.1. Upper pathway genes and metabolic intermediates in PCB and biphenyl degradation. Adapted from Furukawa and Fujihara (2008).

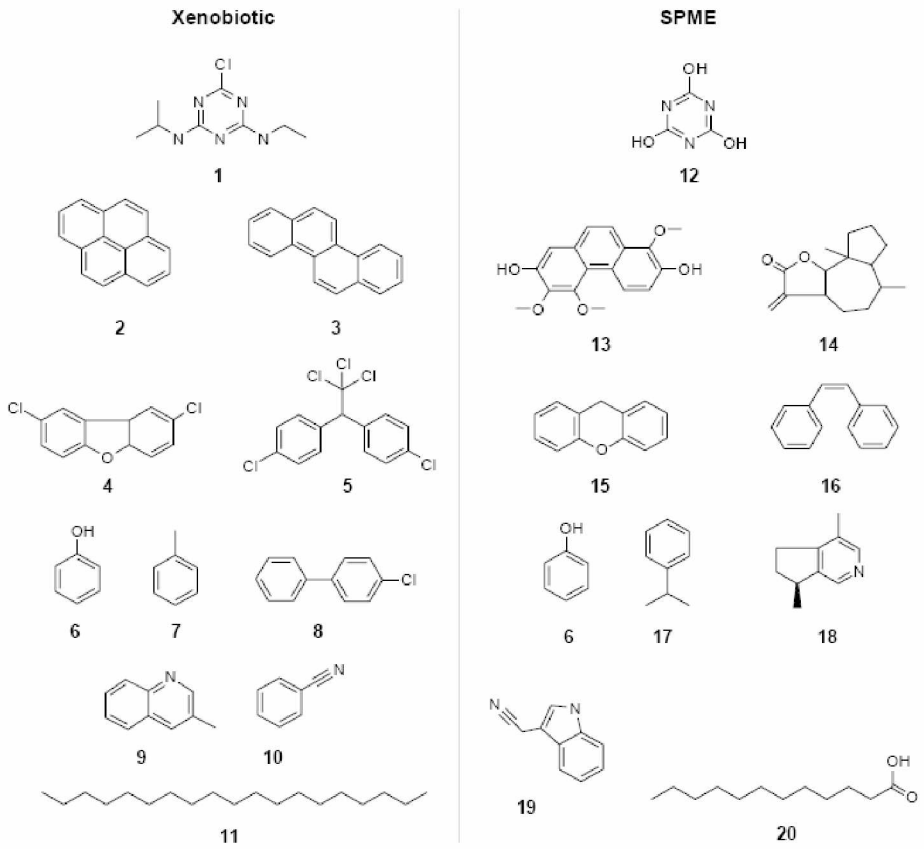

Figure 1.2. Comparison of xenobiotic structures with SPME structures

(1) atrazine; (2) pyrene; (3) chrysene; (4) 3,8-dichlorodibenzo-p-dioxin; (5) p,p-dichlorodiphenyl-trichloroethane (DDT); (6) phenol; (7) toluene; (8) 4-chlorobiphenyl (PCB); (9) 3-methylquinoline; (10) benzonitrile; (11) n-nonadecane; (12) cyanuric acid; (13) confusarin; (14) pseudoguiaianolide; (15) xanthone; (16) stilbene; (17) cumene; (18) actinidine; (19) indole-3-acetonitrile; (20) farnesol. Reprinted from Singer et al. (2003).

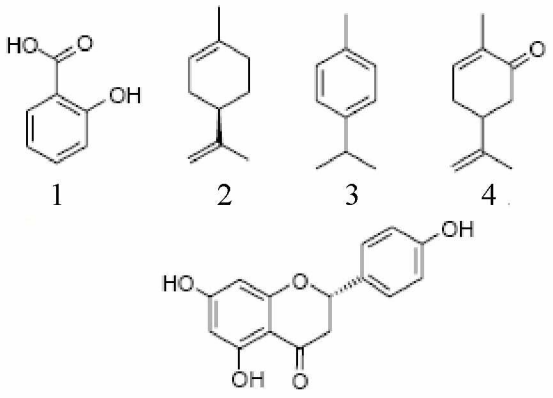

Figure 1.3. Examples of a variety of SPME thought to induce PCB cometabolism (1) salicylate, (2) R-limonene, (3) cymene, (4) carvone, (5) naringin.

1.10 References

Abramowicz, D.A., Aerobic and anaerobic PCB biodegradation in the environment. Environmental Health Perspectives, 1995. 103(Suppl 5): p. 97.

Bedard, D.L. and M.L. Haberl, Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microbial Ecology, 1990. 20(1): p. 87-102.

Bedard, D.L., et al., Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Applied and Environmental Microbiology, 1986. 51(4): p. 761.

Beranová, K., et al., Rhizoremediation for decontamination of long-term PCB contaminated soil with focus on microbial diversity. Journal of Biotechnology, 2007. 131(2S): p. 243-243.

Bertin, C., et al., The role of root exudates and allelochemicals in the rhizosphere. Plant and Soil, 2003. 256(1): p. 67-83.

Brazil, G.M., et al., Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of bph gene expression in the rhizosphere. Applied and Environmental Microbiology, 1995. 61(5): p. 1946-1952.

Chen, S.H. and M.D. Aitken, Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by Pseudomonas saccharophila P15. Environmental Science & Technology, 1999. 33(3): p. 435-439.

Dai, S., et al., Identification and analysis of a bottleneck in PCB biodegradation. Nature Structural & Molecular Biology, 2002. 9(12): p. 934-939.

de Carcer, D.A., et al., Changes in bacterial populations and in biphenyl dioxygenase gene diversity in a polychlorinated biphenyl-polluted soil after introduction of willow trees for rhizoremediation. Applied and Environmental Microbiology, 2007. 73(19): p. 6224.

Dekoning, E. and W. Karmaus, PCB exposure in utero and via breast milk: A review. Journal of Exposure Analysis and Environmental Epidemiology (2000). 10(3): p. 285.

Demnerova, K., et al., Bacteria Degrading PCBs and CBs Isolated from Long-Term PCB Contaminated Soil. Water, Air, & Soil Pollution: Focus, 2003. 3(3): p. 47-55.

Dixon, R.A., et al., Metabolic engineering: prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses—a review. Gene, 1996. 179(1): p. 61-71.

Donnelly, P.K., et al., Growth of PCB-degrading bacteria on compounds from photosynthetic plants. Chemosphere(Oxford), 1994. 28(5): p. 981-988.

Dzantor, E., et al., PCB Dissipation and Microbial Community Analysis in Rhizosphere Soil Under Substrate Amendment Conditions. International Journal of Phytoremediation, 2002. 4(4): p. 283-295.

Dzantor, E.K. and J.E. Woolston, Enhancing dissipation of aroclor 1248 (PCB) using substrate amendment in rhizosphere soil. Journal of environmental science and health. Part A, Toxic/Hazardous Substances & Environmental Engineering, 2001. 36(10): p. 1861.

Erickson, B.D. and F.J. Mondello, Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in Pseudomonas strain LB400. Journal of Bacteriology, 1992. 174(9): p. 2903-2912.

Fava, F. and D. Di Gioia, Soya lecithin effects on the aerobic biodegradation of polychlorinated biphenyls in an artificially contaminated soil. Biotechnology and Bioengineering, 2001. 72(2): p. 177-184.

Fava, F. and D.Di Gioia, Effects of Triton X-100 and Quillaya Saponin on the ex situ bioremediation of a chronically polychlorobiphenyl-contaminated soil. Applied Microbiology and Biotechnology, 1998. 50(5): p. 623-630.

Fava, F. and V.F. Ciccotosto, Effects of Randomly Methylated- -Cyclodextrins (RAMEB) on the bioavailability and aerobic biodegradation of polychlorinated biphenyls in three pristine soils spiked with a transformer oil. Applied Microbiology and Biotechnology, 2002. 58(3): p. 393-399.

Fava, F., et al., Methyl-b-cyclodextrin-enhanced solubilization and aerobic biodegradation of polychlorinated biphenyls in two aged-contaminated soils. Biotechnology and Bioengineering, 2003. 81(4): p. 381-390.

Field, J.A. and R. Sierra-Alvarez, Microbial transformation and degradation of polychlorinated biphenyls. Environmental Pollution, 2008. 155(1): p. 1-12.

Fletcher, J.S. and R.S. Hegde, Release of phenols by perennial plant roots and their potential importance in bioremediation. Chemosphere, 1995. 31(4): p. 3009-3016.

Francova, K., et al., Ability of bacterial biphenyl dioxygenases from Burkholderia sp. LB400 and Comamonas testosteroni B-356 to catalyse oxygenation of ortho-hydroxychlorobiphenyls formed from PCBs by plants. Environmental Pollution, 2004. 127(1): p. 41-48.

Furukawa, K. and H. Fujihara, Microbial degradation of polychlorinated biphenyls: Biochemical and molecular features. Journal of Bioscience and Bioengineering, 2008. 105(5): p. 433-449.

Furukawa, K., et al., Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. Applied and Environmental Microbiology, 1978. 35(2): p. 223-227.

Gilbert, E.S. and D.E. Crowley, Plant compounds that induce polychlorinated biphenyl biodegradation by Arthrobacter sp. strain B1B. Applied and Environmental Microbiology, 1997. 63(5): p. 1933.

Gilbertson, A.W., et al., Transport and survival of GFP-tagged root-colonizing microbes: Implications for rhizodegradation. European Journal of Soil Biology, 2007. 43(4): p. 224-232.

Grbic-Galic, D., Fermentative and oxidative transformation of ferulate by a facultatively anaerobic bacterium isolated from sewage sludge. Applied and Environmental Microbiology, 1985. 50(4): p. 1052.

Hadacek, F., Secondary metabolites as plant traits: current assessment and future perspectives. Critical Reviews in Plant Sciences, 2002. 21(4): p. 273-322.

Hartmann, A., et al., Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. Plant and Soil, 2008. 312(1): p. 7-14.

Hernandez, B.S., et al., Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil. Biodegradation, 1997. 8(3): p. 153-158.

Huisman, M., et al., Neurological condition in 18-month-old children perinatally exposed to polychlorinated biphenyls and dioxins. Early Human Development, 1995. 43(2): p. 165-176.

Ionescu, M., et al., Isolation and characterization of different plant associated bacteria and their potential to degrade polychlorinated biphenyls. International Biodeterioration & Biodegradation, 2009.

Kang, Y., et al., Characterization of genes involved in biosynthesis of a novel antibiotic from Burkholderia cepacia BC11 and their role in biological control of Rhizoctonia solani. Applied and Environmental Microbiology, 1998. 64(10): p. 3939.

Kleckner, N., et al., Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics. Journal of Moleculat Biology, 1977. 116(1): p. 125-59.

Leigh, M.B., et al., Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. Applied and Environmental Microbiology, 2006. 72(4): p. 2331-2342.

Leigh, M.B., et al., Root Turnover: An Important Source of Microbial Substrates in Rhizosphere Remediation of Recalcitrant Contaminants. Environmental Science & Technology, 2002. 36(7): p. 1579-1583.

Luo, W., et al., Organic carbon effects on aerobic polychlorinated biphenyl removal and bacterial community composition in soils and sediments. Chemosphere, 2008. 70(3): p. 364-373.

Lynch, J.M. and J.M. Whipps, Substrate flow in the rhizosphere. Plant and Soil, 1990. 129(1): p. 1-10.

Mackova, M., et al., Biodegradation of polychlorinated biphenyls by plant cells. International Biodeterioration & Biodegradation, 1997. 39(4): p. 317-325.

Mackova, M., et al., Biotransformation of PCBs by plants and bacteria–consequences of plant-microbe interactions. European Journal of Soil Biology, 2007. 43(4): p. 233-241.

Mackova, M., et al., Phytoremediation of polychlorinated biphenyls. Phytoremediation and Rhizoremediation, 2006: p. 143–167.

Master, E.R. and W.W. Mohn, Induction of bphA, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant Pseudomonas strain Cam-1 and mesophilic Burkholderia strain LB400. Applied and Environmental Microbiology, 2001. 67(6): p. 2669-2676.

Mulligan, C.N., et al., Surfactant-enhanced remediation of contaminated soil: a review. Engineering Geology, 2001. 60(1-4): p. 371-380.

Narasimhan, K., et al., Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. Plant Physiology, 2003. 132(1): p. 146.

Novakova, M., et al., Cloning the bacterial bphC gene into Nicotiana tabacum to improve the efficiency of PCB phytoremediation. Biotechnology and Bioengineering, 2009. 102(1): p. 29-37.

Ohtsubo, Y., et al., BphS, a Key Transcriptional Regulator of bph Genes Involved in Polychlorinated Biphenyl/Biphenyl Degradation in Pseudomonas sp. KKS102. Journal of Biological Chemistry, 2001. 276(39): p. 36146-36154.

Ohtsubo, Y., et al., Expression of the bph genes involved in biphenyl/PCB degradation in Pseudomonas sp. KKS102 induced by the biphenyl degradation intermediate, 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoic acid. Gene, 2000. 256(1-2): p. 223-228.

Ohtsubo, Y., et al., Novel Approach to the Improvement of Biphenyl and Polychlorinated Biphenyl Degradation Activity: Promoter Implantation by Homologous Recombination. Applied and Environmental Microbiology, 2003. 69(1): p. 146-153.

Olson, P., et al., Natural attenuation/phytoremediation in the vadose zone of a former industrial sludge basin. Environmental Science and Pollution Research, 2001. 8(4): p. 243-249.

Parales, R. E. and C. S. Harwood, Characterization of the genes encoding beta-ketoadipate: succinyl-coenzyme A transferase in Pseudomonas putida. Journal of Bacteriology, 1992. 174(14): p. 4657.

Plangklang, P. and A. Reungsang, Effects of rhizosphere remediation and bioaugmentation on carbofuran removal from soil. World Journal of Microbiology and Biotechnology, 2008. 24(7): p. 983-989.

Rein, A., et al., Degradation of PCB congeners by bacterial strains. Applied Microbiology and Biotechnology, 2007. 77(2): p. 469-481.

Siebert, M., et al., Genetic Engineering of Plant Secondary Metabolism (Accumulation of 4-Hydroxybenzoate Glucosides as a Result of the Expression of the Bacterial ubiC Gene in Tobacco). Plant Physiology, 1996. 112(2): p. 811.

Singer, A.C., et al., Secondary plant metabolites in phytoremediation and biotransformation. Trends in Biotechnology, 2003. 21(3): p. 123-130.

Singer, A.C., et al., Bioremediation of polychlorinated biphenyl-contaminated soil using carvone and surfactant-grown bacteria. Applied Microbiology and Biotechnology, 2000. 54(6): p. 838-843.

Singer, A.C., et al., Contribution of earthworms to PCB bioremediation. Soil Biology and Biochemistry, 2001. 33(6): p. 765-776.

Singer, A.C., et al., Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil. Environmental Toxicology and Chemistry, 2003. 22(9): p. 1998-2004.

Sylvestre, M., et al., Transgenic plants to improve rhizoremediation of polychlorinated biphenyls (PCBs). Current Opinion in Biotechnology, 2009. 20(2): p. 242-247.

Taiz, L. and E. Zeiger, Secondary metabolites and plant defense. Plant Physiology. Sinauer Associates, Inc., Sunderland, MA, 2002: p. 283-308.

Thompson, I.P., et al., Bioaugmentation for bioremediation: the challenge of strain selection. Environmental Microbiology, 2005. 7(7): p. 909-915.

Van den Berg, M., et al., The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicological Sciences, 2006. 93(2): p. 223.

Van Dyke, M.I., et al., Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil. Journal of Industrial Microbiology and Biotechnology, 1993. 11(3): p. 163-170.

van Pée, K.H., Biosynthesis of halogenated metabolites by bacteria. Annual Reviews in Microbiology, 1996. 50(1): p. 375-399.

Verpoorte, R., et al., Metabolic engineering of plant secondary metabolite pathways for the production of fine chemicals. Biotechnology Letters, 1999. 21(6): p. 467-479.

Villacieros, M., et al., Polychlorinated Biphenyl Rhizoremediation by Pseudomonas fluorescens F113 Derivatives, Using a Sinorhizobium meliloti nod System To Drive bph Gene Expression. Applied and Environmental Microbiology, 2005. 71(5): p. 2687.

Wink, M., Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. The Plant Vacuole, 1997: The Plant Vacuole: p. 141.

Wittich, R.M. and P. Wolff, Growth of the genetically engineered strain Cupriavidus necator RW112 with chlorobenzoates and technical chlorobiphenyls. Microbiology, 2007. 153(1): p. 186.

Chapter 2: Assessment of an Alaskan willow, *Salix alaxensis*, with potential for PCB rhizoremediation[1]

2.1 Abstract

Certain plant species may enhance polychlorinated biphenyl degradation through cometabolism due to release of aromatic plant compounds that foster the growth of aromatic compound-degrading bacteria in the root zone. *S. alaxensis* (willow) root crushates were added to microcosms containing soil spiked with PCBs to simulate the release of plant compounds by fine root turnover. After 180 days, soil treated with willow root crushates showed up to 30% greater PCB loss than untreated soil for some PCB congeners, including the toxic congeners, PCB 77, 105 and 169. Biphenyl is known to stimulate PCB degradation, and biphenyl treated microcosms showed a similar PCB loss pattern (in both extent of degradation and congeners degraded) to willow treated microcosms, suggesting that willow may be a useful plant for PCB remediation. Treatments with *Picea glauca* (white spruce) roots or salicylate did not stimulate PCB loss. Using the bioassay Microtox the soil toxicity was assessed before and after treatments showing that the willow treatment led to a less toxic soil environment. The biphenyl utilizing bacterium, *Cupriavidus spp.* was isolated from the soil, and clone libraries revealed that uncultured PCB degrading bacteria may also be present in the soil. The findings suggest that *S. alaxensis* may be an effective plant for rhizoremediation by enhancing the loss of some PCB congeners and reducing the toxicity of the soil

[1]Slater, H., Gouin, T., and Leigh, M.B. University of Alaska Fairbanks. 2010. Assessment of an Alaskan willow, *Salix alaxensis*, with potential for PCB rhizoremediation. Prepared for submission to Environmental Science and Technology.

2.2 Introduction

Polychlorinated biphenyls (PCBs) are toxic chemicals used industrially for decades prior to a worldwide ban in the 1970s and 1980s. While in use, over 1.5 million tons of PCBs were produced and a large percentage has been released to the environment. Several of the 209 possible congeners are highly toxic and PCBs are considered probable human carcinogens. They are also implicated in several developmental disorders in children (Huisman et al. 1995). Due to these health risks, PCBs are now major targets for remediation efforts in many areas of the world.

Current remediation strategies include capping known polluted sites and excavation or incineration of contaminated soil. These approaches are often prohibitively expensive and impractical, especially in remote regions. *In situ* bioremediation would be a more desirable solution due to costs, public opinion and environmental impact (Bouwer et al. 1994). Many studies have suggested that rhizoremediation is a possible approach for inexpensive and effective bioremediation of PCBs (Gilbert and Crowley 1997; Leigh et al. 2002; Beranová et al. 2007).

Rhizoremediation is based on the idea that the roots of certain plants may promote the growth and activity of pollutant degrading bacteria. Some plants release secondary compounds from their roots, especially during fine root turnover (root death) that PCB-degrading bacteria can use as carbon and energy sources and/or as inducers of the biphenyl degradation pathway (Donnelly et al. 1994; Ohtsubo et al. 2000; Master and Mohn 2001; Singer et al. 2003). These compounds could promote the growth and

activity of pollutant-degrading bacteria, thereby biostimulating the cometabolic degradation of PCBs (Master and Mohn 2001). Root-released surfactants may also enhance PCB degradation by increasing the bioavailability of PCBs for microbial uptake and degradation (Singer et al. 2000).

Identifying plant species that accelerate microbial PCB degradation is the first step towards developing a successful rhizoremediation strategy. Interior Alaskan plants are attractive candidates for investigation because they often have high concentrations of secondary defense compounds (Jefferies et al. 1994). Furthermore, many remote, high latitude areas, including Alaska, are contaminated with PCBs and rhizoremediation using plants adapted to extreme environmental conditions such as nutrient limitation and low temperatures would be advantageous. In this experiment, we assess the PCB rhizoremediation potential of two common Alaskan species, *Salix alaxensis* (feltleaf willow) and *Picea glauca* (white spruce).

Willow was a focus of the study because willows are known to release salicylate, a metabolic intermediate and stimulatory carbon source for some aromatic compound degrading bacteria (Grund et al. 1992; Chen 1999). Previous studies found high numbers of biphenyl-utilizing bacteria associated with the root zone of another willow, *Salix caprea*, growing in a PCB-contaminated site in central Europe (Leigh et al. 2006). Results of several studies suggest that willow is a potentially useful plant for PCB rhizoremediation (de Carcer et al. 2007a; de Carcer et al. 2007b; Rein et al. 2007; Ionescu et al. 2009). *Salix alaxensis* has phenolic concentrations that are greater than similar

willows of European origin (Bryant et al. 1989). *Picea glauca* (white spruce) was assessed as another potential candidate for PCB rhizoremediation. A previous, preliminary study detected high numbers of culturable biphenyl-utilizing bacteria in the root zone of white spruce relative to other tree species *Betula papyrifera* (paper birch), and *Populus balsamifera* (balsam poplar) in Fairbanks, Alaska (Leigh, unpublished data). Both feltleaf willow and white spruce are broadly distributed across Alaska and Canada.

This study investigated whether fine root turnover of *Salix alaxensis* (feltleaf willow) or *Picea glauca* (white spruce), simulated using crushed roots, promoted PCB biodegradation and detoxification in contaminated soil microcosms. To investigate one possible chemical mechanism, an amendment with salicylate was also tested. Biphenyl, unamended and abiotic controls were run for comparison. Microbial community fingerprinting and sequence analyses were conducted to investigate community dynamics associated with PCB degradation and the addition of root crushates and chemical ammendments. This study aims to identify promising plant species for PCB rhizoremediation and provide mechanistic insight into the roles of root turnover, plant secondary chemistry and microbial community dynamics in this process.

2.3 Experimental Methods

2.3.1 Soil contamination

The experimental soil was a 60:40 peat:silt mix (Great Northwest Landscaping Fairbanks, AK, USA) classified as a member of the order Inceptisol and further classified as a Typic Eutrocryept using the USDA soil taxonomic system (Mulligan 2004). The soil

had 25.5% moisture and 6.5% organic carbon. After collection, soil was passed through a 2 mm sieve and stored at 4°C until used, about 1 month later. The soil was then spiked with a mixture of PCB congeners (Ultra Scientific North Kingstown, RI, USA) ranging from 2 to 6 chloro substitutions. The congeners selected were DiCB-8, -9, -10, TriCB-18, -28, -30 (internal standard), TetraCB-44, -52, -66, -77, -81, PentaCB-95, -97, -105, -118, -126, HexaCB-149, -153 and -169, which were chosen for the patterns of chlorination, range of toxicity and environmental relevance. Solid PCB congeners were dissolved in acetone (HPLC grade VWR, West Chester, PA, USA) and then combined, resulting in a PCB mixture of 18 congeners that was then applied to 2 kg of soil. The PCB mixture was sprayed onto the soil in an unused, clean, 5 gallon, stainless steel paint can and shaken on a large paint can shaker for ten 3 minute cycles. The acetone was allowed to evaporate by opening the paint can in a fume hood for 8 h, and mixing the soil periodically. To determine the initial PCB concentration and homogeneity of spiked soil, three 5-g samples were taken from random locations in the paint can and subjected to PCB quantification described below. Initial soil PCB concentration was between 37.0 and 38.6 mg total PCB/kg soil. Soil was stored in the sealed paint can until microcosm setup a few days later.

2.3.2 Plant candidates

The plant species of interest, *Picea glauca* (referred to as spruce) and *Salix alaxensis* (referred to as willow), were collected from sites with no known contamination predominantly populated with each test species. Willow roots were collected south of

Fairbanks (64°79'84" N 147°89'89" W) and spruce roots were collected on the campus of the University of Alaska Fairbanks (64°86'18" N 147°85'10" W). In order to remove excess soil, roots were rinsed with tap water and stored at 4□ C until use, one week later. Fine roots (1 to 5 mm in length) were removed from larger roots and patted dry before weighing. Fine roots were frozen and crushed under liquid nitrogen, and immediately added to microcosms described below.

2.3.3 Microcosm setup

Acetone-washed glass serum bottles (125 mL Wheaton, Millville, NJ, USA) were used for microcosm setup. All microcosms contained 10 g of PCB spiked soil moistened with 400 µL sterile water. For plant treatments, 1 g crushed fine roots (willow or spruce) were added. Biphenyl treated microcosms contained 5 mg biphenyl (Acros Organics Geel, Belgium), in crystalline form, which was deposited by pipetting a solution of acetone and biphenyl onto the interior walls of the serum bottles and then allowing acetone to evaporate completely prior to the addition of soil. Salicylic acid (Acros Organics Geel, Belgium) was dissolved in sterile water and 5 mg total was added to each salicylate-treated microcosm, with water volume adjusted to achieve equivalent moisture levels as other microcosms. Unamended control microcosms were also constructed. All serum bottles were vortexed for a few seconds to mix contents. Soil that formed clumps was broken up before the microcosms were sealed with Teflon caps and aluminum crimp tops (Wheaton, Millville, NJ, USA). Sterile controls for each treatment were produced by autoclaving sealed microcosms immediately following setup. A 16 gauge needle was

inserted through the septum of all microcosms to allow for gas exchange. Microcosms were incubated in the dark at ambient room temperature (approx. 22°C) and destructively harvested after 30, 90, and 180 days. Six replicates at each time point were set up for live tests including untreated controls, while three replicates at each time point were set up for autoclaved tests. After incubation, microcosms were destructively harvested and soil was stored at -80°C until chemical, toxicological and microbial analyses were performed.

2.3.4 PCB extraction and quantification

The Soxhlet method was used to extract PCBs from soil. Cellulose thimbles (Whatman, Piscataway, NJ, USA) were pre-extracted in 1:1 (v/v) acetone-hexane for 3 h before adding soil to thimbles. Two grams of soil were thawed and immediately mixed with 5 g Na_2SO_4 (VWR West Chester, PA, USA), added to the thimble and allowed to extract overnight for 18 h using 100 mL of 1:1 (v/v) acetone-hexane mixture (HPLC grade, VWR West Chester, PA, USA). Three replicates from each live soil treatment were analyzed at one time, alternating with two replicates from each treatment from dead soil. Either a blank (no soil) or recovery test (Na_2SO_4 in thimble spiked with PCB mixture of known concentration) was run in the third space.

After extraction, the solvent was evaporated down to about 2 mL using a rotary evaporator. The contents were transferred to a test tube with three washes of hexane. Samples were further reduced in volume to 1 mL using a stream of nitrogen gas. If necessary, a Na_2SO_4 column was used to remove excess water from sample, and extract was once again blown down to 1 mL. All samples were cleaned on silica-alumina

columns prepared as follows. Silicic acid (VWR West Chester, PA, USA) was baked and stored at 120°C at all times, and 3 g of 3% deactivated silicic acid was added to a glass column as a slurry with hexane. Then, 2 g of 6% deactivated aluminum oxide (VWR West Chester, PA, USA) was added on top of, but without disturbing the silicic acid layer. Finally, about 1 cm of Na_2SO_4 was added on top to protect column and remove any remaining water. Before addition of the sample, the column was cleaned with 30 mL hexane. Sample was added to the column, followed by three washes of the test tube with hexane, and the sample was eluted into an amber vial with 30 mL hexane. Nitrogen blowdown reduced the volume to about 0.5 mL. A solvent exchange step to isooctane (HPLC grade, VWR West Chester, PA, USA) followed with three washes of isooctane to remove hexane from sample. At this point, 100 µL of 30 ng/µL standard PCB-30 was then added and used as internal standard. The final volume for GC autosampler vials was 2.00 mL, and vials were sealed with screw caps and Teflon-lined septa. Samples were refrigerated in autosampler vials until analysis up to a few days later.

Samples in isooctane were run on an Agilent (Santa Clara, CA, USA) 6890N GC equipped with an ECD detector and an autosampler. A J&W Scientific (Folsom, CA, USA) DB-5 column 30 meters in length, ID 0.250 mm, film of 0.25 µm was used. The oven program was as follows: initial temperature of 90°C, hold for 1 min, temperature raised 10°C per minute to 160°C, then raised 3°C per min to 280°C hold for 2 min, 1.0 µL sample was injected. Helium was used as the carrier gas. After the run, peaks on the chromatograph were manually integrated and peak area was used to determine amount of

PCBs present using PCB-30 as an internal standard and a standard curve for each congener analyzed.

2.3.5 Isolation of biphenyl-utilizing microbes

Culturable biphenyl-utilizing microbes from soil were grown on agar plates with biphenyl as the sole carbon source and further screened for biphenyl utilization using a clearing zone assay using methods previously described (Leigh et al. 2007). Eleven colonies producing zones of clearing were then streaked for isolation onto $1/8^{th}$ strength plate count agar (PCA) plates. Finally, isolates were grown in basal mineral liquid with biphenyl crystals as the sole carbon source until cultures appeared turbid in order to verify growth on biphenyl (Leigh et al. 2007). Only one of eleven colonies retained the ability to utilize biphenyl throughout the isolation procedure and this colony was identified by 16S rRNA gene sequence analysis as described below.

2.3.6 DNA extraction

To extract total soil DNA the Bio101 Fast DNA Spin Kit for soil (MP Biomedicals, Solon, OH, USA) was used. DNA was extracted from 0.5 g soil following kit instructions. The DNeasy kit for blood and tissue (Qiagen, Valencia, CA, USA) was used to extract DNA from pure cultures using the manufacturer's protocols.

2.3.7 T-RFLP

Community profiling using terminal restriction fragment length polymorphism (T-RFLP) was performed on DNA extracted from the soil of 5 microcosms from each treatment. PCR reactions for T-RFLP were performed using 20 pmol each of 27F-FAM

(5'AGAGTTTGATCMTGGCTCAG) and 1392R (5'ACGGGCGGTGTGTRC) primers and 1 μL template DNA with a total reaction volume of 25 μL. Thermal cycler conditions were as follows: denaturation at 95°C for 10 minutes followed by 25 cycles of 95°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute 40 seconds and a final extension step of 72°C for 10 minutes. After PCR, amplification of a product of the correct size (~1380 base pairs) was confirmed using agarose gel electrophoresis. PCR products were then purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) and quantified. PCR products were not detected following several attempts at amplification of extracts obtained from autoclaved soil. All PCR products were standardized to the same DNA concentration with sterile, nuclease free water, and then 80 μg PCR product was digested for 3 h at 37°C using Hha I restriction enzyme, with *Pseudomonas stutzeri* DNA run as a control to verify complete digestion. After digestion, fragments were precipitated overnight using sodium acetate, glycogen, and ethanol as described in Gallagher et al. (2005). Pellets were resuspended in 1.0 μL H_20, and mixed with 0.5 μL of Rox 500 ladder and 9.5 μL of deionized formamide, then run on an ABI 3100 Genetic Analyzer with size standard ROX 500, Dye set D (6-FAM, VIC, NED, and ROX) on a 50 cm array with a 9000 second run time.

Fragments less than 50 base pairs long and more than 505 base pairs long were excluded from analysis. The baseline cutoff for peak height was set at 50 fu. Manual binning to determine fragment length consisted of rounding to the nearest integer. Additionally, sequences obtained from the clone library were digested *in silico* and if a

range of peak heights were possible for one OTU, peaks in that range were binned together.

2.3.8 Clone Libraries

Separate clone libraries were constructed for soils treated with biphenyl, willow or salicylate. Equal portions of DNA from the five replicate microcosms (the same DNA extracts used for T-RFLPs) were pooled and then subjected to cloning and sequence analysis. Purified 16S rRNA gene PCR products were obtained using the same conditions as for T-RFLPs. Immediately prior to cloning, a 3' poly A tail was added to all samples by incubating 25 µL reactions using 20 µL purified amplicon (50 ng/ul), 25 µmol dATP, 0.5 U taq DNA polymerase and 75 µmol $MgCl_2$ in 10X taq buffer at 72°C for 10 minutes. Cloning was performed with a TOPO-TA Cloning Kit for Sequencing with pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA). Plasmid purification followed by single extension Sanger sequencing with primer 27F was performed by Macrogen, Inc. (Seoul, Korea). Purified PCR product from the biphenyl-utilizing isolate, generated using primers 27F and 1392R was also sequenced by Macrogen with single extension sequencing from primer 27F.

Sequences were then trimmed, aligned and taxonomically identified using the Ribosomal Database Project II (RDP II) pipeline. Sequences with less than 80% sequence similarity were considered unclassified at that taxonomic level. *In silico* digestions of clone sequences were conducted by RDP to estimate predicted T-RF lengths for each sequence.

2.3.9 Microtox assays

In order to compare the toxicity of soils incubated for 180 days with different amendments and controls, a basic solid phase test with the bioassay Microtox (Strategic Diagnostics Inc., Newark, DE, USA) was utilized. This bioassay measures the toxicity of many compounds against the bioluminescent bacterium *Vibrio fisheri* by measuring changes in light production. In short, 3.5 grams of soil and 17.5 mL solid phase test diluent were stirred with a stir bar for 10 minutes. A serial dilution was then performed where the soil slurry was further diluted to the following concentrations: 99%, 49.5%, 24.75%, 12.37%, 6.18%, 3.09%, 1.5%, 0.77%, 0.39% and just diluent for 0% w/v. A 10 µL aliquot of the reconstituted reagent (*V. fischeri*) was added to 500 µL diluent and initial light levels (I_0) were recorded. Immediately following, 500 µL of each dilution of the soil slurry was added to the cuvette containing the reagent and samples were incubated for 30 minutes when the light intensity was again measured (I_{30}). A curve was generated using the log of the sample concentration vs. percentage of light decrease in 30 minutes. Percentage of light decrease in 30 minutes was calculated as a normalized value taking into account the amount of light lost when only diluent and no soil was added to the reagent (0%). These values were plotted and the data was interpolated to determine the effective concentration 50 (EC_{50}). An average EC_{50} was determined from duplicate assays of one soil treatment and three replicates of each soil treatment for a total of six replicates for each soil treatment. Clean soil set aside before PCBs were added was used as a reference sample.

2.3.10 Statistical analyses

Independent samples t-tests were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) in order to determine statistical significance.

T-RFLP data was normalized such that peak height was represented as a percentage of total peak height in each sample. The different bacterial communities were then subjected to a cluster analysis using a Bray-Curtis distance with the open source statistical application PAST (Hammer et. al. 2001).

2.4 Results and Discussion

2.4.1 PCB degradation in treated soil

Statistically significant ($p<0.05$) losses in PCB congeners beyond what was observed in untreated soil were detected for soils treated with willow root crushates or biphenyl, but not for spruce root crushates or salicylate (Figure 2.1). Untreated soil represents an important control that accounts for losses from volatilization, sorption, baseline biodegradation, and other processes that would occur in newly polluted soil that has not undergone weathering. Autoclaved soil is an abiotic negative control for chemical and physical processes that may lead to an apparent reduction in PCB concentration, however artifacts resulting from autoclaving are possible, including increased volatilization and sorption. Ultimately, the chemical data were represented as a percent of the PCBs remaining in untreated soil rather than sterile soil because there were significant differences between the two controls for several congeners (Figure 2.2), and

using unamended live soil yielded a more conservative estimate of the extent to which PCBs were degraded.

Significant losses were observed for congeners with a range of chlorination substitution patterns and extents in the treated microcosms (Figure 2.1). The overall patterns of congener loss in willow-treated soil and biphenyl-treated soil were similar, with many of the same congeners being lost from both samples and often to similar extents. In willow and/or biphenyl-treated soils, PCB congeners with 2 (PCB-8), 3 (PCB-18), 4 (PCB-77), 5 (PCB-105) and 6 (PCB-153 and -169) chloro substitutions were significantly degraded (Figure 2.1). These congeners represent non-ortho substituted (PCB-77 and -169), mono-ortho substituted (PCB-8 and -105) and di-ortho substituted (PCB-18 and -153). Notably, degradation occurred for the toxic congeners PCB-77, -81, -105 and -169 (Figure 2.1). These findings indicate that microbial populations stimulated may possess enzymes capable of a variety of dioxygenase attack locations.

The lack of significantly enhanced degradation in spruce-treated soil indicates that the apparent stimulatory effect is plant species specific and that willows possess one or more stimulatory chemicals lacked by spruce. Willows commonly possess salicylate, which has been hypothesized to biostimulate the the cometabolism of PCBs. However, when salicylate was introduced to the soil, PCB degradation was not induced, rather PCB degradation appeared to be inhibited relative to losses observed in untreated soils (Figure 2.1). This may indicate that salicylate is not the active compound in willow roots that leads to enhanced biodegradation of PCBs. Another possible explanations for the effect

observed include that the concentration of salicylate used in the soil was high enough to be either directly toxic to some soil microbes, chelate iron in a way that inhibited the activity of biphenyl dioxygenase, an iron-dependent protein or to lower pH substantially. Given that spruce produces acidic litter, inhibition due to pH reduction is a factor that warrants further investigation.

In a recent pot study, historically contaminated soils that were planted with *Salix caprea* had a 32% loss of total PCBs over 6 months compared with 18% in unplanted soil (Ionescu et al. 2009). This mirrors what we observed with several congeners being degraded up to 30% more extensively than in untreated soils during a 6 month incubation period. Total PCB concentrations in our study showed that with one application of crushed plant roots, 40% of PCBs were degraded whereas untreated soils lost 25%, a difference of 15% (data not shown). The difference in observed PCB degradation between the two studies is likely due to our use of freshly contaminated soils versus historically contaminated soils used in the pot study. Total PCB concentrations were still exhibiting a downward trend at 180 days, indicating that with more time, degradation may continue to occur (Figure 2.3).

2.4.2 Soil Toxicity

The Microtox dose response curves for contaminated soil (Figure 2.4) were used to determine EC_{50} values (Table 2.1). Microtox EC_{50} values showed that willow-treated soil was significantly less toxic than untreated soil and biphenyl-treated soil. Willow-treated soil toxicity was in the same range as the reference (uncontaminated) soil.

Biphenyl-treated soil was the most toxic despite having a lower PCB concentration overall, probably due to the fact that biphenyl itself is toxic and the system contained large amounts of the compound. It is clear that the treatment with willow roots led to a less toxic soil environment, probably as a result of degradation of acutely toxic, co-planar congeners PCB-77, -81, -105 and -169 (Figure 2.1). The less toxic soil environment may also indicate a lack of accumulation of PCB degradation intermediates that might be more toxic to microbes than the parent compounds, although metabolites were not quantified in this study.

Calculated TEQ (toxic equivalency quotient) values show no significant difference between any soil toxicity levels (Table 2.1). TEF (toxic equivalency factor) values for select PCB congeners account for dioxin-like activity and toxicity in humans. Many congeners that could disrupt microbial cell membranes, resulting in the toxic effects detectable with Microtox assay, do not have associated TEF values. As a result, a soil that showed no or little change in TEQ after treatment may still be less toxic to soil microbes as was observed in this study.

Over the incubation time course (Figure 2.3), total PCB concentrations displayed a downward trend in willow treatments, indicating that biodegradation was not yet complete. Thus, longer incubation times may result in lower TEQ values because microbes will have more time to degrade these highly toxic congeners. In a rhizoremediation setting using perennial trees, treatment via root turnover cycles could easily continue for many years beyond the 180-day timeframe of this bench study,

resulting in a long-term sustainable treatment strategy. Longer term pot and field studies are warranted to further assess treatment times needed to reduce TEQ.

2.4.3 Microbial community analyses

The biphenyl-utilizing organism isolated from our soil (Table 2.2) was identified as *Cupriavidus sp*. The genus *Cupriavidus* is among the β-Proteobacteria and is closely related to the genus *Burkholderia. Cupriavidus necator* H850 (formerly *Ralstonia eutrophus* H850 and *Alcaligenes eutrophus* H850) is a well studied PCB-degrader with notable degradation abilities (Furukawa and Fujihara 2008).

T-RFLP results show that several treatments resulted in shifts in microbial community composition and structure. In biphenyl-treated soils, several T-RFs were present and prevalent that did not appear in initial soil or as a result of other treatments (Figure 2.5). These include T-RFs with lengths of 155 (*Acidovorax spp.*), 200, 202 (*Micrococcaceae arthrobacter*) and 367 base pairs (bp) long. In salicylate treated soil, a 205 bp T-RF appeared in every soil community and was rarely detected in other treatments, only being present in one willow-treated soil community and one spruce-treated soil community. Similarly, a 343 bp T-RF was consistently present in willow-treated soil that appeared in several other soil communities, but not consistently. There were also several T-RFs that were common to nearly all communities, such as 58, 61, 75, and 81 bp. Because clone library rarefaction curves indicated that the community was not fully sampled, several T-RFs do not have taxonomic identification are referred to by base pair length.

Cluster analysis of T-RFLPs showed that the bacterial communities of biphenyl-treated soils clustered together and the salicylate-treated soil clustered together (Figure 2.6). The other clustering patterns were not as distinct. Two willow-treated soils clustered together, probably due to the presence of a T-RF of 505 bp. From this analysis it is evident that the different amendments differentially affected the microbial community structure, and that biphenyl and salicylate treatments in particular produced the most reproducible changes in community profile. Notably, biphenyl and salicylate treatments did not result in similar shifts in the microbial community, which might have been expected under the hypothesis that salicylate induces *bph* gene expression and co-metabolism of PCBs (Furukawa et al. 1983; Master and Mohn 2001).

Several genera were detected in clone libraries (Table 2.2) that have been previously implicated in biphenyl and PCB degradation using culture dependent and independent techniques. Previously isolated biphenyl utiltizing bacteria associated with willow trees include members of *Pseudomonas* (Leigh et al. 2006) which was detected in our clone library (Table 2.2). Culture independent techniques, such as stable isotope probing (SIP), have revealed numerous bacteria involved in biphenyl degradation that were not previously identified using culture dependent methods. Many of these genera were detected in our clone libraries. For example, in willow-treated soil, *Variovorax*, *Polaromonas*, *Methylovorus*, and *Methylophilus* are all present and previously identified as biphenyl-utilizing bacteria (Leigh et al. 2007; Uhlik et al. 2009). *Polaromonas* and *Methylophilus* are both detected in biphenyl-treated soil. In salicylate-treated soil,

Paenibacillus and *Methylophilus* were detected. Each SIP study using biphenyl to probe for PCB degrading bacteria have implicated new genera in PCB degradation, and likely many more are undiscovered (Uhlík et al. 2009). Several relatives of known PCB degrading bacteria were present in our soils, and these bacteria might be responsible for the degradation that we observed. Further studies, such as SIP, are needed to determine whether the genera detected in the clone library played an active role in the observed PCB degradation.

Chao 1 richness estimates of operational taxonomic units (OTUs), defined as 97% sequence similarity, in clone libraries were 948, 133, and 505 for biphenyl, salicylate, and willow clone libraries respectively. Clone library rarefaction curves indicated that the microbial communities were not fully sampled, meaning that the relative abundances of different genera do not accurately represent the soil community. Because of this, they may not accurately reflect community structure however their linkage to T-RFLP profiles can provide an indication of relative abundance of different taxa.

Salix alaxensis is a promising candidate for rhizoremediation of PCB contaminated soil and this is especially true for contaminated sites in Alaska and Canada. It is important to identify native species with rhizoremediation potential and this willow species represents one with a large range in Alaska and Canada. The observed PCB degradation and soil detoxification that occurred when soils were incubated with willow roots indicates that the species has the capability to significantly enhance biodegradation rates by indigenous microflora. Microbial analyses demonstrated that relatives of known

PCB degrading bacteria were present in the contaminated soils after treatment. Larger scale, long term studies are warranted to investigate the rhizoremediation capabilities of willow under field conditions and to better understand the mechanisms underlying the plant-microbial interactions.

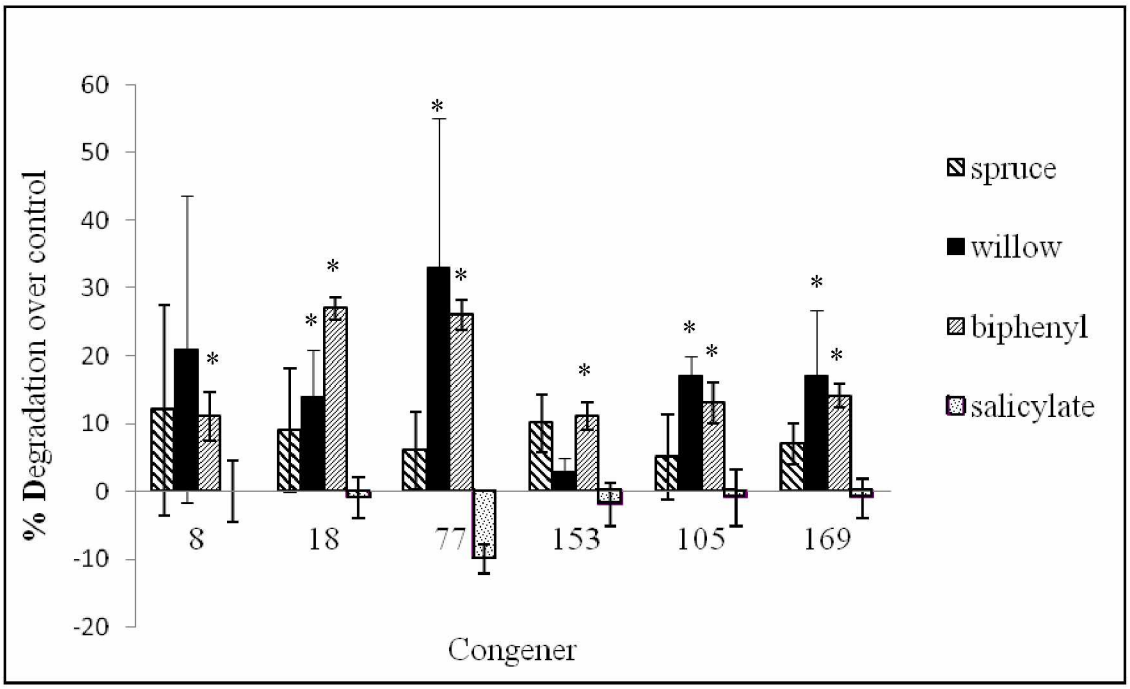

Figure 2.1. Percent degradation of PCB congeners over untreated soil after 180 days Error bars represent one standard deviation. Statistical significance ($P < 0.05$) denoted by *. For each treatment n=6.

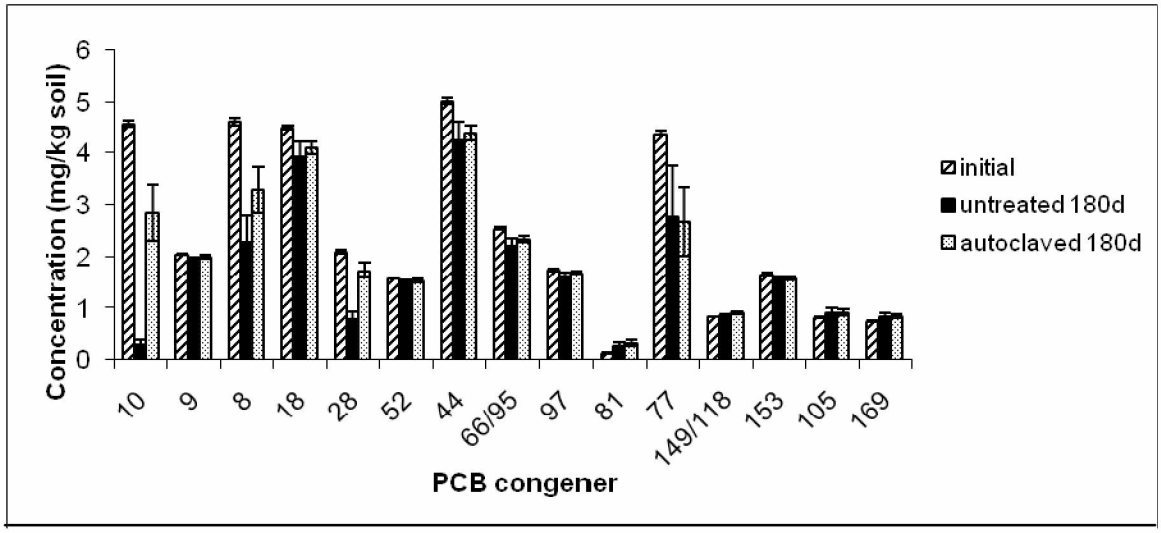

Figure 2.2. Absolute PCB congener concentrations in soil samples

Error bars represent one standard deviation (n=3 for initial and autoclaved, n=6 for untreated).

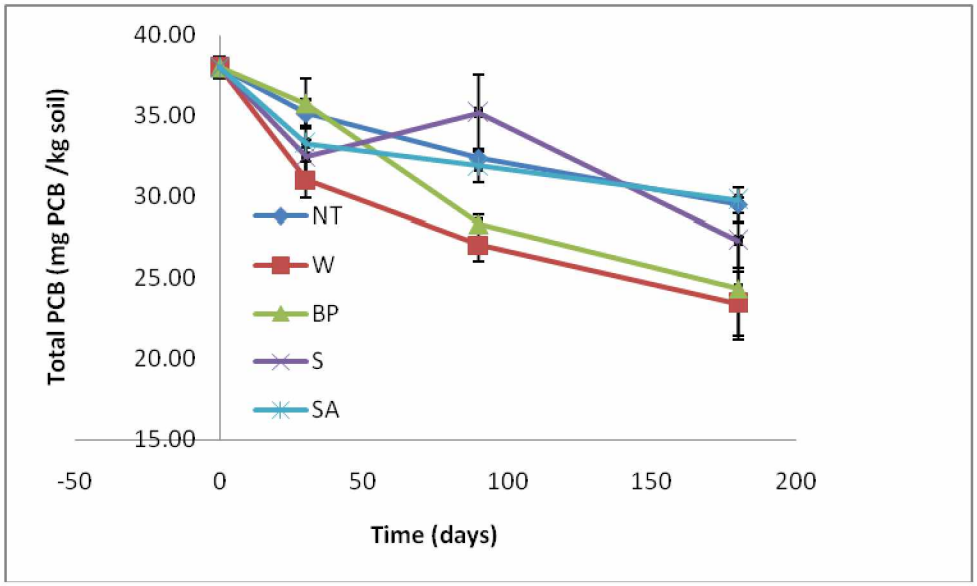

Figure 2.3. Total PCB concentration in soils decreases over time.

For each treatment, n=6.

Table 2.1. EC_{50} and TEQ values for contaminated soils after 180 days (std dev). Superscript denotes statistical significance ($P < 0.05$). For each calculation and treatment, n=6.

	Reference	Initial	No treatment	Willow	Biphenyl	Spruce	Salicylate
$LogEC_{50}$	1.8290^{a} (0.1979)	0.9780^{b} (0.0947)	1.5220^{c} (0.1000)	1.9654^{a} (0.1499)	0.8605^{d} (0.0438)	n/a	n/a
TEQ	n/a	0.0229^{A} (<0.0001)	0.0253^{A} (<0.0002)	0.0223^{A} (<0.0002)	0.0253^{A} (<0.0001)	0.0257^{A} (<0.0001)	0.0278^{A} (<0.0001)

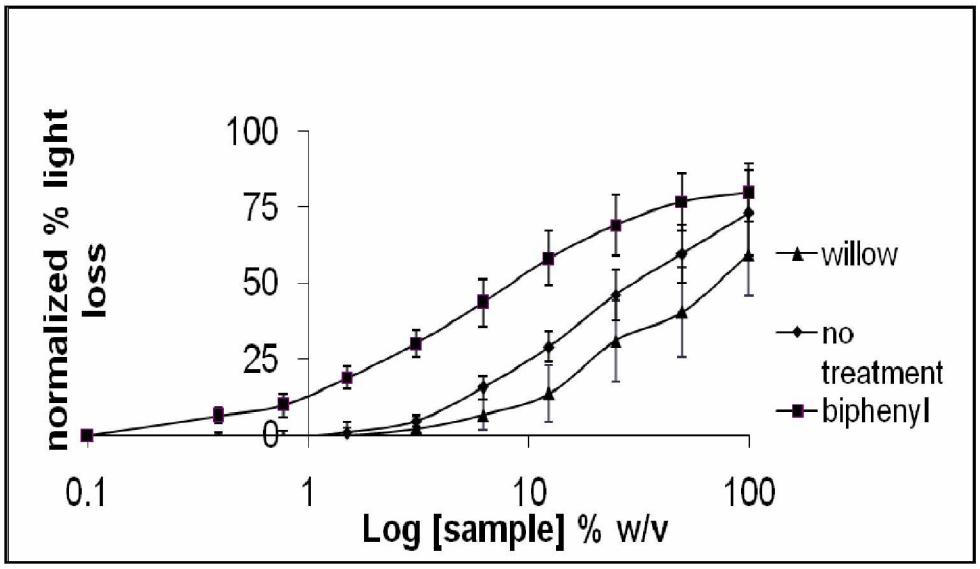

Figure 2.4. Dose response curves for contaminated soils after 180 days

Error bars represent one standard deviation (n=6).

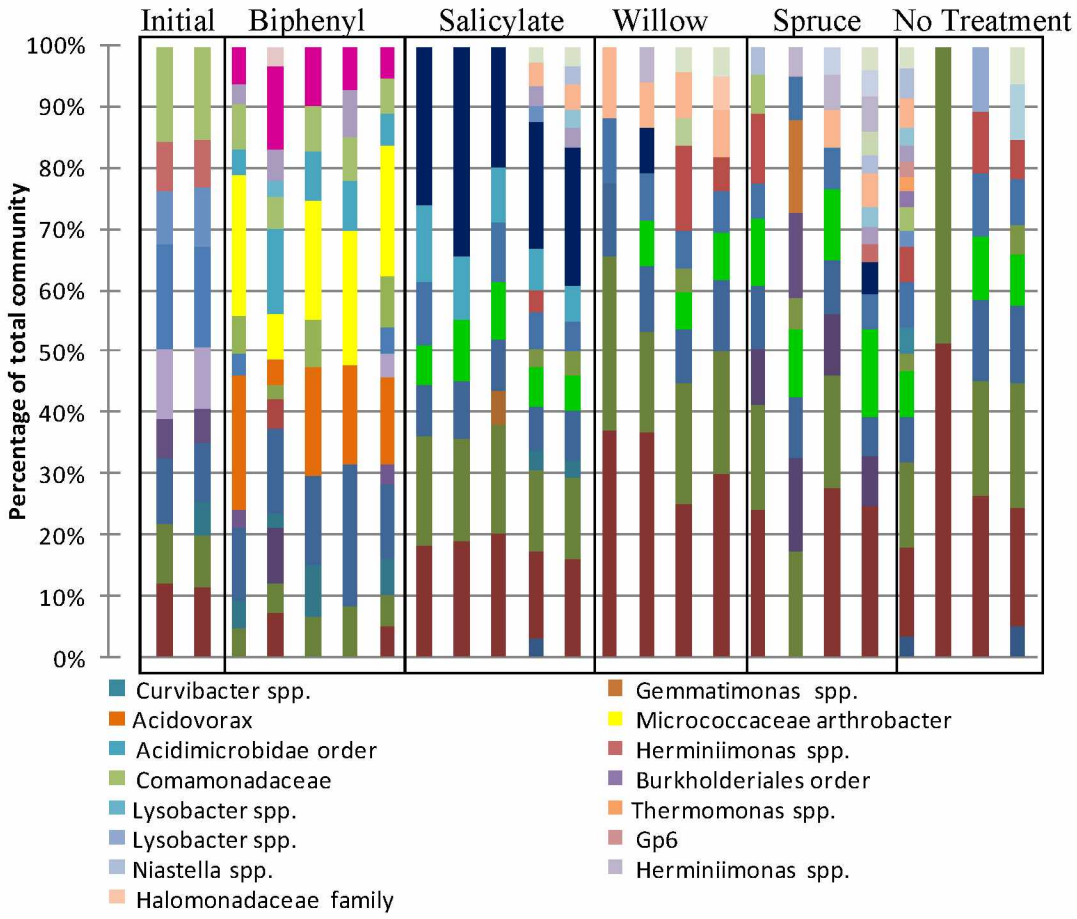

Figure 2.5. Soil bacterial communities represented by T-RF length (different colors) and abundance (relative size).

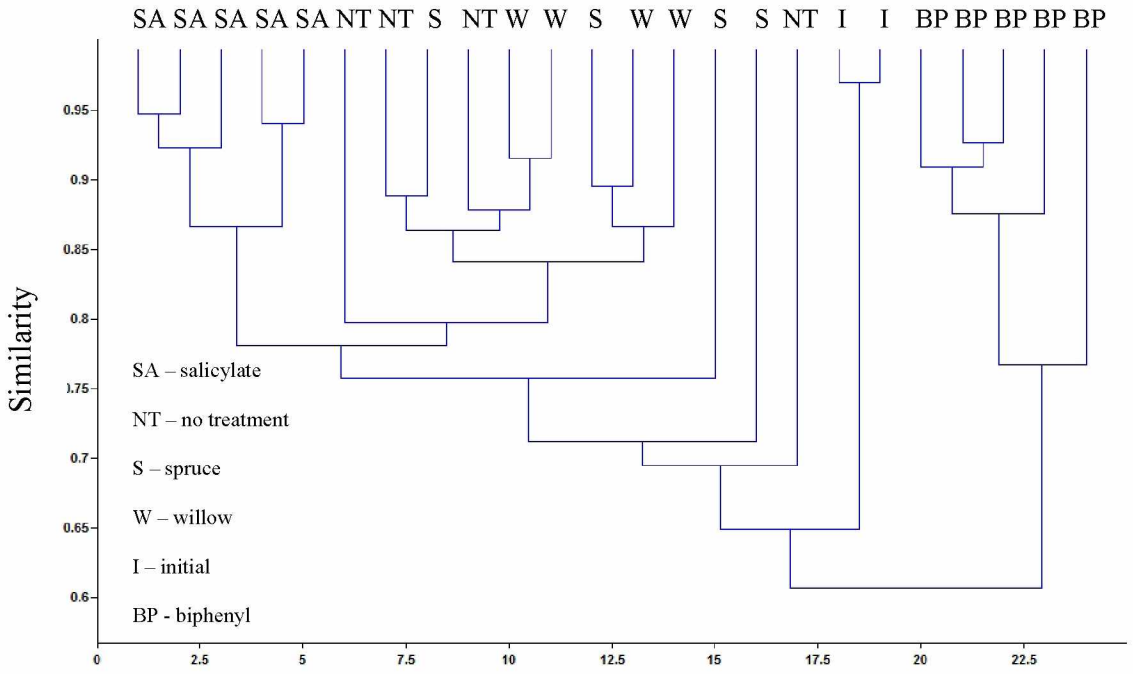

Figure 2.6. Cluster analysis based on T-RF length and abundance in soil samples Bray-Curtis distance was used to determine clusters.

Table 2.2. Taxonomic identification of bacterial16S rRNA gene clones and isolates using RDPII phylogenetic assignments. BP is biphenyl-treated soil, SA is salicylate-treated soil and W is willow-treated soil.

Phylum	**family**	**genera**	**BP**	**SA**	**W**	**isolate**
Acidiobacteria	Acidobacteriaceae	*Gp3*		1		
		Gp4		2	1	
		Gp6	4	11	2	
		Gp7		1		
		Gp10	1			
		Gp16		2	1	
	Unclassified Acidiobacteria			1		
Actinobacteria	Actinomycetales	*Micrococcineae*	18	7	13	
		Micromon			1	
		Propionibacterineae	4	2	4	
		Frankineae	1	1		
		Unclassified Actinomycetales		1		
		Streptomycineae		2	4	
	Rubrobacterales	*Rubrobacterineae*	3	1		
	Unclassified Actinobacteria		10	2	4	
Bacteroidetes	Crenotrichaceae	*Chitinophaga*			1	
		Unclassified Crenotrichaceae	1		1	
	Flavobacteriaceae	*Flavobacterium*		1	1	
	Flexibacteraceae	*Adhaeribacter*	2			
		Niastella	1	2		
		Unclassified Flexibacteraceae			4	
	Saprospiraceae	*Lewinella*		1	1	
	Unclassified Bacteroidetes		1	1	1	
Chloroflexi	Caldilineales	*Caldilineacea*	1	4	2	
	Unclassified Chloroflexi			1	4	

Table 2.2 continued.

	Rhodoferax			1
	Simplicispira	1		
	Variovorax			1
	Unclassified Comamonadaceae	4		1
Coxiellaceae	*Aquicella*		1	
Hyphomicrobiaceae	*Devosia*	2	6	1
	Blastochloris	1		
	Hyphomicrobium			1
	Rhodoplanes			1
	Unclassified Hyphomicrobiaceae	1	4	5
Methylophilaceae	*Methylovorus*			8
	Methylophilus	1	1	3
	Unclassified Methylophilaceae	4		
Moraxelaacea	*Acinetobacter*		1	
Oxalobacteraceae	*Collimonas*			2
	Herbaspirillum			1
	Herminiimonas	2	1	4
	Massilia		1	
	Unclassified Oxalobacteraceae	1	1	1
Phyllobacteriaceae	*Phyllobacterium*		1	
	Mesorhizobium		1	
	Aquamicrobium	1		
	Unclassified Phyllobacteriaceae		1	1
Pseudomonadaceae	*Pseudomonas*	3	7	1
Rhodobacteraceae	*Rhodobacter*			1
	Dinoroseobacter	1		
	Unclassified Rhodobiaceae	1	1	1
Rhodocyclaceae	*Denitratisoma*			1
Rhizobiaceae	*Rhizobium*	1		

Table 2.2 continued

	Sphingomonadaceae	*Novosphingobium*		1	1
		Sphingobium			1
		Porphyrobacter	1		1
		Unclassified Sphingomonadaceae	2		
	Sorangineae	*Polyangiaceae*	1	3	2
		Unclassified Sorangineae		1	1
	Xanthomonadaceae	*Lysobacter*	5	3	7
		Stenotrophomonas	1		1
		Thermomonas	3		1
		Unclassified Xanthomonadeaceae	7		
	Unclassified				
	Alphaproteobacteria		3	3	4
	Unclassified				
	Betaproteobacteria		9	1	1
	Unclassified				
	Deltaproteobacteria		1	10	3
	Unclassified				
	Gammaproteobacteria		3	3	3
	Unclassified Proteobacteria		1	2	2
	Unclassified				
Thermomicrobia	Thermomicrobia		1		
TM7	Unclassified TM7		8	2	8
	Unclassified				
Verrucomicrobia	Verrucomicrobiales			2	
Unclassified					
Bacteria			13	7	3
Total sequences analyzed			157	128	151	1

2.5 References

Beranová, K., et al., Rhizoremediation for decontamination of long-term PCB contaminated soil with focus on microbial diversity. Journal of Biotechnology, 2007. 131(2S): p. 243-243.

Bouwer, E., et al., Degradation of xenobiotic compounds in situ: capabilities and limits. FEMS Microbiology Reviews, 1994. 15(2-3): p. 307-317.

Bryant, J.P., et al., Biogeographic evidence for the evolution of chemical defense by boreal birch and willow against mammalian browsing. The American Naturalist, 1989. 134(1): p. 20-34.

Chen, S.H. and M.D. Aitken, Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by Pseudomonas saccharophila P15. Environmental Science & Technology, 1999. 33(3): p. 435-439.

de Carcer, D.A., et al., Changes in bacterial populations and in biphenyl dioxygenase gene diversity in a polychlorinated biphenyl-polluted soil after introduction of willow trees for rhizoremediation. Applied and Environmental Microbiology, 2007a. 73(19): p. 6224.

de Carcer, D.A., et al., The introduction of genetically modified microorganisms designed for rhizoremediation induces changes on native bacteria in the rhizosphere but not in the surrounding soil. The ISME Journal, 2007b. 1(3): p. 215-223.

Donnelly, P., et al., Growth of PCB-degrading bacteria on compounds from photosynthetic plants. Chemosphere (Oxford), 1994. 28(5): p. 981-988.

Furukawa, K. and H. Fujihara, Microbial degradation of polychlorinated biphenyls: Biochemical and molecular features. Journal of bioscience and bioengineering, 2008. 105(5): p. 433-449.

Furukawa, K., et al., Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in Pseudomonas paucimobilis. Journal of Bacteriology, 1983. 154(3): 1356.

Gallagher E., et al., 13C-carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. Applied and Environmental Microbiology, 2005. 71: 5192–5196.

Gilbert, E.S. and D.E. Crowley, Plant compounds that induce polychlorinated biphenyl biodegradation by Arthrobacter sp. strain B1B. Applied and Environmental Microbiology, 1997. 63(5): p. 1933.

Grund, E., B. et al., Naphthalene degradation via salicylate and gentisate by Rhodococcus sp. strain B4. Applied and Environmental Microbiology, 1992. 58(6): p. 1874-1877.

Hammer, Ø., et al., PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica, 2001. 4(1): 9.

Huisman, M., et al., Neurological condition in 18-month-old children perinatally exposed to polychlorinated biphenyls and dioxins. Early Human Development, 1995. 43(2): p. 165-176.

Ionescu, M., et al., Isolation and characterization of different plant associated bacteria and their potential to degrade polychlorinated biphenyls. International Biodeterioration & Biodegradation, 2009. 63(6): 667-672.

Jefferies, et al., Vertebrate herbivores and northern plant communities: reciprocal influences and responses. Oikos, 1994. 71(2): p. 193-206.

Leigh, M.B., et al., Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. Applied and Environmental Microbiology, 2006. 72(4): p. 2331-2342.

Leigh, M.B., et al., Root Turnover: An Important Source of Microbial Substrates in Rhizosphere Remediation of Recalcitrant Contaminants. Environmental Science & Technology, 2002. 36(7): p. 1579-1583.

Leigh, M. B., et al., Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). The ISME Journal, 2007. 1(2): p. 134-148.

Master, E.R. and W.W. Mohn, Induction of bphA, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant Pseudomonas strain Cam-1 and mesophilic Burkholderia strain LB400. Applied and Environmental Microbiology, 2001. 67(6): p. 2669-2676.

Mulligan, D., Soil Survey of Greater Fairbanks Area, Alaska. USDA, NRCS, 2004.

Ohtsubo, Y., et al., Expression of the bph genes involved in biphenyl/PCB degradation in Pseudomonas sp. KKS102 induced by the biphenyl degradation intermediate, 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoic acid. Gene, 2000. 256(1-2): p. 223-228.

Rein, A., et al., Degradation of PCB congeners by bacterial strains. Applied Microbiology and Biotechnology, 2007. 77(2): p. 469-481.

Singer, A.C., et al., Bioremediation of polychlorinated biphenyl-contaminated soil using carvone and surfactant-grown bacteria. Applied Microbiology and Biotechnology, 2000. 54(6): p. 838-843.

Singer, A.C., et al., Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil. Environmental toxicology and chemistry, 2003. 22(9): p. 1998-2004.

Uhlik, O., et al., Biphenyl-Metabolizing Bacteria in the Rhizosphere of Horseradish and Bulk Soil Contaminated by Polychlorinated Biphenyls as Revealed by Stable Isotope Probing. Applied and Environmental Microbiology, 2009. 75(20): p. 6471.

Chapter 3: Supplemental studies[1]

3.1 Abstract

Microbial degradation of PCBs is a well studied field, but many questions remain that may be answered using emerging methods such as chiral PCB analyes and stable isotope probing. Enantiospecific degradation of chiral PCB congeners could provide proof of microbial activities as well as insight into congener specificity and enzyme activity. No enantiospecific degradation occurred during the short study time (180 days), but this may be due to the high degree of chlorination of the selected congeners and the length of the study. Stable Isotope Probing (SIP) is a molecular technique that links microbial metabolic function with genetic identification without the use of culturing. During the study, a shoulder of Heavy (labeled) DNA was detected, but the difference from controls was not enough to continue the analyses. Nutrient additions to the soil may help with the incorporation of labeled substrate into microbial DNA.

[1]Slater, H., Gouin, T., and Leigh, M.B. University of Alaska Fairbanks. 2010. Supplemental studies. Prepared for submission to Environmental Science and Technology.

3.2 Introduction

PCBs are prevalent and ubiquitous in the environment, which is problematic due to their toxicity and ability to accumulate in the food chain. Biodegradation of PCBs is one promising remediation strategy. One hallmark of biodegradation of PCBs is enantioselective degradation of chiral congeners, such as PCB 95 and 149 (Asher et al. 2007). In aged soils, non-racemic signatures of these congeners are often detected, indicating that selective biodegradation of on enantiomer has occurred (Robson and Harrad 2004). In this study, two chiral congeners, PCB 95 and 149, were included in a biodegradation study. If non-racemic signatures of these congeners are detected, this would confirm that the PCBs were biologically degraded and not lost to other physical processes such as volatilization or irreversible sorption.

The microbial community that results in the degradation of PCBs is also an important part of understanding how PCBs are degraded in soils. In the past, stable isotope probing (SIP) has been successfully utilized to identify PCB degrading bacteria in contaminated soils (Leigh et al. 2006). In DNA based SIP, soils are incubated with a ^{13}C labeled substrate and the label is incorporated into active organisms' DNA. The DNA is then separated using ultracentrifugation and the Heavy (^{13}C labeled) DNA can be used in downstream applications such as clone libraries, sequencing, and community fingerprint analyses. Biphenyl is chosen as the labeled substrate in this study because PCBs are cometabolized through the biphenyl pathway, and microbes shown to derive carbon from

biphenyl are probable PCB degraders (Ohtsubo et al. 2000). This knowledge would clarify which individuals in a microbial community are involved in PCB degradation.

3.3 Experimental methods

3.3.1 Chiral PCB analysis

Soil contamination, treatment, and PCB extraction are the same as was previously described. For chiral PCB-95 and -149, soil extracts described above were then run on a Chirasil-Dex column 25 m in length, ID .25 mm, DF .25 um (Vairan Inc., Palo Alto, CA, USA). The oven program was an initial temperature of 60°C held for 2 minutes, then increased temperature 10°C/min to 150°C then increased 1°C/min to 225°C held for 20 minutes, then decreased 10°C/min to 30°C and held for 30 minutes. All other conditions were the same as described above for PCB quantification. Technical standards for PCB-95 and PCB-149 were run several times throughout the day and between samples. Enantiomeric fractions (EFs) were determined for samples and standards by dividing the area of the first peak by the sum of the area of the first and second peaks.

3.3.2 Stable Isotope Probing (SIP) microcosms

Microcosms for SIP were set up in sterile 125 mL glass serum bottles. Before addition of soil ^{13}C biphenyl (Isotec, Miamisburg, OH, USA) or unlabeled biphenyl was dissolved in acetone and a volume was added to each microcosm by pipeting the solution on to the inside of the serum bottle and allowing the acetone to evaporate in a fume hood, leaving behind 1mg biphenyl crystals. Added to each serum bottle were 5 g of PCB contaminated soil and 400 μL sterile water. The bottles were then sealed with a Teflon

stopper and aluminum crimp top. Duplicate microcosms were set up for each treatment (labeled or unlabeled biphenyl) and each time point. Microcosms were then incubated at room temperature in the dark until destructive harvesting. An aliquot of soil without ^{13}C biphenyl added was frozen at -80°C to serve as the time 0 (T0) sample for comparison with incubated samples. At the time of harvest for each microcosm, a 1-mL headspace gas sample was collected through the Teflon stopper with a sterile needle and syringe, which was then injected into an airtight gas sampling tube (12 mL) equilibrated with ambient lab air at the same time microcosms were sealed. These gas samples underwent further isotopic analysis described below. Microcosms were harvested at 1, 4, 14, and 28 days (T1, T4, T14, and T28 respectively) and soil was stored at -80°C until further analysis.

3.3.3 Headspace gas isotope analysis

The δ ^{13}C was determined for headspace CO_2 gas samples using a Thermo Finnigan GasBench II carbonate analyzer with a Delta^{Plus}XP Mass Spectrometer. Reactions were run at 70˚C with a He flow rate of 120 mL/min and a GC oven temp of 50˚C. Samples were first placed into clean exetainer tubes, with NIST and IAEA standards being placed in round bottom exetainer tubes. Standard sample tubes were purged for 20 minutes with UHP Helium and then 0.2 mL of 85% H_3PO_4 was manually added to the standards by syringe to the sample through the septum in the lid of the tubes. CO_2 gas samples were analyzed as is, with no headspace flushing or acid addition. Headspace gases were then transferred to the GasBenchII where the water was removed

through a nafion dryer. CO_2 was separated chromatographically from the other gases present and then transferred to the isotope ratio mass spectrometry (IRMS), carbon were measured. $\delta^{13}C_{PDB}$ values are reported in reference to international isotope standards. Quality control measures were taken by analyzing a blank every twenty samples. The laboratory working standard was calcium carbonate (Merck, Suprapur, 99.95% Lot # B510959 313).

3.3.4 DNA extraction

To extract total soil DNA the Bio101 Fast DNA Spin Kit for soil (MP Biomedicals, Solon, OH, USA) was used. DNA was extracted from 0.5 g soil following kit instructions. The tubes containing soil and the lysing matrix were shaken using a FastPrep Mini-beadbeater instrument (BioSpec product Inc., Bartlesville, OK, USA) in order to break up cell walls more efficiently. This method was used for all soil extracts.

3.3.5 Isopycnic centrifugation

Density gradient centrifugation was performed on the DNA extracted from the time courses (T0-T28) in order to separate the ^{13}C-DNA from microbes that utilized the labeled carbon source from the rest of the soil community unlabeled DNA. A solution of cesium trifluoroacetate (CsTFA) (GE Healthcare, Buckinghamshire, UK) and sterile water was added until it reached a buoyant density (BD) of approximately 1.60 g/mL. BD was measured gravimetrically by pipeting a known volume onto an analytical balance. This solution was placed into 5.1 mL volume Beckman centrifuge tubes (part number 342412) and equal volumes of DNA extract were added to each tube to total 2.0

μg of DNA per gradient. Duplicate gradients of each sample were run concurrently and blank gradients that contained water in place of DNA were also run. Gradients were produced by centrifugation for 3 days (72 h) in a Beckman-Coulter Optima L-100 XP Ultracentrifuge using a NVT-100 rotor at 134260 g (45600 rpm) at 25°C. Gradients were then fractionated into twenty fractions of 250 μL each. Blank gradients containing no DNA were fractionated and density was measured in order to determine the density of each fraction for all gradients run in parallel. DNA was then precipitated from fractions containing DNA using isopropanol (Sigma, St. Louis, MO, USA) and pellets were resuspended in 30 μL DNase free sterile water. Fractions were frozen at -20°C until further analysis.

3.3.6 Quantitative PCR (qPCR) screening of SIP fractions

To locate bacterial DNA in SIP fractions, qPCR was performed on every fraction for each time point in duplicate. Universal bacterial primers targeting 16S rDNA 1108F and 1132R were used for qPCR. A master mix containing SYBR green (Applied Biosystems, Foster City, CA, USA), primers (5μM), BSA (10mg/mL), and water was added to 1.0 μL template for a total reaction volume of 15 μL. Thermocycler conditions were an initial temperature of 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative quantity of bacterial DNA was determined in relation to standard curves (10 ng, 1 ng, 0.1 ng and 0.01 ng), consisting of genomic DNA extracted from pure culture of *Burkholderia xenovorans* LB400. After qPCR, the results were graphed and fractions containing ^{13}C-labeled

bacterial DNA were identified. At the same time, fractions from the T0 gradient and ^{12}C biphenyl treated microcosms were analyzed in order to identify any background DNA contamination present throughout gradients.

3.4 Results and Discussion

3.4.1 Chiral PCB degradation

The calculated EF values for both PCB 95 and PCB 149 indicate that in all soil samples were racemic (0.500) for the selected congeners. This would imply that no biological degradation of those congeners occurred (Table 3.1). This is in agreement with chemical data which showed that no significant losses for the selected congeners occurred over autoclaved controls, in which no biological transformation could occur. PCB-95 is a pentachlorobiphenyl and PCB-149 is a hexachlorobiphenyl. The high degree of chlorination could prevent PCB degrading bacteria from acting on these molecules during the short time of our study.

3.4.2 SIP experiments

Isotopic headspace analysis indicated that a small amount of labeled CO_2 was produced by microorganisms in the SIP microcosms (Figure 3.1). When qPCR was performed on the SIP fractions to locate Heavy (^{13}C labeled) DNA, no Heavy fractions were identified. Small shoulders of possible Heavy DNA on the large unlabeled DNA peaks were detected, but the difference from unlabeled controls was not great enough to continue analyses (Figure 3.2). Due to the low organic carbon content of the soils used, it is possible that microbial biomass able to derive carbon from the substrate was too low to

detect. In order to successfully detect biphenyl-utilizing microbes in this way, nutrient additions may be necessary.

3.4.3 Final remarks

The results of these studies show that no biological degradation of chiral PCB congeners occurred during the study. The chiral congeners included where highly chlorinated, which could have contributed to the lack of biodegradation that occurred. In future studies, lower chlorinated chiral congeners may be a better choice because they are more quickly degraded. A longer incubation time may also lead to degradation of chiral congeners and an observable difference in EF.

SIP experiments were not conclusive due to the lack of clearly labeled Heavy DNA peaks being detected. This could be due to a lack of nutrients in the soil. In the past, the addition of mineral salts medium to soil before the introduction of a labeled substrate led to more Heavy DNA being detected (Ondrej Uhlik, personal communication). The addition of nutrients to the soil may increase the incorporation of labeled substrate.

Table 3.1. Calculated average EF values for select soil PCB extracts and the standard deviations. No significant differences between EF values were observed.

	standard	autoclaved	untreated	willow
average EF	0.4853	0.4921	0.4920	0.4923
standard deviation	0.0138	0.0020	0.0015	0.0007

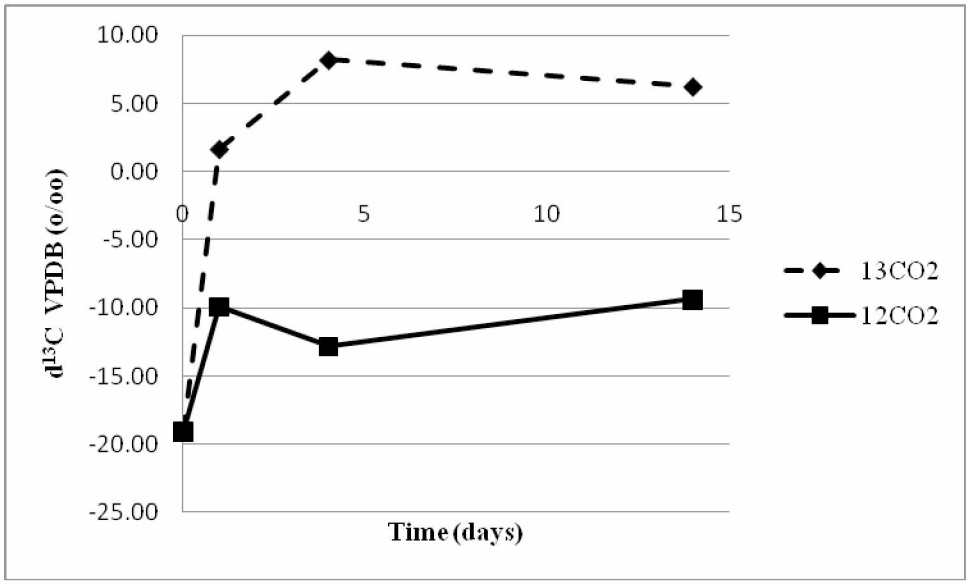

Figure 3.1. Detection of labeled CO_2 after soil incubation with ^{13}C biphenyl

This figure shows that there was a small difference in the isotopic ratio of CO_2 evolving from microcosms incubated with ^{13}C labeled biphenyl (dashed line $^{13}CO_2$) and ^{12}C unlabeled biphenyl negative controls (solid line $^{12}CO_2$).

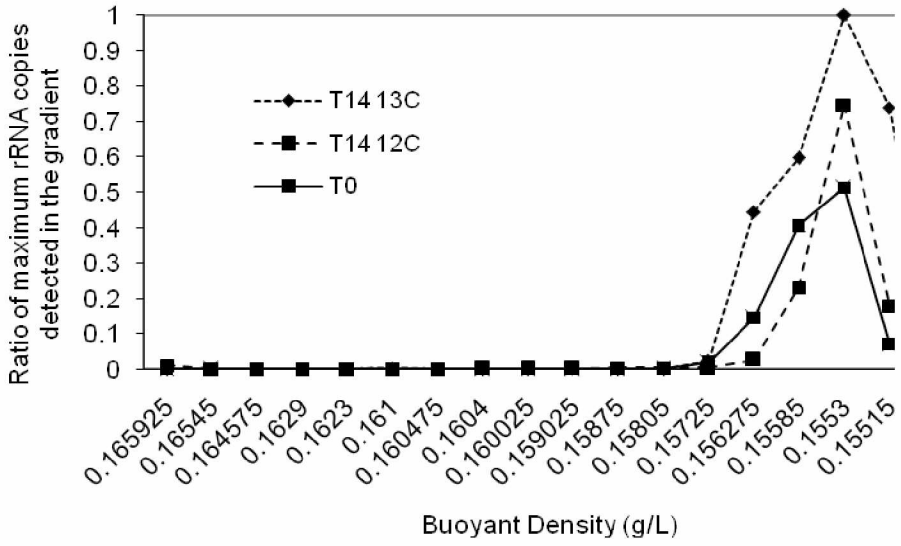

Figure 3.2. Detection of DNA in gradient fractions for time 0 and time 14 days

The slight shoulder seen on the T14 13C peak may indicate the presence of Heavy DNA because the shoulder is not detected in unlabeled or T0 gradients.

3.5 References

Asher, B. J., et al., Chiral Source Apportionment of Polychlorinated Biphenyls to the Hudson River Estuary Atmosphere and Food Web. Environmental Science & Technology 2007. 41(17): p. 6163-6169.

Leigh, M. B., et al., Polychlorinated Biphenyl (PCB)-Degrading Bacteria Associated with Trees in a PCB-Contaminated Site. Applied and Environmental Microbiology 2006. 72(4): p. 2331-2342.

Ohtsubo, Y., et al., Expression of the bph genes involved in biphenyl/PCB degradation in Pseudomonas sp. KKS102 induced by the biphenyl degradation intermediate, 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoic acid. Gene 2000. 256(1-2): p. 223-228.

Robson, M. and S. Harrad, Chiral PCB Signatures in Air and Soil: Implications for Atmospheric Source Apportionment. Environmental Science & Technology 2004. 38(6): p. 1662-1666.

Conclusion

This assessment of the rhizoremediation potential of two Alaskan tree species indicates that *S. alaxensis* (willow) root turnover promotes microbial degradation of PCBs, resulting in increased extent of PCB disappearance relative to untreated soils and a reduction of soil toxicity. The capacity to biostimulate PCB degradation and detoxification was found to be plant-specific, as it was not shown by *P. glauca* (white spruce). Although willow treated soil led to PCB degradation, salicylate, a willow secondary compound did not stimulate PCB loss. Thus, the active compound present in willow roots is not clear and further studies are needed to elucidate the mechanism of PCB rhizoremediation by this willow. Future studies are also needed to identify the active PCB degrading microorganisms and to screen additional plant candidates for PCB rhizoremediation potential.

Rhizoremediation is a promising technique to treat PCB contaminated soils *in situ*, but success of this technology for high latitude regions requires the identification of effective plant species adapted to cold climates. This study clearly identifies a common plant native to Alaska and Canada with the potential to promote PCB rhizoremediation. *S. alaxensis* thus warrants further investigation. Long term field scale studies are needed in order to determine if the degradation observed in some lab studies is transferable to actual contaminated sites with variable soil types, ambient temperatures and environmental conditions.