Published in final edited form as:

Chemosphere. 2011 June ; 84(2): 199–206. doi:10.1016/j.chemosphere.2011.04.058.

Assessing the potential for rhizoremediation of PCB contaminated soils in northern regions using native species

Heather Slater¹, Todd Gouin¹, and Mary Beth Leigh^{*,2}

¹University of Alaska Fairbanks, Department of Chemistry & Biochemistry, P.O. Box 756160, Fairbanks, AK 99775-6160

²University of Alaska Fairbanks, Institute of Arctic Biology, P.O. Box 757000, Fairbanks, AK 99775-6160

Abstract

Rhizosphere bioremediation of polychlorinated biphenyls (PCBs) offers a potentially inexpensive approach to remediating contaminated soils that is particularly attractive in remote regions including the Arctic. We assessed the abilities of two tree species native to Alaska, *Salix alaxensis* (felt-leaf willow) and *Picea glauca* (white spruce), to promote microbial biodegradation of PCBs via the release of phytochemicals upon fine root death. Crushed fine roots, biphenyl (PCB analogue) or salicylate (willow secondary compound) were added to microcosms containing soil spiked with PCBs and resultant PCB disappearance, soil toxicity and microbial community changes were examined. After 180 d, soil treated with willow root crushates showed a significantly greater PCB loss than untreated soils for some PCB congeners, including the toxic congeners, PCB 77, 105 and 169, and showed a similar PCB loss pattern (in both extent of degradation and congeners degraded) to biphenyl-treated microcosms. Neither *P. glauca* (white spruce) roots nor salicylate enhanced PCB loss, indicating that biostimulation is plant species specific and was not mediated by salicylate. Soil toxicity assessed using the Microtox bioassay indicated that the willow treatment resulted in a less toxic soil environment. Molecular microbial community analyses indicated that biphenyl and salicylate promoted shifts in microbial community structure and composition that differed distinctly from each other and from the crushed root treatments. The biphenyl utilizing bacterium, *Cupriavidus spp.* was isolated from the soil. The findings suggest that *S. alaxensis* may be an effective plant for rhizoremediation by altering microbial community structure, enhancing the loss of some PCB congeners and reducing the toxicity of the soil environment.

Keywords

Rhizoremediation; PCBs; Bioremediation; Microbial degradation

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding author contact: mbleigh@alaska.edu, Tel: (907) 474-6656, FAX: (907) 474-6967.

Supplementary Material Supplementary material includes taxonomic identifications of bacterial 16S rRNA gene clones and biphenyl-utilizing isolates, EC₅₀ and TEQ values for contaminated soils following incubation for 180 d and absolute PCB congener concentrations in initial and control soil samples.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Environmental contamination with polychlorinated biphenyls (PCBs) is both widespread and particularly challenging to remediate in the Arctic. Whereas concern regarding the long-range transport of PCBs to the Arctic has been the focus of a number of studies (Macdonald et al., 2000; Wania, 2003), the influence of local point sources, such as the large number of sites associated with the Distant Early Warning (DEW) line in Canada, and formerly used defense sites (FUDS) in Alaska, is of continuing concern to residents who live in close proximity to those sites (Ap O'Hehir et al., 2005), many of whom rely on wildlife for subsistence. While a great deal of effort has been directed towards remediation of several DEW and FUDS contaminated sites (Poland et al., 2001; Stow et al., 2005), with clean-up efforts resulting in lower exposures of PCBs (Brown et al., 2009), sites that remain contaminated continue to influence PCB levels in wildlife (Anthony et al., 2007; Ricca et al., 2008; Miles et al., 2009).

In general, a combination of the remoteness of contaminated FUDS sites in the Arctic with the expense of remediation has resulted in a limited number of FUDS being remediated. According to the US Army Corps of Engineers, there are currently only 132 FUDS that have been remediated out of 312 that have been identified for environmental clean-up in the state of Alaska (<http://www.poa.usace.army.mil/hm/default.htm>). Typical methods to remediate PCB contaminated sites include excavation of soils and subsequent destruction by incineration or burial in secure landfills. These methods can be prohibitively expensive to undertake, particularly when considering the remote locations of FUDS, and additional challenges relating to the Arctic environment must be overcome, such as excavation of permafrost soils and preventing the migration of PCBs along the surface bedrock (Poland et al., 2001). Many studies have suggested that *in situ* bioremediation methods, such as rhizoremediation, may provide an inexpensive and effective alternative to *ex-situ* remediation techniques (Gilbert and Crowley, 1997; Beranova et al., 2007; Leigh et al., 2002).

Rhizoremediation is a method that capitalizes on the ability of roots of certain plants to promote the growth and activity of pollutant degrading bacteria. Some plants have been demonstrated to promote PCB loss from the rhizosphere soil, which may occur via a number of different mechanisms such as cometabolism, increased bioavailability and improved aeration (Van Aken et al., 2004). One hypothesized mechanism is the release of plant secondary compounds from their roots, especially during fine root turnover (i.e. root death), that can be used by PCB-degrading bacteria as carbon and energy sources and/or as inducers of the biphenyl degradation pathway (Donnelly et al., 1994; Singer et al., 2004). The release of these secondary compounds by roots of a particular species could promote the growth and activity of pollutant-degrading bacteria, thereby biostimulating the co-metabolic degradation of PCBs over years of fine root growth and dieback.

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 many possess high concentrations of secondary defense compounds (Jefferies et al., 1994), which could potentially function as cometabolites. Additionally, their adaptation to extreme environmental conditions, such as nutrient limitation and low temperatures, represents additional advantages when attempting to remediate contaminated sites in remote Alaska and Canada. In this study, we assess the potential of two common Alaskan tree species, *Salix alaxensis* (feltleaf willow) and *Picea glauca* (white spruce), to promote PCB degradation in contaminated soil.

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). The root zones of some willows have been found to harbor increased numbers of PCB degrading bacteria relative to other species of bacteria (Leigh et al., 2006). Additionally, a diversity of biphenyl dioxygenase genes have been detected in the root zones of certain willows (de Carcer et al., 2007a). Willows also are known to produce salicylate, which may induce biphenyl (Master and Mohn, 2001) and polyaromatic hydrocarbon degradative gene expression (Chen and Aitken, 1999). *S. alaxensis* has been identified as having phenolic concentrations greater than similar willows of European origin (Bryant et al., 1989). Spruce possesses an array of terpenoids, and some terpenoids have been thought to induce PCB degradation (Singer et al., 2000). Both willow and spruce are broadly distributed across the boreal forest of Canada and Alaska, and therefore may be good candidates for *in situ* rhizoremediation of PCB-contaminated FUDS.

We assess the rhizoremediation potential of these tree species by simulating fine root turnover by amending contaminated soils with crushed fine roots and subsequently monitoring the loss of PCBs, changes in soil toxicity and microbial community dynamics associated with PCB degradation. This study aims to identify promising plant species for PCB remediation for contaminated sites in circumpolar boreal areas such as Alaska and Northern Canada, and provides mechanistic insight into the roles of root turnover and plant secondary chemistry in rhizoremediation processes.

2. Methods

2.1 Contaminated soil

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 PCB congeners spiked were DiCB 8 (2,4'), 9 (2,5), 10 (2,6), TriCB 18 (2,2',5), 28 (2,4,4'), TetraCB 44 (2,2',3,5'), 52 (2,2',5,5'), 66 (2,3',4,4'), 77 (3,3',4,4'), 81 (3,4,4',5), PentaCB 95 (2,2',3,5',6), 97 (2,2',3,4',5'), 105 (2,3,3',4,5), 118 (2,3',4,4',5), HexaCB 149 (2,2',3,4',5',6), 153 (2,2',4,4',5,5') and 169 (3,3',4,4',5,5'), selected for their patterns of chlorination, range of toxicity and environmental relevance. Standard solutions of individual PCB congeners (Ultra Scientific North Kingstown, RI, USA) were dissolved in acetone (HPLC grade VWR, West Chester, PA, USA) and then combined, resulting in a PCB mixture of 18 congeners in 0.150 L of solution that was then applied to 2 kg of soil. The PCB mixture was combined with the soil in a solvent-rinsed 20 L stainless steel can, which was then sealed and rigorously shaken for 10 3-min cycles. The acetone was allowed to evaporate by opening the 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 randomly sampled and extracted following the method described below. Initial soil PCB concentrations of between 37 and 38 mg total PCB kg⁻¹ soil were observed. Soil was stored at room temperature (22 °C) in the sealed can for 3 d prior to being used in individual microcosms.

2.2 Plant material

The plant species of interest, *P. glauca* (hereafter referred to as spruce) and *S. alaxensis* (hereafter referred to as willow), were collected from sites with no known contamination that were 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 mm diameter, 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 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 to individual microcosms. The crushed roots simulate fine root turnover when secondary compounds are released into soil in relatively large quantities. Additionally, control microcosms were also established, including biphenyl treated microcosms, which contained 5 mg biphenyl (Acros Organics Geel, Belgium), which was spiked 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, and 5 mg of salicylic acid (Acros Organics Geel, Belgium), which was dissolved in sterile water and added to soil in each of the salicylate-treated control microcosms, with water volume adjusted to achieve moisture levels equivalent to other microcosms. Untreated control microcosms were also constructed. All serum bottles were vortexed for a few seconds to mix contents. Soil clumps were broken up using a sterile spatula 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. These autoclaved microcosms may account for apparent PCB loss due to physical or chemical processes, including sorption to plant roots. 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 d. 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.

2.4 PCB extraction and quantification

At the end of 30, 90, and 180 d, 2 g of soil taken from each microcosm was Soxhlet extracted in cellulose thimbles (Whatman, Piscataway, NJ, USA) that were pre-extracted in 1:1 (v/v) acetone-hexane for 3 h before adding soil to thimbles. Soil samples were mixed with 5 g Na₂SO₄ (VWR West Chester, PA, USA), added to the pre-extracted thimble and extracted 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 autoclaved soil. Blank (no soil) and recovery tests (Na₂SO₄ in thimble spiked with PCB mixture of known concentration) were also included throughout the extraction of microcosm samples.

After extraction, the solvent was rotary evaporated to 2 mL, and transferred to a 5 mL test tube with three washes of hexane. Samples were further reduced in volume to 1 mL using a gentle stream of nitrogen gas, and cleaned-up using 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, followed by 1 g of Na₂SO₄ added to protect the 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. Samples were then volume reduced to about 0.5 mL, and solvent exchanged into isooctane (HPLC grade, VWR West Chester, PA, USA). PCB 30 (2,4,6) was then added as an internal standard.

Samples were run on an Agilent (Santa Clara, CA, USA) 6890N GC equipped with an ECD detector and an autosampler. A sample volume of 1.0 μL was injected onto a J&W Scientific (Folsom, CA, USA) DB-5 column 30 m in length, id 0.250 mm, film of 0.25 μm was used. The oven program was as follows: initial temperature of 90 $^{\circ}\text{C}$, held for 1 min, temperature raised 10 $^{\circ}\text{C min}^{-1}$ to 160 $^{\circ}\text{C}$, then raised 3 $^{\circ}\text{C min}^{-1}$ to 280 $^{\circ}\text{C}$ held for 2 min. Helium as the carrier gas. PCBs were identified based on retention time and quantified using standard curves of each PCB congener with PCB 30 used as an internal standard for each sample.

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, including sorption to plant roots; 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 (Supplementary Material (SM), Fig. SM-1), and using untreated live soil yielded a more conservative estimate of the extent to which intrinsic PCB degradation was affected by the amendments.

2.5 Isolation of biphenyl-utilizing microbes

Culturable biphenyl-utilizing microbes from soil were grown on basal mineral agar plates with biphenyl as the sole carbon source and further screened for biphenyl utilization using a clearing zone assay, methods previously described (Leigh et al., 2007). Eleven colonies producing zones of clearing were then streaked for isolation onto 1/8th 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.

2.6 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.

2.7 Terminal restriction fragment length polymorphism (T-RFLP) profiling

Community profiling using T-RFLP was performed on DNA extracted from the soil of 5 microcosms from each treatment. T-RFLP methods previously described (Leigh et al., 2007) were recreated with the exception that 80 μg PCR product was digested for 3 h at 37 $^{\circ}\text{C}$ using Hha I restriction enzyme. PCR products were not detected following several attempts to amplify extracts obtained from autoclaved soil. Fragments were analyzed using 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 (bp) long and more than 505 bp 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 operational taxonomic unit (OTU), peaks in that range were binned together.

2.8 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 PCR amplification of 16S rRNA genes cloning and sequence analysis as was previously described (Leigh et al., 2007). Plasmid purification followed by single extension Sanger sequencing was performed by Macrogen, (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.

2.9 Microtox assays

In order to compare the toxicity of amended and control soils incubated for 180 d, a basic solid phase test with the bioassay Microtox (Strategic Diagnostics, Newark, DE, USA) was utilized. This bioassay measures the toxicity of many compounds against the bioluminescent bacterium *Vibrio fischeri* by measuring changes in light production. In short, 3.5 g of soil and 17.5 mL solid phase test diluent were stirred with a stir bar for 10 min. 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 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 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 min when the light intensity was again measured. A curve was generated using the log of the sample concentration vs. percentage of light attenuation in 30 min. Percentage of light attenuation in 30 min 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 log effective concentration 50 (log EC₅₀). An average log EC₅₀ 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.

2.10 Statistical analyses

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

3. Results and Discussion

3.1 PCB disappearance

Significant PCB losses were observed for congeners with a range of chlorination substitution patterns in our soil microcosms (Figs. 1 and SM-1). Soils treated with willow root crushates or biphenyl showed significantly greater losses ($p < 0.05$) in several PCB congeners beyond what was observed in untreated soils, while spruce and salicylate treatments did not (Figs. 1 and 2). 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, concentrations of PCB congeners with 2 (2,4'), 3 (2,2',5), 4 (3,3',4,4'), 5 (2,3,3',4,5) and 6 (2,2',4,4',5,5' and 3,3',4,4',5,5') chloro substitutions were significantly reduced (Fig. 1). These congeners represent non-ortho substituted (PCB 77 and 169), mono-ortho substituted (PCB 8 and 105) and di-ortho substituted PCBs (PCB 18 and 153). Notably, significant losses occurred for the toxic congeners PCB 77, 105 and 169 (Fig. 1). These findings suggest that microbial populations stimulated by both willow and biphenyl possess enzymes capable of a variety of dioxygenase attack locations. The apparent degradation of highly chlorinated congeners is particularly notable, and should be further investigated in future studies by identifying possible metabolites as additional evidence of biotransformation.

The difference in PCB disappearance among willow and spruce treated soils indicates that a plant species-dependent characteristic is responsible for the apparent biostimulation effect. One possible explanation for this difference is that willows possess one or more stimulatory chemicals lacked by spruce. Willow and spruce differ substantially in their secondary chemistry (Glasby, 1991), and willows commonly possess salicylate, which has been reported to induce PCB degradative gene expression (Master and Mohn, 2001; Fujihara et al., 2006). However, when we introduced pure salicylate into soil, PCB degradation appeared to be inhibited relative to losses observed in untreated soils (Fig. 1) indicating that salicylate is not the active component of willow roots that promoted the enhanced disappearance of PCBs. Further studies are warranted to systematically fractionate and identify the biostimulatory component(s) of willow, an approach which has revealed phytochemicals from root crushates that biostimulate PAH degradation (Yi and Crowley, 2007).

The cause of the apparent inhibition of PCB degradation by salicylate is unclear, but may be due to the concentration being too high, resulting in reduced pH, toxicity to some soil microbes and/or chelation of iron that may inhibit the activity of iron-dependent dioxygenase enzymes. However, the pH of salicylate-treated soils (6.8-6.9) was not significantly different than the pH of untreated soil (6.9-7.1) using a 1:1 suspension test, leading us to believe that pH was not responsible for the apparent inhibition by salicylate.

Total PCB concentrations were still exhibiting a downward trend at the end of the 180 d incubation, suggesting that with more time, degradation would continue to occur (Fig. 2). The extent of PCB disappearance we observed parallels a recent study in which historically contaminated soils planted with *S. caprea* showed a 32% loss of total PCBs over 6 months compared with 18% in unplanted soil (Ionescu et al., 2009). Total PCB concentrations in our study showed that with one application of crushed willow roots, 40% of PCBs were degraded whereas untreated soils lost 25%, a difference of 15% (data not shown). The greater PCB loss observed in our study is likely due to our use of freshly contaminated soils versus historically contaminated soils used in the pot study as well as our experimental design maximizing the exposure of soil to simulated fine root lysates in a short time period. Additionally, the two soils have different compositions and microbial communities which could contribute to the varying extent of PCB loss during respective treatments.

3.2 Microbial community analyses

Cluster analysis of T-RFLP data showed that the bacterial communities of biphenyl-treated soils and the salicylate-treated soils formed distinct, separate clusters from the other samples (Fig. 3). From this analysis it is evident that the different amendments with pure compounds differentially affected the microbial community structure and produced more reproducible changes in community profile than root crushates, which contain a diverse array of organic compounds. 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).

In biphenyl-treated soils, several T-RFs were present and prevalent that were not detected in initial soil or as a result of other treatments (Fig. 4). These include T-RFs with lengths of 155 (*Acidovorax* spp.), 200, 202 (*Arthrobacter* spp.) and 367 bp long. Salicylate-treated soil communities shifted on a different trajectory than other treatments, with a 205 bp T-RF detected in every salicylate microcosm that was rarely detected in other treatments, only being present in one willow-treated soil community and one spruce-treated soil community.

The willow-treated community did not show a clear change in microbial community structure or composition that linked it clearly to communities treated with biphenyl, although the willow treatment displayed similar degradative capacity to biphenyl. There was not a clear change in the willow community that paralleled changes seen in salicylate treatments, although salicylate is a willow phytochemical. Willow also did not exhibit a distinctive community structure that differentiated it consistently from the spruce treatment, which did not display similar PCB biodegradative abilities. A 343 bp T-RF was consistently present in willow-treated soil that appeared in several other soil communities, but not consistently. Because willow roots did not appear to enrich any of the bacterial taxa specifically associated with biphenyl, the apparent PCB degradation in biphenyl and willow treatments was either performed by different microbial populations or by minor members of the communities not detectable with T-RFLP.

The difference in PCB disappearance among the willow and spruce treated microcosms may be due to differences in PCB biodegradation resulting from biostimulation, bioaugmentation, bioavailability, or some combination of these factors. Root crushates of both plant species may have introduced new microbial consortia into the microcosms, causing differential microbial community responses in the soil of both treatments. However, the repeated freeze-thaw and grinding of roots in liquid nitrogen is likely to have reduced the viable microbial populations substantially, which may account for the lack of distinctive, consistent, differences in willow and spruce community structure (Fig. 3). Alternatively, willow may contain phytochemicals that function as surfactants, increasing the bioavailability of PCBs for biodegradation by microbes in the microcosms derived from the willow rhizosphere and/or the test soil.

The biphenyl-utilizing organism isolated from our soil (Table SM-1) 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), and our isolate has > 97% similarity for the sequenced 16S rRNA fragment. Given that PCB degradative abilities and congener specificities vary widely among even closely related strain, future studies are needed to assess the specific degradative capabilities of this isolate. *In silico* digestion of the 16S rRNA gene from the isolate did not match any peaks detected in T-RFLPs, indicating that it is a minor member of the microbial community.

Several genera were detected in clone libraries (Table SM-1) that have been previously implicated in biphenyl and PCB degradation using culture dependent and independent techniques. 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 have previously been identified as biphenyl-utilizing bacteria (Leigh et al., 2007; Uhlik et al., 2009b). *Polaromonas* and *Methylophilus* were both detected in biphenyl-treated soil. In salicylate-treated soil, *Paenibacillus* and *Methylophilus* were detected (Leigh et al., 2007; Uhlik et al., 2009b). Each SIP study using biphenyl to probe for PCB degrading bacteria has implicated new genera in PCB degradation, and likely many more remain undiscovered (Uhlik et al., 2009a). Previously isolated biphenyl utilizing bacteria associated with willow trees include members of *Pseudomonas* (Leigh et al., 2006) which was detected in our clone library (Table SM-1). The relatives of known PCB degrading bacteria present in our soils might have been responsible for the degradation that we observed, however further studies, such as SIP, are needed to determine whether these genera detected in our clone libraries play an active role in PCB degradation in these soils.

Chao 1 richness estimates of OTUs in clone libraries, defined as sequence clusters sharing 97% sequence similarity in the 16S rRNA gene sequence compared, 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, however their associated T-RFs can provide an indication of relative abundance of certain taxa. We were unable to assign taxonomic identities to all T-RFs using *in silico* digestion of our clone libraries, as a result of incomplete sampling of the diverse soil communities, however many major T-RFs were identified (Fig. 4).

3.3 Soil Toxicity

The Microtox dose-response curves were used to determine log EC₅₀ values (Table SM-2) for contaminated soils. Soil treated with willow root crushates for 180 d (log EC₅₀ 2.0 ± 0.2) was significantly less toxic ($p < 0.05$) than untreated soil (log EC₅₀ 1.5 ± 0.1) and biphenyl-treated soil (log EC₅₀ 0.86 ± 0.04). Willow-treated soil toxicity was in the same range as the reference (uncontaminated) soil (log EC₅₀ 1.8 ± 0.2) (Table SM-1). Biphenyl-treated soil was the most toxic despite having a lower PCB concentration overall, probably due to the fact that biphenyl itself is toxic. It is clear that the treatment with willow roots led to a less toxic environment for the Microtox assay test species, probably as a result of degradation of acutely toxic, co-planar congeners PCB 77, 105 and 169 (Fig. 1). The reduction in soil toxicity observed may also indicate a lack of accumulation of PCB degradation intermediates that might be more toxic to the test species than the parent compounds, although metabolites were not quantified in this study. In future studies, a focus on metabolite identification and quantification may answer this question.

Calculated toxic equivalency quotient (TEQ) values show no significant difference between any soil toxicity levels (Table SM-2). Toxic equivalency factor (TEF) 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 little or no change in TEQ after treatment may still be less toxic to soil microbes, as was observed in this study. Over the incubation time course (Fig. 2), 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.

4. Conclusions

Our 180-d *in vitro* assessment of rhizoremediation potential indicates that *S. alaxensis* is a promising candidate for rhizoremediation of PCB contaminated soils that warrants further investigation. Simulated root turnover of *S. alaxensis* appears to promote increased rates of PCB degradation and detoxification over intrinsic rates in untreated soils or those treated with spruce root crushates. The biostimulatory effect of willow appears not to be mediated by salicylate, but instead may be caused by other phytochemicals present in the fine roots. *S. alaxensis* holds particular promise for remediation of contaminated sites in northern climates, such as FUDS or other sites in Alaska and Canada, since this willow species is naturally distributed across a wide geographic range within the circumpolar boreal forest biome, grows robustly, and readily colonizes disturbed environments. Larger scale, long term field studies with weathered soil are warranted to assess the abilities of willow to promote PCB degradation and detoxification and to better understand and optimize the mechanisms underlying PCB rhizoremediation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study has benefited from guidance from Todd O'Hara and members of the Leigh lab. This project was supported by the NSF under Award 0626544, the University of Alaska Fairbanks Institute of Arctic Biology and a graduate fellowship from Alaska Idea Network for Biomedical Research Excellence (INBRE).

References

- Anthony RG, Miles AK, Ricca MA, Estes JA. Environmental contaminants in bald eagle eggs from the Aleutian Archipelago. *Environ Toxicol Chem.* 2007; 26:1843–1855. [PubMed: 17702538]
- Ap O'Hehir C, Scrudato D, Golodergin R. Polychlorinated biphenyls in serum of the Siberian Yupik people from St. Lawrence Island, Alaska. *Int J Circumpol Heal.* 2005; 64:322–335.
- Army Corps of Engineers. May 5. 2010 <http://www.poa.usace.army.mil/hm/default.htm>
- Beranová K, Kochánková L, Macek T, Macková M. Rhizoremediation for decontamination of long-term PCB contaminated soil with focus on microbial diversity. *J Biotech.* 2007; 131:243–243.
- Brown TM, Sheldon TA, Burgess NM, Reimer KJ. Reduction of PCB contamination in an Arctic coastal environment: A first step in assessing ecosystem recovery after the removal of a point source. *Environ Sci Technol.* 2009:251–263.
- Bryant JP, Tahvanainen J, Sulkioja M, Julkunen-Tiitto R, Reichardt P, Green T. Biogeographic evidence for the evolution of chemical defense by boreal birch and willow against mammalian browsing. *Am Nat.* 1989; 134:134, 20–34.
- Chen SH, Aitken MD. Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. *Environ Sci Technol.* 1999; 33:435–439.
- de Cárcer DA, Martín M, Karlson U, Rivilla R. Changes in bacterial populations and in biphenyl dioxygenase gene diversity in a polychlorinated biphenyl-polluted soil after introduction of willow trees for rhizoremediation. *Appl Environ Microb.* 2007a; 73:6224–6232.
- de Cárcer DA, Martín M, Mackova M, Macek T, Karlson U, Rivilla R. The introduction of genetically modified microorganisms designed for rhizoremediation induces changes on native bacteria in the rhizosphere but not in the surrounding soil. *ISME J.* 2007b; 1:215–223.

- Donnelly PK, Hegde RS, Fletcher JS. Growth of PCB-degrading bacteria on compounds from photosynthetic plants. *Chemosphere*. 1994; 28:981–988.
- Fujihara H, Yoshida H, Matsunaga T, Goto M, Furukawa K. Cross-regulation of biphenyl- and salicylate-catabolic genes by two regulatory systems in *Pseudomonas pseudoalcaligenes* KF707. *J Bacteriol*. 2006; 188:4690–4697. [PubMed: 16788178]
- Furukawa K, Fujihara H. Microbial degradation of polychlorinated biphenyls: Biochemical and molecular features. *J Biosci Bioeng*. 2008; 105:433–449. [PubMed: 18558332]
- Furukawa K, Simon JR, Chakrabarty AM. Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. *J Bacteriol*. 1983; 154:1356–1362. [PubMed: 6343352]
- Gilbert ES, Crowley DE. Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter* sp. strain B1B. *Appl Environ Microb*. 1997; 63:1933–1938.
- Glasby, JS. Dictionary of Plants Containing Secondary Metabolites. Taylor & Francis; London: 1991.
- Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for education and data analysis. *Palaeontol Electron*. 2001; 4:1–9.
- Ionescu M, Beranova K, Dudkova V, Kochankova L, Demnerova K, Macek T, Mackova M. Isolation and characterization of different plant associated bacteria and their potential to degrade polychlorinated biphenyls. *Int Biodeter Biodegr*. 2009; 63:667–672.
- Jefferies RL, Klein DR, Shaver GR. Vertebrate herbivores and northern plant communities: Reciprocal influences and responses. *Oikos*. 1994; 71:193–206.
- Leigh MB, Fletcher JS, Fu X, Schmitz FJ. Root turnover: An important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants. *Environ Sci Technol*. 2002; 36:1579–1583. [PubMed: 11999069]
- Leigh MB, Prouzova P, Mackova M, Macek T, Nagle DP, Fletcher JS. Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. *Appl Environ Microb*. 2006; 72:2331–2342.
- Leigh MB, Pellizari VH, Uhlik O, Sutka R, Rodrigues J, Ostrom N, Zhou J, Tiedje J. Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). *ISME J*. 2007; 1:134–148. [PubMed: 18043623]
- Macdonald RW, Barrie LA, Bidleman TF, Diamond ML, Gregor DJ, Semkin RG, Strachan WMJ, Li YF, Wania F, Alaee M. Contaminants in the Canadian Arctic: 5 years of progress in understanding sources, occurrence and pathways. *Sci Total Environ*. 2000; 254:93–234. [PubMed: 10885446]
- Master ER, Mohn WW. Induction of bphA, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant *Pseudomonas* strain Cam-1 and mesophilic *Burkholderia* strain LB400. *Appl Environ Microb*. 2001; 67:2669–2676.
- Miles AK, Ricca MA, Anthony RG, Estes JA. Organochlorine contaminants in fishes from coastal waters west of Amukta Pass, Aleutian Islands, Alaska, USA. *Environ Toxicol Chem*. 2009; 28:1643–1654. [PubMed: 19374473]
- Mulligan, D. Soil Survey of Greater Fairbanks area. USDA, NRCS; Alaska: 2004.
- Poland JS, Mitchell S, Rutter A. Remediation of former military bases in the Canadian Arctic. *Cold Reg Sci Technol*. 2001; 32:93–105.
- Rein A, Fernqvist MM, Mayer P, Trapp S, Bittens M, Karlson UG. Degradation of PCB congeners by bacterial strains. *Appl Microbiol Biot*. 2007; 77:469–481.
- Ricca MA, Keith Miles A, Anthony RG. Sources of organochlorine contaminants and mercury in seabirds from the Aleutian archipelago of Alaska: Inferences from spatial and trophic variation. *Sci Total Environ*. 2008; 406:308–323. [PubMed: 18692865]
- Singer AC, Gilbert ES, Luepromchai E, Crowley DE. Bioremediation of polychlorinated biphenyl-contaminated soil using carvone and surfactant-grown bacteria. *Appl Microbiol Biot*. 2000; 54:838–843.
- Singer AC, Thompson IP, Bailey MJ. The tritrophic trinity: A source of pollutant-degrading enzymes and its implications for phytoremediation. *Curr Opin Microbiol*. 2004; 7:239–244. [PubMed: 15196490]
- Stow JP, Sova J, Reimer KJ. The relative influence of distant and local (DEW-line) PCB sources in the Canadian Arctic. *Sci Total Environ*. 2005; 342:107–118. [PubMed: 15866270]

- Uhlík O, Jecná K, Leigh MB, Macková M, Macek T. DNA-based stable isotope probing: A link between community structure and function. *Sci Total Environ.* 2009a; 407:3611–3619.
- Uhlík O, Jecná K, Macková M, Vlcek C, Hroudová M, Demnerová K, Paces V, Macek T. Biphenyl-metabolizing bacteria in the rhizosphere of horseradish and bulk soil contaminated by polychlorinated biphenyls as revealed by stable isotope probing. *Appl Environ Microb.* 2009b; 75:6471–6477.
- Van Aken B, Correa PA, Schnoor JL. Phytoremediation of polychlorinated biphenyls: New trends and promises. *Environ Sci Technol.* 2004; 44:2767–2776.
- Wania F. Assessing the potential of persistent organic chemicals for long-range transport and accumulation in polar regions. *Environ Sci Technol.* 2003; 37:1344–1351.
- Yi H, Crowley DE. Biostimulation of PAH degradation with plants containing high concentrations of linoleic acid. *Environ Sci Technol.* 2007; 41:4382–4388. [PubMed: 17626440]

Highlights

- > We assessed the abilities of willow and spruce to promote microbial PCB degradation.
- > We examined effects of crushed roots on soil PCB content, toxicity and microflora.
- > Willow-treated soil showed greater loss of PCBs than spruce, salicylate or controls.
- > The Microtox bioassay indicated that willow treatment resulted in less toxic soil.
- > *Salix alaxensis* (willow) may be an effective plant for rhizoremediation of PCBs.

Fig. 1.

Soil concentration of congeners showing significant losses due to treatment with root crushates and/or biphenyl after 180 days (a) and percent degradation of PCB congeners over untreated soil after 180 days (b). Error bars represent one standard deviation. Statistical significance ($P < 0.05$) denoted by *. For each treatment $n=6$.

Fig. 2. Total PCB concentration in soils decreases over time for all treatments. NT is no treatment, W is willow-treated, BP is biphenyl-treated, S is spruce-treated and SA is salicylate-treated soil. For each treatment, n=6.

Fig. 3. Cluster analysis based on T-RF length and abundance in soil samples Bray-Curtis distance was used to determine clusters.

Fig. 4.

Soil bacterial communities represented by T-RF length (different colors) and abundance (relative size). Bars within one treatment group are from replicate microcosms. Taxonomic identification for certain T-RFs are presented, others could not be identified.

Note: Figure 4 is intended for color reproduction on the web and in print