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RHIZOSPHERE ECOLOGY OF PCB-DEGRADING BACTERIA AT A CONTAMINATED SITE AND IMPLICATIONS FOR BIOREMEDIATION

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

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DOCTOR OF PHILOSOPHY

By
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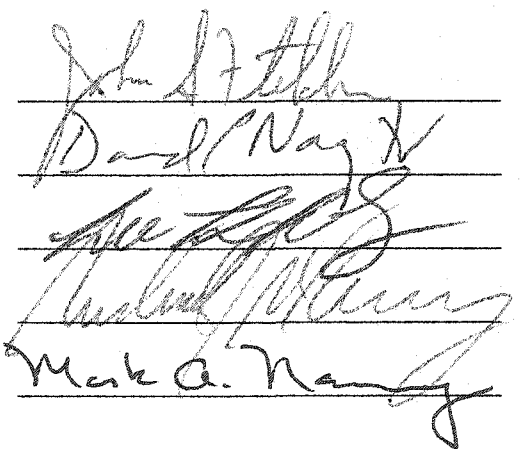
RHIZOSPHERE ECOLOGY OF PCB-DEGRADING BACTERIA AT A CONTAMINATED SITE AND IMPLICATIONS FOR BIOREMEDIATION

A Dissertation APPROVED FOR
THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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After a certain high level of technical skill is achieved, science and art tend to coalesce in esthetics, plasticity, and form. The greatest scientists are always artists as well.
- Albert Einstein

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PREFACE

Polychlorinated biphenyls (PCBs) are hazardous compounds posing human health and ecological risks worldwide due to the release of an estimated 100,000 tons of PCBs into the biosphere. The expense of current cleanup technologies like incineration and landfills exerts an enormous economic burden on nations, especially in economically depressed regions of the world with severe contamination problems like central Europe. Rhizosphere bioremediation, which relies on plants to promote bacterial degradation of contaminants in the root-zone, could provide an inexpensive, alternative approach to cleanup with the additional benefit of facilitating ecosystem restoration in contaminated areas.

Previous work in our laboratory has demonstrated that some plant species may enhance bacterial biodegradation of PCBs by releasing aromatic compounds from their roots that promote the growth and degradative activities of PCB-degrading bacteria. With this foundation of mechanistic evidence established, the next phase of research necessitated field investigations to determine whether some plants actually enhance PCB-degrading bacteria in the root-zone under field conditions. This project was designed with the aim of identifying promising plant species for rhizoremediation by conducting an ecological study of plants, PCB-degrading bacteria and their interactions at PCB-contaminated sites.

Chapter 1. The soil PCB concentrations and associated natural vegetation are characterized and mapped for two PCB-contaminated sites in the Czech Republic in order to identify PCB-tolerant plant species that could be considered candidates for

rhizoremediation. Chapter 1 is the first study to describe the natural vegetation at a PCB-contaminated site, and also represented the first report of PCB tolerance for many plant species. This article was published in 2001 as an article in the book entitled *Phytoremediation, Wetlands and Sediments* (Sixth International *In Situ* and On-Site Bioremediation Symposium, San Diego, CA, June 4-7, 2001, A. Leeson, A., E. A. Foote, M. K. Banks, V. Magar (ed.), Battelle Press, Columbus, OH, p. 61-68).

Chapter 2. The second chapter presents comprehensive analyses of the influences of different plant species and abiotic factors on microbial communities in the root zone and on the rhizoplane of five different tree species and in nonrooted soil at one contaminated site. The primary objective of this work was to identify promising plant species for rhizoremediation by finding plants that foster increased numbers of PCB-degrading bacteria in the root zone under actual field conditions. This was the first thorough enumeration of PCB-degraders across a contaminated site as well as being the first report of the presence of PCB-degrading bacteria associated with plants growing *in situ* in contaminated soil. This chapter was prepared in the format for the journal *Applied and Environmental Microbiology.*

Chapter 3. The PCB-degrading bacteria that were enumerated in Chapter 2 were further investigated to determine their diversity, association with different plant species and PCB-degradative abilities. The goal was to determine which taxa were most prevalent in the root zones of plants, and to better understand their degradative potential. This work represents the first characterization of the diversity of PCB-degrading bacteria associated with plant roots, and also is the first to evaluate relative abundance of different

PCB-degraders present in contaminated soil. Chapter 3 was prepared in the format for the journal *Applied and Environmental Microbiology*.

Combined, the three components of this field study represent the first ecological investigation of a PCB-contaminated area, and provide new information regarding rhizosphere ecology that has significant implications for the field of bioremediation. The project demonstrates that many plant species (trees, shrubs, grasses and forbs) are capable of thriving in contaminated soil, and two tree species alter the soil microbial community structure in favor of indigenous populations of PCB-degrading bacteria that harbor strong degradative abilities. These two most effective tree species, pine and willow, are known to produce large quantities of aromatic compounds, including terpenoids and salicylate derivatives, as well as flavonoids and other phenolics. Along with previous work from this laboratory, this study suggests that plant secondary chemistry is one of the driving forces impacting populations of bacteria that degrade both plant aromatic compounds and aromatic soil contaminants. Because one of the major rate-limiting factors affecting the disappearance of PCBs is the quantity of PCB-degrading bacteria present, this work indicates that the growth of appropriate plant species at PCB-contaminated sites may accelerate the biodegradation of PCBs, providing an inexpensive and sustained strategy for the *in situ* bioremediation of PCB-contaminated soils.

ABSTRACT

Environmental contamination with polychlorinated biphenyls (PCBs) is a worldwide problem that would cost billions of dollars to remediate using conventional cleanup methods such as incineration or specialized landfills. Rhizosphere bioremediation represents a potentially inexpensive, alternative cleanup strategy that relies on plants to promote the growth and activity of contaminant-degrading microorganisms in the root zone. The objectives of this study were to identify PCB-tolerant plant species with the potential to enrich indigenous PCB-degrading bacteria in their root zones and to identify taxa of bacteria significant to rhizoremediation. To address these objectives, ecological investigations were conducted at two naturally-vegetated field sites that were contaminated with polychlorinated biphenyls (PCBs) in the Czech Republic.

Vegetation analyses of the two PCB-contaminated field sites revealed a diverse array of trees, grasses and forbs, totaling 51 different plant species from 23 families, which were rooted in soil contaminated with PCBs ranging in concentration from 1-500 mg/kg PCBs. The presence of a biodiverse community of healthy, older plants and fungi at these sites indicates that PCBs are not toxic to a broad spectrum of plant and fungal species at the concentrations encountered in this study.

Five tree species were selected for a focused investigation of their influence on populations of root-associated PCB-degrading bacteria. Austrian pine (*Pinus nigra*) and goat willow (*Salix caprea*) fostered increased numbers of PCB-degrading bacteria in their root zones due to an enrichment effect, in which a significantly higher percentage of the

total bacterial community was comprised of PCB-metabolizers in the root zones of pine and willow than that of other plant species. On the rhizoplane, numbers of PCB-metabolizers did not differ significantly among plant species. Populations of PCB-degraders in soil were not significantly influenced by soil moisture (ranging from 1.8–18.5%) or soil PCB concentrations (0-500 mg/kg). The results indicate that some plant species can alter microbial community structure in the bulk, root-zone soil, in favor of PCB-degrading bacteria. Therefore, the selection of appropriate plant species is important to the development of successful rhizoremediation strategies for the cleanup of PCB-contaminated soil.

The diversity of PCB-degrading bacteria, the relative abundance of different taxa and their degradative abilities were examined in the root zones and on the rhizoplanes of six plant species at the site. Of the 163 PCB-metabolizing bacteria isolated from root-zone soils and 80 isolates from the rhizoplane, 93% and 100% of them were Gram-positive, respectively. Using 16S rDNA sequence analyses, PCB-degrading bacterial isolates were identified as *Rhodococcus* spp, *Arthrobacter oxydans, Bacillus sp., Microbacterium oxydans, Pseudomonas* spp., *Williamsia murale* and a possible relative of *Rhodanobacter lindanoclasticus*. Rhodococci were the most prevalent taxa of PCB-degraders at the site, and were isolated from the roots of every plant species investigated, including Austrian pine and willow, which have been shown to enrich for PCB-degraders. Several isolates exhibited high PCB degradation rates and broad congener specificities when subjected to a PCB degradation assay. Thus, rhodococci have been identified as an important group of bacteria in the *in situ* rhizosphere bioremediation of PCB-contaminated soils.

This research represents the first comprehensive study of the botany, microbiology and rhizosphere ecology of PCB-contaminated sites. The findings that under field conditions, some plant species enrich populations of indigenous bacteria with outstanding PCB-degradation abilities in the root-zone support the rhizoremediation concept that growing certain plants in contaminated sites can provide a sustained, ecologically-stable strategy for the bioremediation of PCBs.

CHAPTER 1

Vegetation and fungi at Czech PCB-contaminated sites as bioremediation candidates

ABSTRACT

Vegetation analyses were conducted at two polychlorinated biphenyl (PCB)-contaminated field sites in the Czech Republic to identify plant and fungal species that grow well in contaminated soil under field conditions. Both sites had become vegetated through natural processes and a diverse array of trees, grasses and forbs, totaling 51 different plant species from 23 families are now present. Soil excavation in the root zone followed by soil PCB analyses confirmed that these plants were rooted in contaminated soil ranging from 1-500 mg/kg PCBs. At one site, six different species of mushrooms, representing four different families of basidiomycetes were also found growing in 15-262 mg/kg PCBs. The presence of a biodiverse community of healthy, older plants and fungi at these PCB-contaminated sites shows that PCBs are not toxic to a broad spectrum of plant and fungal species. Thus, these species are candidates for use in the bioremediation of PCBs, providing that some or all of these species promote degradative processes within the soil.

INTRODUCTION

Phytoremediation is an attractive alternative to current methods for the cleanup of soil contaminated with recalcitrant aromatic organic compounds (Macek, *et al.*, 2000). Plants may contribute to remediation in several ways, including reducing off-site leaching

of contaminants, aerating soil and by releasing compounds from the roots that selectively foster indigenous contaminant-degrading microorganisms (Fletcher *et al.,* 1995). Before proceeding with the development of *in situ* phytoremediation technology, it is critical to identify plants that can survive and flourish for extended periods in contaminated soils under field conditions. Vegetation analysis of plants growing naturally in a PAH-contaminated former sludge disposal basin revealed a diverse array of tolerant plants and also provided valuable evidence that some plants facilitated microbial degradation of the contaminants (Olson, *et al.*, 2001, Olson and Fletcher, 2000). The same approach can be applied to sites contaminated with polychlorinated biphenyls (PCBs) to screen for PCB-tolerant species that are potentially capable of enhancing PCB degradation.

Little is known about the toxicity of PCBs toward plants. Some growth inhibition has been documented for algae, soybean, beets and pigweed (*Amaranthus retroflexus* L.) (Strek and Weber, 1982), however effects on trees, grasses and other non-crop plants have not been reported. Trees are of particular interest to phytoremediation because of their extensive root systems that can penetrate large volumes of soil (Olson and Fletcher, 1999). Fungi are also potentially important components in soil bioremediation, since several mycorrhizal fungi have been shown to degrade chlorinated aromatic contaminants (Gaskin and Fletcher, 1997, Donnelly and Fletcher, 1995).

OBJECTIVE

Catalogue the plant and macroscopic fungal species growing naturally in PCB-contaminated sites in the Czech Republic as a means of identifying PCB-tolerant species that may stimulate *in situ* PCB degradation.

MATERIALS AND METHODS

Two PCB contaminated sites located in geographically different regions of the Czech Republic were examined in this study. The first area was located on the grounds of the Colorlak paint production plant in Staré Mesto near Uherské Hradište in South Moravia. Empty barrels that had contained a variety of solvents and the commercial PCB mixture, Delor 106 (similar to Aroclor 1260) were stored in this area from the 1950s to 1988, during which time spillage of residual amounts of solvents occurred. The second site was located in Jablonné nad Orlicí, North Moravia, at a capacitor production plant formerly operated by the now defunct company, ISOLIT. The soil is contaminated with a combination of Delor 106 and Delor 103 (similar to Aroclor 1242), as well as crude oils. Although the exact age of the contamination is not known, it can be conservatively estimated at greater than 15 years, since the use of PCBs was discontinued in 1986 in the Czech Republic.

Data were collected at both sites on three occasions in 2000, when vegetation was photographed and collected for taxonomic identification and soil samples were taken for PCB analysis. Colorlak was sampled on June 22, August 23-24 and November 21-22, and ISOLIT was sampled on July 12, August 29 and November 30. Taxonomic identifications of plants and fungi were performed by Dr. Věra Hadincová (Institute of Botany, Academy of Sciences of the Czech Republic, Prague, Czech Republic) and the Czech Mycological Society (Prague, Czech Republic), respectively.

The age and size of trees growing at each site were determined by taking trunk cores and diameter measurements at breast height (DBH). In some cases, the radius of trees exceeded the length of the 20 cm coring device. Because of the large trunk

diameters, the age of some trees was estimated by multiplying the average number of rings per cm of core by one half the diameter of the tree at breast height (minus 4 cm for bark).

Soil sampling was conducted by excavation within the root zones of different plant species. Samples were collected for analysis of PCB concentration from depth intervals of 0-20 cm, 20-40 cm, and when possible also 40-60 cm and 60-80 cm. Samples were wrapped in aluminum foil and kept cool during transport.

In the laboratory, samples were air-dried for 24 hours at room temperature then homogenized with mortar and pestle and sieved through 1 mm mesh. One-gram aliquots of soil were extracted in hexane for four hours using a micro-scale modification of the EPA method for Soxhlet extraction (EPA Method 3540C). Extracts were concentrated under nitrogen stream and were subjected to florisil cleanup (EPA Method 3620B) prior to analysis. Samples were analyzed using a Hewlett-Packard 5890A gas chromatograph with an electron capture detector and a fused silica capillary column (30 m, 0.20 mm inner diameter) coated with 0.25 μm stationary phase SE054 with nitrogen as the carrier gas (flow rate 1 ml/min). The column temperature was programmed to increase from 50 to 195°C at a rate of 25°C/min, then increased to 205°C at 1°C/min, held at 205°C for 5 min, then increased to 280°C at 3°C/min and held for 15 min. Total PCB concentrations were estimated using an external standard method with 7 indicator congeners (EPA method 8082).

RESULTS AND DISCUSSION

Both contaminated sites were vegetated with a wide variety of plant species, including trees, grasses and forbs that were rooted in soils ranging from 1-500 mg/kg PCBs. Table 1 lists all plant species identified from each site, totaling 51 different species representing 23 different families. Although the ISOLIT site was smaller in size, it showed greater diversity with 34 different plant species compared to 24 species at Colorlak.

In total, seven different tree species were identified (Table 2) that were rooted in soil ranging from 1-500 mg/kg PCBs. Vegetation maps of the two sites (Figure 1) show the location of the trees. All trees found are natives to central Europe except for black locust (*Robinia pseudoacacia*) growing at Colorlak, an invasive species introduced from North America. The most frequently observed tree species at both sites was ash (*Fraxinus excelsior*), and three ash trees at ISOLIT were also the oldest trees found, ranging in age from 70-76 years. These trees predate the PCB contamination, since the Delor mixtures of PCBs were not produced until 1959. Numerous ash, Norway maple and alder saplings were observed growing in the understory, confirming that these species are capable of germination and growth in contaminated soil. At Colorlak, the oldest tree identified was Austrian pine (24 years), followed by the weeping birch and ash trees (11-14 years). Thus, the trees present at Colorlak are presumed to have germinated and grown after the soil had been contaminated with PCBs.

All plants observed appeared to be growing robustly and showed no visible signs of stress such as etiolation or leaf spotting, wrinkling or burned margins. Normal

reproduction was observed in annuals and perennials at both sites. Grasses and forbs were mowed occasionally, but between cuttings bloomed robustly. Flowers and/or fruits were observed on raspberries, goat willow, black locust, alder and maple, and the pine produced numerous cones.

The distribution and abundance of vegetation appeared to be influenced more by physical aspects of the sites rather than the concentration of PCBs. Factors such as shading, mowing and soil depth limited growth of some plants. Heavy shading occurred under the large trees at ISOLIT where little ground cover was found, although in comparable PCB concentrations (22 mg/kg) but with full sun, a variety of grasses, shrubs and forbs flourished. At both sites, mowing of the grasses and forbs appeared to prevent invasion by trees, although trees were permitted to grow along fence lines. The dominant ground cover at Colorlak was a mixture of grasses and yarrow (*Achillea millefolium*), which grew similarly in a range of PCB concentrations from 5-262 mg/kg PCBs. Poor growth of these plants was only observed in an area of shallow, rocky soil with a relatively low PCB concentration (50 mg/kg). At both sites, the most highly contaminated spots were vegetated by healthy trees (Figure 1).

Beneath the trees at ISOLIT, the upper 20 cm of soil had a very high density of tree fine roots. Roots were observed in gradually decreasing density as soil depth increased down to 80 cm, where excavation stopped. At Colorlak, tree fine roots occurred in highest density in the upper 40 cm of soil, but some were also present down to 60 cm, where excavation ceased due to the presence of underground cables. At both sites, grass and forb roots were seldom found beneath 20 cm deep. Root avoidance of

PCB-contaminated soil layers was not observed, and in many cases root density was the highest at the most highly contaminated soil depth.

At Colorlak in late November, six different species of basidiomycotan fungal fruiting bodies were found (*Conocybe tenera, Psathyrella prona, Stropharia melasperma* or *coronilla, Clitocybe pityophila, Collybia asema and Lyophyllum fumosum*) that represent four different families (Bolbitaceae, Coprinaceae, Strophariaceae and Tricholomataceae). Locations of the fungi are indicated on the vegetation map (Figure 1). The mushrooms were growing in surface soils with PCB concentrations ranging from 15-262 mg/kg, and exhibited no visible signs of morphological abnormalities. To our knowledge, this is the first report of macroscopic fungi growing naturally in PCB-contaminated soil.

CONCLUSIONS

Vegetation analyses of two PCB contaminated sites in the Czech Republic identified 51 different species of plants from 23 different families growing robustly in PCB-contaminated soil ranging in concentrations of 1-500 mg/kg. It was confirmed by excavation and soil PCB analysis that these plants were rooted in contaminated soil. Six different species of basidiomycotan fungi were also found growing in soil ranging from 15-262 mg PCBs/kg. This is the first characterization of plant or fungal communities growing in PCB-contaminated soil. No visible signs of toxicity were observed either for the plants or the fungi. These results show that PCBs are not toxic to the sustained healthy growth of several different plant and fungal species under field conditions. Thus,

these species are candidates for use in the bioremediation of PCBs, providing that some or all of these species promote degradative processes within the soil.

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TABLE 1. List of plant species growing at two PCB contaminated sites in the Czech Republic.

Family	Species	Common name(s)
COLORLAK		
Apiaceae	*Chaerophyllum aromaticum*	Chervil
	Heracleum sphondylium	Cow parsnip
Asteraceae	*Achillea millefolium*	Yarrow
	Artemisia vulgaris	Mugwort
	Cirsium arvense	Creeping thistle
	Stenactis annua	Tall Fleabane
	Tripleurospermum maritimum	Scentless mayweed, Sea mayweed
Betulaceae	*Betula pendula*	European White Birch, Weeping Birch
Boraginaceae	*Echium vulgare*	Viper's bugloss
Convolvulaceae	*Convolvulus arvensis*	Field bindweed
Fabaceae	*Robinia pseudoacacia*	Black locust
	Trifolium repens	White clover
Geraniaceae	*Geranium pratense*	Meadow crane's bill
Lamiaceae	*Lamium album*	White dead nettle
Oleaceae	*Fraxinus excelsior*	Ash
Pinaceae	*Pinus nigra*	Austrian Pine
Plantaginaceae	*Plantago lanceolata*	Ribwort Plantain
	Plantago major	Greater Plantain
Graminaceae	*Agropyron repens*	Couch Grass
	Agrostis alba (stolonifera)	White Bent
	Calamogrostis epigeios	Wood Small-reed
	Lolium perenne	Perennial ryegrass
Polygonaceae	*Rumex obtusifolius*	Broad-leaved Dock
Salicaceae	*Salix caprea*	Goat willow
Scrophulariaceae	*Verbascum thapsus*	Great Mullein, Aaron's Rod
ISOLIT		
Aceraceae	*Acer platanoides*	Norway maple
Apiaceae	*Angelica sylvestris*	Wild angelica
	Anthriscus sylvestris	Cow parsley
	Chaerophyllum aromaticum	Chervil
	Heracleum sphondylium	Cow parsnip
Asteraceae	*Artemisia vulgaris*	Mugwort
	Cirsium arvense	Creeping thistle
	Cirsium oleraceum	Cabbage thistle
	Crepis biennis	Rough Hawk's-beard
	Leontodon autumnalis	Autumnal hawkbit
	Solidago gigantea	Giant goldenrod
	Tripleurospermum maritimum	Scentless mayweed
Balsaminaceae	*Impatiens parviflora*	Small Balsam
Betulaceae	*Alnus glutinosa*	Alder
Boraginaceae	*Symphytum officionale*	Comfrey
Campanulaceae	*Campanula trachelium*	Nettle-leaved Bellflower
Equisetaceae	*Equisetum arvense*	Field Horsetail
Fabaceae	*Medicago lupulina*	Black medick
	Trifolium repens	White clover
	Vicia cracca	Tufted vetch
Lamiaceae	*Glechoma hederacea*	Ground ivy
Oleaceae	*Fraxinus excelsior*	Ash
	Syringa vulgaris	Common lilac
Graminaceae	*Agrostis canina*	Velvet Bent
	Calamogrostis epigeios	Wood Small-reed
	Dactylis glomerata	Cock's foot
	Festuca gigantea	Giant Fescue
	Poa palustris	Swamp meadow grass
	Trisetum flavescens	Spike trisetum, Yellow oat grass
Ranunculaceae	*Ranunculus acris*	Crowfoot
Rosaceae	*Potentilla anserina*	Silverweed
	Rubus idaeus	Raspberry
Rubiaceae	*Galium album*	Hedge Bedstraw
Salicaceae	*Salix caprea*	Goat willow
Urticaceae	*Urtica dioica*	Stinging nettle

TABLE 2. List of trees at two PCB contaminated sites in the Czech Republic including tree age, trunk diameter and PCB concentration of soil in which tree is rooted.

Site	Common name	Species	Age (years)	Trunk Diameter (cm)	Soil PCB conc. (mg/kg)
Colorlak					
	Ash	*Fraxinus excelsior*	14	[a]19.5, 18.0, 16.5	15
			4	5.0	82
	Weeping birch	*Betula pendula*	11	15.0	153
			13	21.0	153
	Austrian Pine	*Pinus nigra*	24	24.5	15
	Black Locust	*Robinia pseudoacacia*	3	[b]N/A	153
	Goat Willow	*Salix caprea*	5	[b]N/A	470
ISOLIT					
	Alder	*Alnus glutinosa*	[c]N/A	56.2	135
	Ash	*Fraxinus excelsior*	76	35.4	4
			72	43.3	3
			70	48.9	2
	Norway Maple	*Acer platanoides*	53	60.5	22

[a] Trunk branched into three
[b] DBH measurement not possible due to multiple small trunks
[c] Tree rings not countable

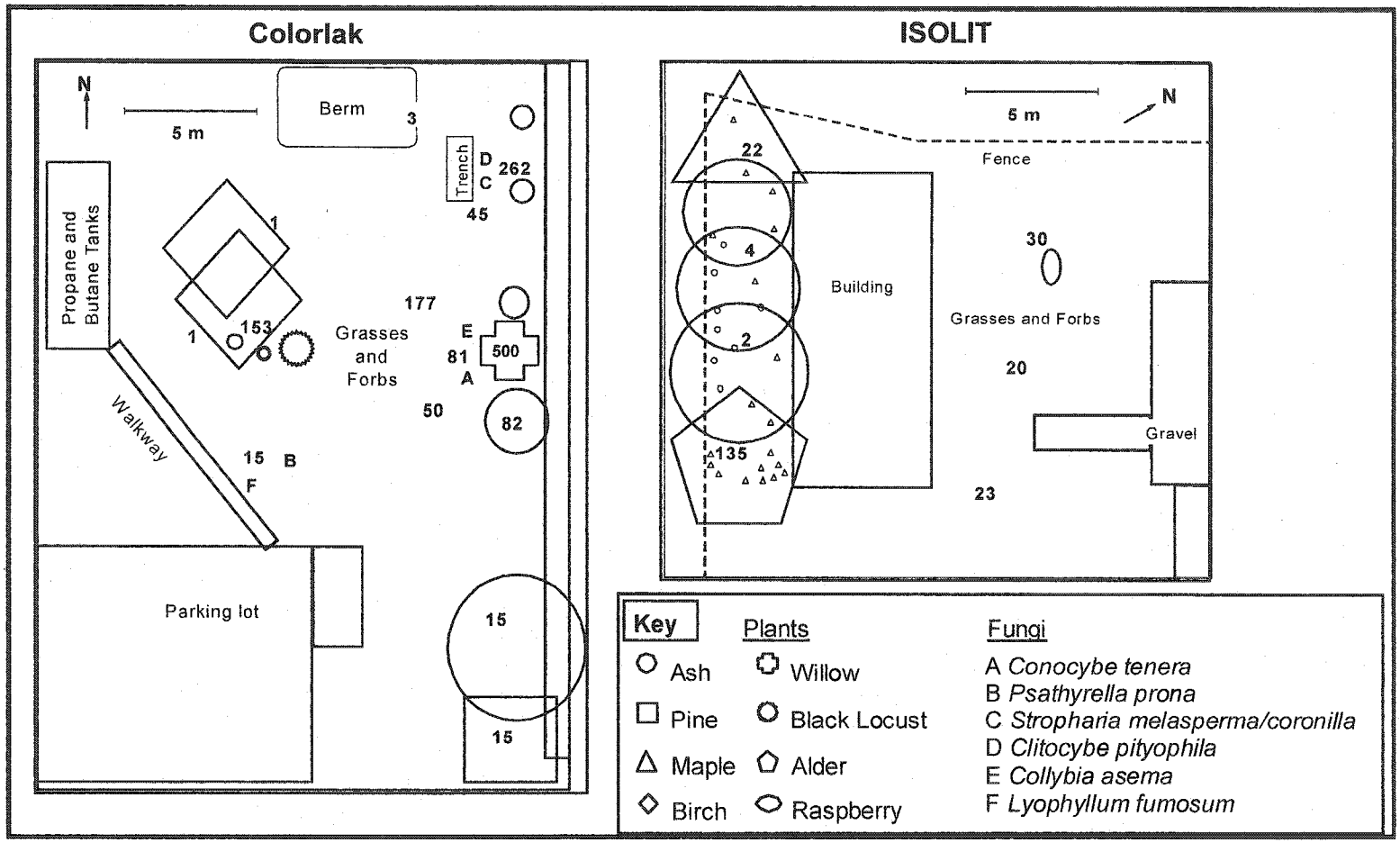

FIGURE 1. Vegetation maps of PCB-contaminated sites investigated. Size of symbol represents canopy size of trees. Numbers indicate PCB concentration of soil (mg/kg) measured at depths of 0-20 cm at ISOLIT and 20-40 cm at Colorlak, except for beneath fungi where surface soil (0-5cm) was analyzed.

CHAPTER 2

Rhizosphere enrichment of PCB-metabolizing bacteria *in situ* at a Czech PCB-contaminated site

ABSTRACT

Using a field-based approach, five tree species growing naturally in a PCB-contaminated site were screened for their potential to facilitate the rhizosphere bioremediation of polychlorinated biphenyls (PCBs). Austrian pine (*Pinus nigra*) and goat willow (*Salix caprea*) were found to foster significantly higher numbers of PCB-metabolizing bacteria in the root zone than other plant species or nonrooted soil. An enrichment effect was observed in the pine and willow root zones, in which a significantly higher percentage of the total bacterial community was comprised of PCB-metabolizers than that in the root zones of other plant species. Populations of PCB-degraders in the root zones of different plants fluctuated seasonally and were significantly higher beneath Austrian pine (*Pinus nigra*) than all other plant species in the autumn and spring. Populations of PCB-metabolizers did not decline with depth down to 60 cm, and actually increased with depth beneath pine and willow. Numbers of PCB-metabolizers were not significantly influenced by soil moisture (ranging from 1.8–18.5%) or soil PCB concentrations (0-500 mg/kg). Numbers of total bacteria were not influenced by soil PCB concentrations; however they shifted with seasonal fluctuations in soil moisture. On the rhizoplane, populations of PCB-metabolizers did not differ significantly among plant species. These results indicate that some plant species can influence microbial community structure in the bulk, root-zone soil. Therefore selection of appropriate plant

species for rhizoremediation is important in efforts to manipulate the soil microbial community in favor of contaminant degradation.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are priority pollutants that persist in the environment despite the presence of indigenous PCB-degrading bacteria. A promising approach to increasing *in situ* degradation rates is by analogue enrichment, in which a selective substrate is added that promotes the growth of bacteria that degrade contaminants by cometabolism (11, 12). Provision of the analogous substrate, biphenyl, to soil microcosms has been found to enrich aerobic PCB-degrading bacteria (13, 47) and to enhance PCB-degradative rates in soils (5, 13) and sediments (18). However, the addition of biphenyl to soil would be an expensive *in situ* treatment strategy because of its low water solubility and the necessity for repeated application. As an alternative to the addition of biphenyl, certain plant species may release plant aromatic compounds from their roots that function as growth substrates for PCB-degraders and thereby provide long-term, sustained enhancement of PCB biodegradation *in situ*.

Several previous rhizosphere bioremediation studies have demonstrated that root-released secondary compounds like flavonoids can serve as growth substrates for PCB-degrading bacteria (6, 28). A major mechanism for the release of these compounds from perennial plant roots is through fine root turnover, a natural process in which small diameter (<1 mm) roots die back seasonally, providing substrates to indigenous PCB-degrading bacteria in the soil microbial community. It is hypothesized that over time, as

fine roots explore the soil and die back (28, 34), populations of degradative bacteria are enhanced, resulting in increased degradative rates throughout the plant root zone (9, 10).

It is critical to recognize that different plant species possess different quantities and varieties of aromatic secondary compounds in their roots (37). For this reason, not all plant species should be expected to promote rhizodegradation of PCBs. A greenhouse screening study of 17 different plant species indicated that only 2 plants, mulberry (*Morus rubra*) and Osage orange (*Maclura pomifera*), released phenolic compounds from their roots that stimulated the growth of PCB-degrading bacteria (9). Because there are likely many other promising plants in existence, further studies are needed to identify effective plant species for rhizoremediation.

Screening studies conducted by looking for short-term losses in recalcitrant contaminants in the presence of plants are problematic for several reasons. Fine roots are estimated to only occupy <1% of soil (20), and hence the stimulatory effects of roots (either living or dead) on the total soil microbial community will be small in any one year. Sampling and analysis of PCB-contaminated soil that has only been 1% treated will likely result in either undetectable or statistically insignificant decreases in contaminant concentrations. An alternative means of evaluating plants for rhizoremediation potential is to examine mechanistic features that underlie rhizoremediation, such as bacterial numbers and degradative potential in the root zone.

In this work, a novel approach was developed to screen plant species for their potential to foster rhizoremediation of PCBs. The method involves the quantitation of soil microflora in the root zones of several taxonomically different, mature plants that have grown under field conditions in aged, PCB-contaminated soil for 3-24 years. This

method addresses a number of important issues in the identification of potentially successful plant species. First, the plants naturally growing in contaminated soil have already been selected for tolerance to PCBs by virtue of their growth at the site. Secondly, the method identifies plants that actually maintain increased numbers of degradative bacteria in their root zones over time. Thus, this approach identifies plant species capable of coping with both contaminant exposure and fluctuating environmental stressors (drought, abrupt temperature shifts, etc.) that reflect normal field and ecological conditions over time, but generally do not prevail in conventional greenhouse studies.

The objectives of this study were to determine the influence of different tree species on numbers of total bacteria and PCB-metabolizing bacteria associated with roots after growing naturally for 3-24 years at a PCB-contaminated site. This study examines the plants' effects on the root zone, defined as the bulk soil beneath the trees, and on the rhizoplane, both of which were examined incrementally down to a depth of 60 cm. In order to better understand the impact of abiotic factors on the microbial community and to distinguish them from plant effects, the influences of soil PCB concentration, moisture content and season were also evaluated. To our knowledge, this is the first study of indigenous bacterial populations in the root zone of plants growing *in situ* in PCB-contaminated soil.

MATERIALS AND METHODS

Site description

The study site was located on the grounds of the Colorlak paint production plant in Staré Město near Uherské Hradiště in South Moravia, Czech Republic. Empty barrels

that had contained the commercial PCB mixture Delor 106, which is analogous to Aroclor 1260, were stored in this area from the 1950s to 1988, during which time inadvertent spillage of residual PCBs occurred. Hexane-washed soil samples from seven locations across the site (20-40 cm depth) were analyzed for pH, nutrient content (nitrate-N, P, K) and soil texture by the Oklahoma Cooperative Extension Service, Soil, Water and Forage Analytical Laboratory (Stillwater, OK), and additional nutrient analyses and oxidizable carbon content were determined by the Czech Agricultural University, Department of Agricultural Chemistry (Prague, Czech Republic). The soil pH ranged from 7.5 to 8.0 across the site and averaged 7.7. Nitrate-nitrogen content ranged from 2 to 24 mg/kg and averaged 8 mg/kg. Average nutrient contents (± SD) were as follows: 40 mg/kg (± 14 mg/kg) P, 256 mg/kg (± 159 mg/kg) K, 4436 mg/kg (± 614 mg/kg) Ca, and 223 mg/kg (± 60 mg/kg) Mg. Soil texture was 61% (± 14%) sand, 16% (± 8%) silt, and 22% (± 6%) clay, with an oxidizable carbon content of 1.7% (± 0.6%).

The area was naturally vegetated with 25 different plant species, including five tree species, which were growing in soil contaminated with PCBs ranging in concentration from 1-500 mg/kg. Trees growing at the site included: a 24-year-old Austrian pine (*Pinus nigra*), a 14-year old ash (*Fraxinus excelsior*), two weeping birch (*Betula pendula*) that were 11 and 13 years old growing adjacent to each other, and a 3-year old black locust (*Robinia pseudoacacia*). The age of trees was established by counting rings in core samples removed from the trunk of each tree (Chapter 1). The ground was covered with a variety of grasses (*Agropyron repens, Agrostis alba (stolonifera), Calamogrostis epigeois, Lolium perenne)* and forbs (predominantly

Achillea millefolium). The complete plant species list and a map of the vegetation were presented in Chapter 1 (27).

Sampling methods

Samples of soil and roots were collected from the root zones of each different tree species and beneath the grass/forb mixture in 1999 on June 22, August 23-24 and November 21-22, and in 2000 on May 28. Soil was excavated with an ethanol-disinfected shovel at three depth increments, 0-20 cm, 20-40 cm and 40-60 cm. Several non-rooted control samples were collected at depths of 20-40 and 40-60 cm from areas in the site vegetated by grasses whose roots only extended to a depth of approximately 15 cm. A non-contaminated control sample was also collected from beneath a large birch tree growing elsewhere within the Colorlak factory grounds. Mounds of soil that were excavated from each depth increment were homogenized, approx. 50 g was placed in plastic bags for microbial analyses, and approx. 20 g was stored in aluminum foil for determination of PCB concentration. For rhizoplane samples, fine roots (approx. 1 mm diameter) were collected from each depth level, loose soil was removed by shaking, and then the roots with tightly bound soil were stored in plastic bags. Samples were kept on ice during transport to the laboratory, and subsequently were stored at 4°C. Samples were not accessible beneath birch or locust in August, because the soil was too dry and hard to permit manual excavation. In May 2000, samples were only collected from pine, willow and birch because of time restrictions related to site access. Samples from the 40-60 cm level were not accessible beneath pine and some grassy areas due to the presence of shallow buried electrical cables.

Determination of soil PCB concentration

Soil samples were prepared for PCB extraction and analysis by air-drying at room temperature for 24 hours, homogenizing with mortar and pestle and passing through a 1 mm screen. One-gram aliquots of soil were extracted in 7 ml of hexane by refluxing at 85°C for 4 hrs using a micro-scale modification of the EPA method for Soxhlet extraction (EPA Method 3540C). Extracts were concentrated under nitrogen stream and then were subjected to florisil cleanup (EPA Method 3620B) prior to analysis. Samples were analyzed using a Hewlett-Packard 5890A gas chromatograph with an electron capture detector and a fused silica capillary column (30 m, 0.20 mm inner diameter) coated with 0.25 μm stationary phase SE054 with nitrogen as the carrier gas (flow rate 1 ml/min). The column temperature was programmed to increase from 50 to 195°C at a rate of 25°C/min, then increased to 205°C at 1°C/min, held at 205°C for 5 min, then increased to 280°C at 3°C/min and held for 15 min. Total PCB concentrations were estimated using an external standard method with 7 indicator congeners (EPA method 8082).

Microbial enumeration

Total and PCB-metabolizing bacteria were enumerated from soil and rhizoplane samples using direct plate count methods modified from Kastner *et al.* (22). One-gram aliquots of soil were placed in sterile, 50-ml screw-cap plastic tubes with approximately 3 g of sterile glass beads (5 mm dia.) and 10 ml of 1% w/v sodium pyrophosphate ($Na_4P_2O_7$) solution. For rhizoplane samples, 0.1 g of fresh roots (approx. 1 mm dia.) was extracted with 10 ml solution in sterile, 15-ml screw-cap plastic tubes without glass

beads. Tubes were shaken horizontally on a reciprocal platform shaker for 1 hour and then allowed to stand upright for 30 min. Serial dilutions were prepared in basal mineral liquid (2.13 g/L Na_2HPO_4, 1.3 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7\ H_20$) and 100 ul aliquots were spread-plated in duplicate onto agar plates. One-eighth strength plate count agar was used to enumerate total culturable bacteria (2.94 g plate count agar and 13.12 g Bacto agar in 1L basal mineral liquid), which was demonstrated experimentally to yield the same colony numbers as full-strength plate count agar. The slower colony growth rate on this medium was advantageous for the PCB-metabolizer screening technique described below. Colonies were counted following one week of incubation at room temperature. For enumeration of PCB-metabolizers, basal mineral agar was prepared using basal mineral liquid and 15 g/L Noble agar (Difco, Detroit, MI) with biphenyl provided as the sole carbon source by sprinkling a few crystals in the lid of the inverted Petri dish. Basal mineral plates were incubated for 3 weeks at room temperature, and then were subjected to a spray assay modified from Sylvestre (44) to screen for colonies that metabolize 4-bromobiphenyl, an inexpensive and readily available analogue of PCBs. A 5% w/v solution of 4-bromobiphenyl in ethyl ether was sprayed onto the agar surface using a TLC-plate sprayer to overlay the plate surface with a thin cloudy film. After additional incubation of 1-2 weeks, zones of clearing formed around colonies capable of metabolizing the compound, which were then counted. Microbial numbers were calculated based on dry weight of soil or root. Following extraction, roots were recovered and dried to determine dry weights, and soil moisture was determined gravimetrically by preparing separate aliquots of 2-5 g fresh soil that were dried at 60°C for 24 hours.

To evaluate the seasonal effects of plants on the soil microflora, four to six replicate plate counts were performed from soil samples from the 20-40 cm depth taken on each sampling date. This depth level was selected because of the high density of roots from the individual tree species being examined, and the absence of roots from forbs and grasses that grew beneath the canopies of trees at the site whose roots prevailed in the 0-20 cm level. For comparative analyses of rooted versus nonrooted soil samples, the 20-40 cm tree samples were conveniently matched with nonrooted control samples collected at the same depth beneath pure stands of grass/forb vegetation growing at the site. Repeated analyses of rhizoplane and root-zone samples at each depth level (0-20 cm, 20-40 cm and 40-60 cm) were performed on samples collected in November because this sampling date provided samples from the largest number of plant species.

Statistical analyses

Statistical analyses of microbial counts were performed using SPSS software (SPSS, Inc.). The distribution of each dataset was first tested for normality using the one-sample Kolmogorov-Smirnov (K-S) test, which indicates whether datasets are distributed normally ($p > 0.05$) or nonparametrically ($p < 0.05$). Normally distributed data were analyzed using ANOVA with Tukey's post-hoc test or Pearson's product-moment correlation test. Nonparametric datasets were analyzed using the nonparametric Kruskall-Wallis test for several independent samples, followed by the Mann-Whitney U test to determine pairwise differences among groups; correlations were tested using the Spearman's rank-order correlation. These tests are the nonparametric equivalents of the ANOVA, the paired t-test and the Pearson's product moment correlation, respectively,

and are considered to be more conservative (7). Significance levels for all comparison and correlation tests were set at $p < 0.05$ unless otherwise stated.

RESULTS

Abiotic factors and relationships to soil bacteria

Soil PCB concentrations were heterogeneous across the site, and ranged from 8-500 mg/kg. PCB concentrations beneath the major trees at 20-40 cm depth were as follows: Austrian pine (*Pinus nigra*) in soil contaminated with 8-15 mg/kg, ash (*Fraxinus excelsior*) in 15-16 mg/kg, weeping birch (*Betula pendula*) in 153 mg/kg, black locust (*Robinia pseudoacacia*) in 13-153 mg/kg, and willow (*Salix caprea*) in 471-500 mg/kg. The nonrooted control soil collected at 20-40 cm beneath grasses and forbs had a PCB concentration of 50 mg/kg. The soil beneath the uncontaminated control birch tree located on the factory grounds but approximately 200 m away from the contaminated study site had a PCB concentration of 0.365 mg/kg. A map of contamination levels and vegetation was presented previously in Chapter 1 (27).

To investigate the overall distribution of the dataset, 96 data points of soil microbial numbers collected at the 20-40 cm depth across the entire site on all sampling dates were analyzed. Numbers of total culturable bacteria ranged from 2.9×10^5 to 7.8×10^7 cells/gDW soil, with a mean of 1.1×10^7 and median of 5.3×10^6 cells/gDW soil (N=96). Culturable PCB-metabolizing bacteria were detected throughout the site at numbers ranging from 1.6×10^2 to 1.0×10^5 cells/gDW soil, with a mean of 1.4×10^4 and a median of 3.3×10^3 cells/gDW (N=96). When all datapoints were examined together, bacterial numbers were not normally distributed, and were right-skewed with g_1

values of 2.6 (total bacteria) and 2.1 (PCB-metabolizers), indicating that the majority of datapoints occurred at the lower ends of the distributions.

The influence of soil PCB concentration on numbers of total bacteria and PCB-metabolizers was investigated using a subset of data from samples that had been analyzed for PCB content (N=49) (Figure 1). There was no significant correlation between soil PCB concentration and numbers of total bacteria in soil ($p=0.219$, Spearman's rho = -0.179, N = 49). There was also no correlation between PCB concentration and numbers of PCB-metabolizers at the 95% confidence level, however a slightly negative correlation was significant at the 90% confidence level ($p = 0.078$, Spearman's rho = -0.254, N = 49) (Figure 1b).

When bacterial numbers beneath the birch tree growing in uncontaminated soil (<1 mg PCBs/kg soil) were compared to those present beneath the birch growing in contaminated soil (153 mg PCBs/kg soil), the median number of total bacteria was 5.59×10^6 colonies/gDW versus 1.02×10^7 colonies/gDW, respectively. These values were not significantly different to the 95% confidence level (Kruskall-Wallis test, $p = 0.077$). In contrast, the numbers of PCB-metabolizing bacteria were found to be lower in the contaminated soil (1.06×10^3 colonies/gDW soil) relative to uncontaminated soil (5.56×10^3 colonies/gDW soil) (Kruskall Wallis test, $p = 0.001$). Several factors other than PCB concentration may have contributed to this difference, with the most notable being that the birch growing in the uncontaminated soil was much larger, presumably older, and rooted in soil that was darker and more friable than the birch growing in PCB-contaminated soil.

Moisture content of soil samples throughout the site (20-40 cm depth) ranged from 1.8 to 18.5% (N=96) and was significantly influenced by the sampling date (Kruskall-Wallis test, $p = 8 \times 10^{-15}$). Median soil moisture values on each sampling date were: 2.3% (June, 1999), 3.8% (August, 1999), 10.5% (November, 1999) and 10.4% (May, 2000). The soil moisture content showed a significant increase with each progressive sampling date (June, August, November) in 1999 (Mann-Whitney U test, $p < 0.001$). Soil moisture during the early spring of the following year (May, 2000) did not differ significantly from November of 1999 ($p=0.216$).

The relationship between soil moisture and numbers of total bacteria throughout the site (20-40 cm depth) was examined (Figure 2a). Total bacterial numbers correlated significantly and positively with percent soil moisture ($p=0.000001$, Spearman's rho = 0.766, N = 96). Sampling date significantly influenced total bacterial populations (Kruskall-Wallis test $p = 0.0000001$), such that total numbers significantly increased from the dry summer months (June and August, 1999) to the wetter fall and spring months (November 1999, May 2000) (Mann-Whitney U test, $p < 0.0005$) (Figure 2a).

The influence of soil moisture on numbers of PCB-metabolizing bacteria was also examined using data points from across the site at the 20-40 cm depth (Figure 2b). There was no significant correlation between soil moisture and numbers of PCB-metabolizers ($p = 0.265$, Spearman's rho = 0.115, N = 96). Sampling date did not have an overall influence on numbers of PCB-metabolizers (Kruskall-Wallis test $p = 0.587$) when data points from across the site were analyzed together. However, significant seasonal fluctuations in numbers of PCB-metabolizers were observed in the root zones of individual plant species as described below (Figure 3b).

Plant species effects on root zone bacteria

Seasonal trends in root-zone bacterial numbers at the 20-40 cm depth beneath different plant species were examined (Figure 3). Numbers of total bacteria in the root zones of all plants increased approximately ten-fold during the study period between June, 1999 and May, 2000 (Figure 3a). Seasonal increases in total counts were statistically significant for all plant species except for pine (ANOVA, $p < 0.05$). Seasonal trends in numbers of PCB-metabolizing bacteria (Figure 3b) did not parallel the upward trends in total bacterial numbers for all plants. While numbers of PCB-metabolizers beneath pine increased significantly over the course of the study, numbers dropped significantly beneath birch (ANOVA, $p < 0.05$) and no significant seasonal differences were detected beneath the other plant species. Beneath willow, high variability prevented the detection at statistical differences, but pronounced seasonal fluctuations in PCB-metabolizers were apparent in which numbers of PCB-degraders increased from June to August, dropped in November, and then increased again by the following May.

Numbers of total and PCB-metabolizing bacteria among the different plant species were compared separately on each respective sampling date. Datasets from each individual sampling date were normally distributed (K-S test, $p < 0.05$), which permitted the use of parametric tests (ANOVA). Numbers of total and PCB-metabolizing bacteria did not differ significantly among any plant species on either June or August sampling dates. However, species differences were observed in November and the following May. In November, total bacterial numbers were significantly higher beneath ash, black locust and pine in comparison to willow (detailed below and in Table 2), and in May, the root zones of pine and willow had higher total counts than birch. Differences in numbers of

PCB-metabolizers were also found among plant species in November and the following May. Pine fostered significantly higher numbers of PCB-metabolizers in its root zone than all other plants tested in both November and May (ANOVA, $p < 0.02$).

Seasonal trends in the microbial community structure beneath different plants were examined by evaluating the percentage of total bacteria that metabolize PCBs on each sampling date (Figure 3c). PCB-metabolizers comprised percentages of the total culturable bacterial community ranging from means of 0.02% (birch, May, 2000) to 1.4% (black locust, June, 1999). Percent degraders did not differ significantly among the plant species on either June or August due to high variabilities in counts. The pine root zone fostered a significantly higher percentage of PCB-degraders than all other plants in November (ANOVA $p < 0.02$) and in May (ANOVA $p < 0.0001$).

Because distinct differences in bacterial numbers were found beneath different plant species in November, this data set was examined to evaluate the influence of soil depth on microbial numbers beneath different plant species (Table 1). Numbers of total bacteria did not differ significantly with depth beneath each individual tree species (Table 1, rows), except for black locust which fostered higher total numbers at the 20-40 cm depth than the 0-20 cm depth. Beneath the grasses, whose roots were only present in the upper 20 cm of soil, total numbers of bacteria declined significantly with greater depth. Significant differences in total bacterial populations were observed among some plant species at each individual depth level (Table 1, columns). At the 20-40 cm depth level, ash and black locust fostered higher total counts than birch or willow, and pine's total counts were also higher than willow. At the 40-60 cm depth, the nonrooted soil had significantly lower numbers of total bacteria than all trees samples at that depth.

Populations of PCB-metabolizers did not decline with depth beneath any trees or in the nonrooted soil, and numbers actually increased with depth beneath pine and willow. Plant species differences in numbers of PCB-metabolizers were not detected at the 0-20 cm depth, where the roots of individual trees occupied the soil mutually with grass and forb roots. However, at the 20-40 cm depth, the pine root zone had significantly higher numbers of PCB-metabolizers (6.8×10^4) than all other trees and the nonrooted control soil. Overall, the highest mean number of PCB-metabolizers (1.2×10^5) was found in the willow root zone at the 40-60 cm depth level. This value was higher than nonrooted soil at the same depth, and was statistically similar to the high counts found beneath pine at the 20-40 cm depth (independent samples t-test, $p = 0.582$). The deep willow root-zone also had a very high percentage of degraders (2.11%), which was even significantly higher than the enriched root zone of pine (0.59%) at the 20-40 cm depth (Independent samples t-test, $p = 0.004$).

Plant species effects on the rhizoplane

The influence of plant species and depth on rhizoplane bacterial populations was examined from samples collected in November (Table 2). The resulting data set was normally distributed and thus was analyzed using parametric tests. Mean numbers of total culturable bacteria on the rhizoplane ranged from 3.5×10^8 (black locust 20-40 cm depth) to 1.7×10^{10} (grass, 0-20 cm depth) colonies/gDW root. At the 0-20 cm depth level, the grass rhizoplane fostered significantly higher numbers of total bacteria than ash, willow and pine. The high total counts on the grass rhizoplane may be attributable to the small diameter of its roots, which resulted in a higher surface area per gram dry weight

than for the tree roots. At the 20-40 cm depth, the pine rhizoplane had the highest mean number of total bacteria, which was significantly higher than birch or black locust rhizoplanes. Populations of total rhizoplane bacteria did not decline with depth beneath any plant species, and actually increased significantly with depth beneath willow and pine.

Average numbers of rhizoplane PCB-metabolizers ranged from 8.4×10^4 (black locust, 20-40 cm depth) to 1.6×10^8 (grass, 0-20 cm depth). Numbers of PCB-metabolizers did not decline with depth beneath any plant with sufficient samples for comparison, and increased with depth beneath willow. Due to high variabilities, no plant species differences were detected at the shallowest depth, but some plant species differed at the 20-40 and 40-60 cm depths. The pine rhizoplane had the highest mean number of PCB-metabolizers at 20-40 cm, however this value only differed significantly from black locust. At 40-60 cm depth, willow rhizoplane fostered significantly higher numbers of PCB-metabolizers than ash or black locust. Unfortunately, pine samples were unavailable at the 40-60 cm depth for comparison due to buried electrical cables, however when the rhizoplanes of pine (20-40 cm) and willow (40-60 cm) were compared they were found to be statistically similar in numbers of PCB-metabolizers (independent samples t-test, $p=0.32$).

To examine community structure on the rhizoplane, the correlation between numbers of total bacteria and PCB-metabolizers was investigated. Numbers of PCB-metabolizers correlated significantly and positively with numbers of total bacteria on rhizoplane samples at the 20-40 cm depth ($p = 0.00005$, Pearson's correlation coefficient $= 0.744$, $N = 23$). In Figure 4, the data points are plotted and a close relationship is

visible. When the percentage of total bacteria that degrade PCBs was calculated and compared for each plant species at 20-40 cm, mean values ranged from 0.05 to 0.32% and there were no significant differences found among plants. When the rhizoplane communities were examined at 40-60 cm depth, PCB-degraders comprised 0.18% of the willow rhizoplane community, which was significantly higher than ash (0.005%) but not black locust (0.07%). These results indicate that although PCB-degrading bacteria were present on the rhizoplanes of all plants, their numbers were not differentially enhanced by plant species except for willow which appears to exert a slight enrichment effect on rhizoplane degraders at the 40-60 cm depth level.

DISCUSSION

Populations of total and PCB-metabolizing bacteria were enumerated with regard to their association with vegetation, temporal fluctuations and vertical distribution in soil at a naturally-vegetated, PCB-contaminated site. Previous studies enumerating bacteria from PCB-contaminated sites have usually relied on composite samples, and did not investigate the distribution of PCB-metabolizing organisms at the study site. Layton *et al.* (26) found total culturable bacteria ranging from approximately 5×10^3 to 5×10^6 CFUs/g soil by plate counts, and detected biphenyl utilizers up to 9×10^2 CFUs/g soil by a hybridization assay of colonies grown from soils contaminated with 40-144 mg/kg PCBs. Fava *et al.* (8) reported higher numbers of 2×10^6 total bacteria and 6×10^5 biphenyl-utilizers in a landfill soil with 350 mg/kg PCBs using direct plate-count methods. In a study of two landfills with 100-10,000 mg/kg PCBs, Walia *et al.* (48) found total bacteria in numbers of 4-8 $\times 10^8$ CFUs/g soil, and PCB–metabolizers ranging

from below detection limits to 8×10^7 cells/g soil using a plate count and spray-assay method similar to ours. In our study, numbers of total culturable bacteria ranged from 2.9 $\times 10^5$ to 7.8×10^7 cells/gDW soil with a median of 5.3×10^6 cells/gDW soil, which are comparable to the previous reports in PCB-contaminated soils (8, 26, 48) as well as in pristine soils (1, 31). These results, as well as our finding no negative correlation between PCB concentration and numbers of total bacteria (Figure 1a) suggest that PCB concentrations between 8-500 mg/kg are not profoundly inhibitory towards the growth of many soil bacteria.

Numbers of PCB-metabolizers in our study ranged from 1.6×10^2 to 1.0×10^5 cells/gDW soil, with a median of 3.3×10^3 cells/gDW, which are similar to reports by Layton *et al.* (26), but are lower than the results of Fava *et al.* (8) and Walia *et al.* (48). Although the soils examined in the latter two studies had higher PCB concentrations (350-10,000 mg/kg) than our site (8-500 mg/kg), PCB concentration is unlikely to be solely responsible for the higher numbers of degraders since most PCB congeners are not good growth substrates for PCB-degraders (4), and our results (Figure 1b) found no positive correlation between PCB-concentration and numbers of degraders in soil. However, the authors did report that landfill soils contained other toxic organic co-contaminants (48) and chlorinated contaminants (8). The co-contaminants may have acted as growth substrates for biphenyl-utilizers, since biphenyl dioxygenase genes are closely related to other aromatic dioxygenases (32) and promiscuous dioxygenase enzymes have been observed that can degrade both biphenyl and other aromatic contaminants (23-25, 36).

The influences of various abiotic factors, including soil PCB concentration, soil moisture, and season on microbial populations at the site were examined. These interactions are important to understand the impact of environmental conditions on PCB bioremediation, and also necessary to distinguish site heterogeneities versus true plant effects on microbial populations. Soil moisture varied with season, and had a significant impact on numbers of total bacteria (Figure 2a). The seasonal fluctuations in total bacterial numbers follow a trend commonly observed in temperate regions, in which the highest total bacterial numbers are found in the wetter autumn and spring seasons, with lower numbers in the dry summer months (1). Soil moisture did not correlate closely with numbers of PCB-metabolizers, suggesting that other factors were more important determinants of degrader population size. These results indicate that populations of PCB-metabolizing bacteria at the site are relatively stable in soil moisture levels ranging from 1.8 to 18.5%, even though the total culturable bacterial population increased during periods of high soil moisture.

Soil PCB concentration did not significantly impact numbers of total bacteria in soil (Figure 1a), indicating that PCBs at levels encountered in this study are neither stimulatory nor highly toxic to the overall microbial community. A slight negative correlation was detected between soil PCB concentration and numbers of PCB-metabolizing bacteria (Figure 1b), suggesting that high PCB concentrations were associated with reduced numbers of degraders. However, a larger number of samples in the upper range of PCB concentrations would be needed in order to conclude this with confidence. The observation that soil PCB concentration did not correlate strongly with increased numbers of PCB-metabolizing bacteria is not surprising, considering the

composition of the PCB mixture present. Delor 106 (analogous to Aroclor 1260) consists primarily of congeners with 4 to 7 chloro-substitutions. Although many known PCB-metabolizing bacteria can partially degrade highly-substituted congeners, it is also known that congeners with more than 1 chloro-substitution generally cannot serve as growth substrates (4), and thus their presence would not be expected to enrich for degradative bacteria.

Some plant species had a significant effect on the number of PCB-metabolizing bacteria in root-zone soil. The plant species effect was not detectable in shallow soil (0-20 cm), probably due to the confounding effect of grass and forb roots that shared the soil with the trees. However at lower depths where tree roots predominated (20-40 and 40-60 cm), definitive differences among tree species emerged. At 20-40 cm, the root zone of Austrian pine (*Pinus nigra*) fostered significantly higher numbers of PCB-metabolizers than all other plant species tested in both November and May. Although the willow (*Salix caprea*) root zone had low numbers of PCB-metabolizers at 20-40 cm depth, the deep root zone (40-60 cm) fostered very high numbers that were similar to pine. The increased numbers of PCB-metabolizers present in both the pine and willow root zones were due to an enrichment effect, in which PCB-metabolizers comprised a higher percentage of the total culturable community than in the root zone of other plants.

The enrichment of PCB-metabolizing bacteria observed in the root zones of pine and willow suggests that these plants provide selective conditions for degradative bacteria in the root zone, perhaps due to release of selective substrates from roots since aromatic compounds present in fine roots have been shown in laboratory studies to support the growth of PCB-degrading bacteria (6, 28). Pine and willow are both known

to produce aromatic secondary compounds that are significant to the degradation of aromatic contaminants like PCBs and PAHs. *Pinus nigra* produces terpenoids (3, 21), simple phenolic compounds (17) and tannins (14). Some terpenoids have been found to induce the PCB-degradative pathway (15, 35) as well as serving as substrates for PCB-degrading bacteria (19, 49). Yu *et al.* (49) found that bacteria capable of growth on two tricyclic diterpenoids (resin acids) that *Pinus nigra* produces in abundance (3) were enriched in hydrocarbon-contaminated soils, and that they also degraded aromatic hydrocarbons including biphenyl. Willows (*Salix* spp.) are widely recognized for their production of the phenolic compound salicylic acid, which is known to induce transcription of naphthalene degradative genes in PAH-degrading bacteria (38), and also reportedly functions as a growth substrate and inducer for a PCB-degrading bacterium (41). Salicylate-degrading bacteria were found to be enriched beneath willows in comparison to other vegetation, indicating it functions as a substrate for aromatic-degrading bacteria in soil (39). Although salicylate itself has not been found in *Salix caprea*, this plant does produce two related compounds, saligenin (salicyl alcohol) and salicin (a glucoside of saligenin) in the leaves and bark (43), which may function similarly to salicylate. Other willows are also know to contain flavonoids and condensed tannins in their aboveground tissues (16, 45), and although the flavonoid content of *Salix caprea* has not been extensively characterized, the flavonoids myricetin and apigenin were identified in another willow species (45), which have been found by Donnelly *et al.* (6) to serve as growth substrates for cultures of PCB-degrading bacteria.

To our knowledge, the presence of aromatic compounds in *Pinus nigra* and *Salix caprea* has only been investigated in the aboveground portions of the plants. It is

unlikely that compounds released from aboveground litter detritus are responsible for the enhancement of PCB-degrading bacteria observed in this study, since the effects were not detected in the upper 20 cm of the soil, but rather were found at 20-40 cm (pine) and 40-60 cm (willow). Although the root chemistry of these two species has not been investigated, studies of related species show that roots often contain the same secondary chemicals as reported in the aboveground portions. For example, the roots of several other pine species are known to contain terpenoids (33, 42) and flavonoids (37), and the roots of other willow species contain tannins (30), salicin and flavonoids (2).

It is not clear from this study whether stimulatory substrates may have been released into the soil by exudation from living roots or by lysis of dead roots during root turnover. However, the seasonal fluctuations in microbial stimulation that were observed among the different plants are consistent with the root turnover hypothesis, since the process of fine root turnover in perennial plants often occurs in seasonal cycles with periodicities and turnover rates that vary among species (46). Because both seasonal root turnover cycles and secondary products accumulated by plants vary among species, it is anticipated that production and release of chemicals by roots will vary temporally among different plants. Thus, the seasonal difference in enhanced PCB-metabolizer numbers beneath pine and willow could be accounted for by an earlier occurrence of fine root turnover for pine than for willow (Figure 3b).

A review of the literature indicates that this is the first comparison of PCB-degrading microbial populations associated with the roots of different plant species. However, in studies investigating rhizoremediation of PAHs, differences in numbers of PAH-degraders have been found in the rhizospheres of different plant species (29, 40).

Siciliano *et al.* (40) also recently reported that the presence of plants (native grass or grass/legume mixtures) in PAH-contaminated soils enhanced numbers of PAH-catabolic genes in bulk soil relative to unplanted soil. These results are in agreement with our observations that some plants can exert a stimulatory influence on microbial populations beyond the immediate rhizosphere into the bulk root-zone soil.

In this work, we have demonstrated that certain plants are capable of selectively enriching indigenous, PCB-metabolizing bacteria in the soil under natural field conditions. By increasing the number of degradative bacteria and hence the number of catalytic units, the growth of appropriate plant species in contaminated soil may enhance the rate of PCB-degradation *in situ* and provide a cost-effective alternative to conventional cleanup methods for PCB-contaminated soil.

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TABLE 1. Influence of soil depth and plant species on numbers of bacteria in the root zone in November, 1999. Mean values are presented with standard deviations in parentheses. Different capital letters indicate statistically significant differences among members of the same column, and different lower-case letters denote differences among the same row (ANOVA $p < 0.05$).

Plant species	N[1]	Bacterial numbers in root-zone soil (Colonies/gDW soil)		
		0-20 cm depth[2]	20-40 cm depth	40-60 cm depth
TOTAL CULTURABLE BACTERIA				
Ash	4, 4, 4	5.7×10^6 (5.1×10^6)$^{AB, a}$	1.7×10^7 (7.8×10^6)$^{A, a}$	8.9×10^6 (5.0×10^6)$^{AB, a}$
Birch	2, 4, 0	1.4×10^6 (1.1×10^5)$^{AB, a}$	4.1×10^6 (3.6×10^6)$^{BC, a}$	na[3]
Black Locust	4, 4, 4	9.8×10^6 (6.2×10^6)$^{A, a}$	1.8×10^7 (1.6×10^6)$^{A, b}$	1.1×10^7 (2.7×10^6)$^{A, ab}$
Pine	2, 4, 0	1.2×10^7 (1.2×10^7)$^{A, a}$	1.4×10^7 (7.2×10^6)$^{AC, a}$	na
Willow	6, 4, 5	3.0×10^6 (2.2×10^6)$^{AB, a}$	2.5×10^6 (1.2×10^6)$^{B, a}$	5.4×10^6 (2.9×10^6)$^{B, a}$
Grass/Nonrooted[4]	8, 4, 8	2.5×10^6 (1.9×10^6)$^{B, a}$	1.5×10^6 (4.3×10^5)$^{B, ab}$	5.2×10^5 (1.9×10^5)$^{C, b}$
PCB-METABOLIZING BACTERIA				
Ash	4, 4, 4	7.3×10^3 (5.2×10^3)$^{D, d}$	2.5×10^4 (2.7×10^4)$^{D, d}$	2.7×10^4 (4.3×10^4)$^{DE, d}$
Birch	2, 4, 0	6.7×10^3 (7.1×10^1)$^{D, d}$	3.3×10^3 (2.0×10^3)$^{D, d}$	na
Black Locust	4, 4, 4	8.1×10^3 (5.2×10^3)$^{D, d}$	1.7×10^4 (1.5×10^4)$^{D, d}$	2.6×10^3 (9.9×10^2)$^{DE, d}$
Pine	2, 4, 0	8.0×10^2 (2.5×10^2)$^{D, d}$	6.8×10^4 (2.7×10^4)$^{E, e}$	na
Willow	6, 4, 5	2.2×10^3 (1.8×10^3)$^{D, d}$	1.3×10^3 (7.6×10^2)$^{D, de}$	1.2×10^5 (9.6×10^4)$^{D, e}$
Grass/Nonrooted	8, 4, 8	3.3×10^3 (3.5×10^2)$^{D, d}$	1.2×10^3 (1.2×10^3)$^{D, d}$	3.9×10^3 (4.9×10^3)$^{E, d}$

[1] Number of replicates is noted for 0-20 cm, 20-40 cm and 40-60 cm depths, respectively.

[2] The 0-20 cm depth level was vegetated with grasses and forbs beneath all trees.

[3] Sample not available

[4] Grass roots were limited to the upper 0-20 cm of soil, thus the 20-40 and 40-60 cm depths were nonrooted control soil from within the contaminated site.

TABLE 2. Influence of soil depth and plant species on numbers of bacteria on the rhizoplane in November, 1999. Mean values are presented with standard deviations in parentheses. Different capital letters indicate statistically significant differences among members of the same column, and different lower-case letters denote differences among the same row (ANOVA $p < 0.05$).

Plant species	N[1]	Bacterial numbers on the rhizoplane (Colonies/gDW root)		
		0-20 cm depth	20-40 cm depth	40-60 cm depth
TOTAL CULTURABLE BACTERIA				
Ash	4, 6, 4	2.8×10^9 (1.3×10^9) $^{A, a}$	2.6×10^9 (2.1×10^9) $^{AB, a}$	4.0×10^9 (1.3×10^9) $^{A, a}$
Birch	0, 4, 0	na[2]	9.5×10^8 (5.2×10^8) ^{A}	na
Black Locust	4, 3, 4	8.6×10^9 (7.2×10^9) $^{AB, a}$	3.8×10^8 (2.9×10^8) $^{A, a}$	1.4×10^9 (5.8×10^8) $^{B, a}$
Pine	6, 6, 0	3.1×10^9 (3.5×10^9) $^{A, a}$	5.8×10^9 (3.1×10^9) $^{B, b}$	na
Willow	4, 4, 3	2.2×10^9 (1.3×10^9) $^{A, a}$	2.8×10^9 (1.2×10^9) $^{AB, ab}$	2.9×10^9 (2.8×10^8) $^{AB, b}$
Grass	8, 0, 0	1.7×10^{10} (7.5×10^9) ^{B}	na	na
PCB-METABOLIZING BACTERIA				
Ash	4, 6, 4	1.6×10^6 (8.6×10^5) $^{D, d}$	1.0×10^6 (9.1×10^5) $^{DE, d}$	2.5×10^5 (1.6×10^5) $^{D, d}$
Birch	0, 4, 0	na	2.0×10^6 (1.5×10^6) ^{DE}	na
Black Locust	4, 3, 4	5.2×10^6 (5.1×10^6) $^{D, d}$	8.4×10^4 (6.8×10^4) $^{D, d}$	7.0×10^5 (3.5×10^5) $^{D, d}$
Pine	6, 6, 0	2.4×10^6 (1.8×10^6) $^{D, d}$	3.0×10^6 (1.8×10^6) $^{E, d}$	na
Willow	4, 4, 3	9.5×10^5 (9.0×10^5) $^{D, d}$	1.4×10^6 (2.5×10^5) $^{DE, d}$	5.3×10^6 (3.1×10^6) $^{E, c}$
Grass	8, 0, 0	1.6×10^8 (2.1×10^8) ^{D}	na	na

[1] Number of replicates is noted for 0-20 cm, 20-40 cm and 40-60 cm depths, respectively.

[2] Sample not available

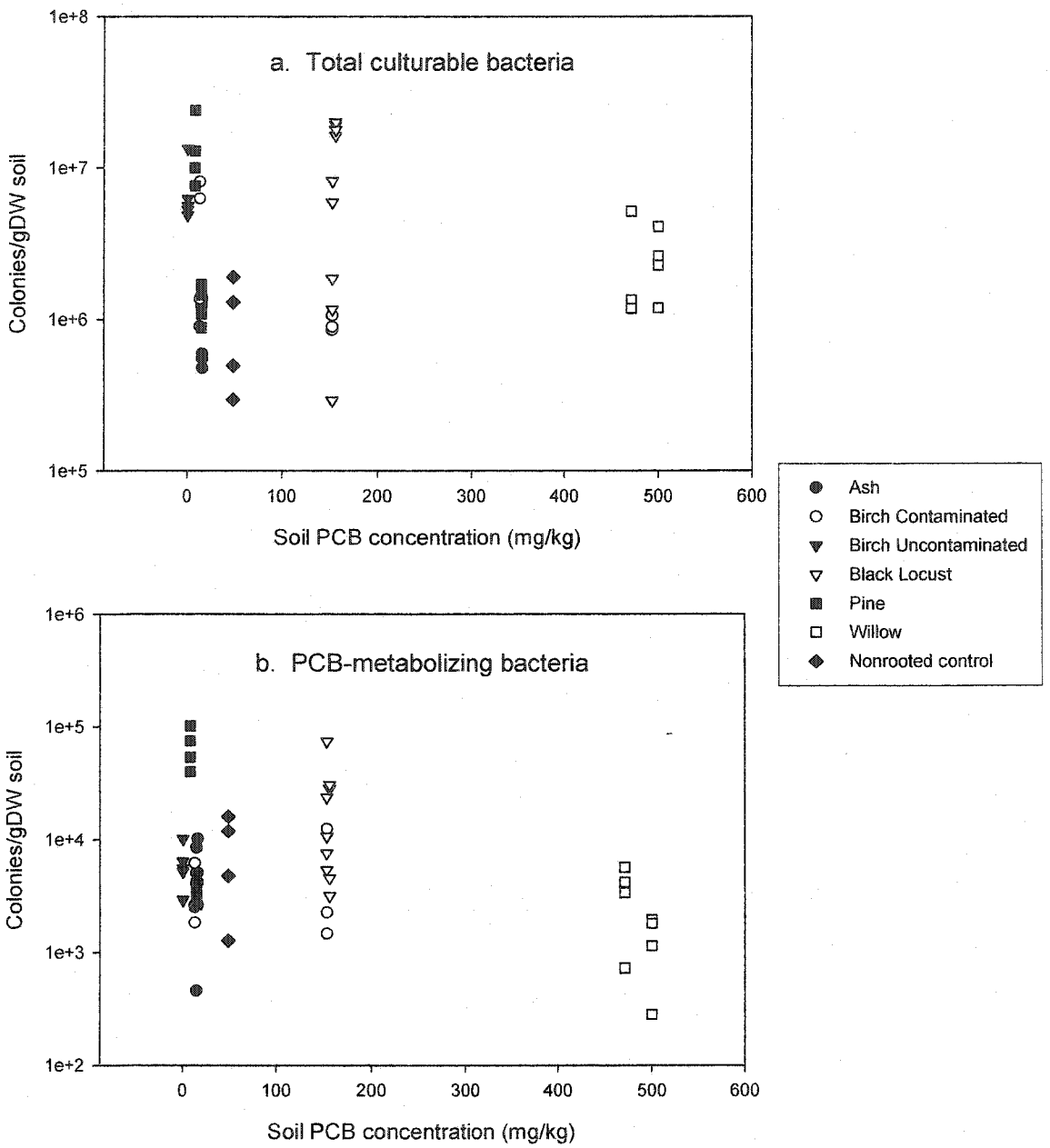

FIGURE 1. Influence of soil PCB concentration on numbers of a) total culturable bacteria and b) PCB-metabolizing bacteria (N=49).

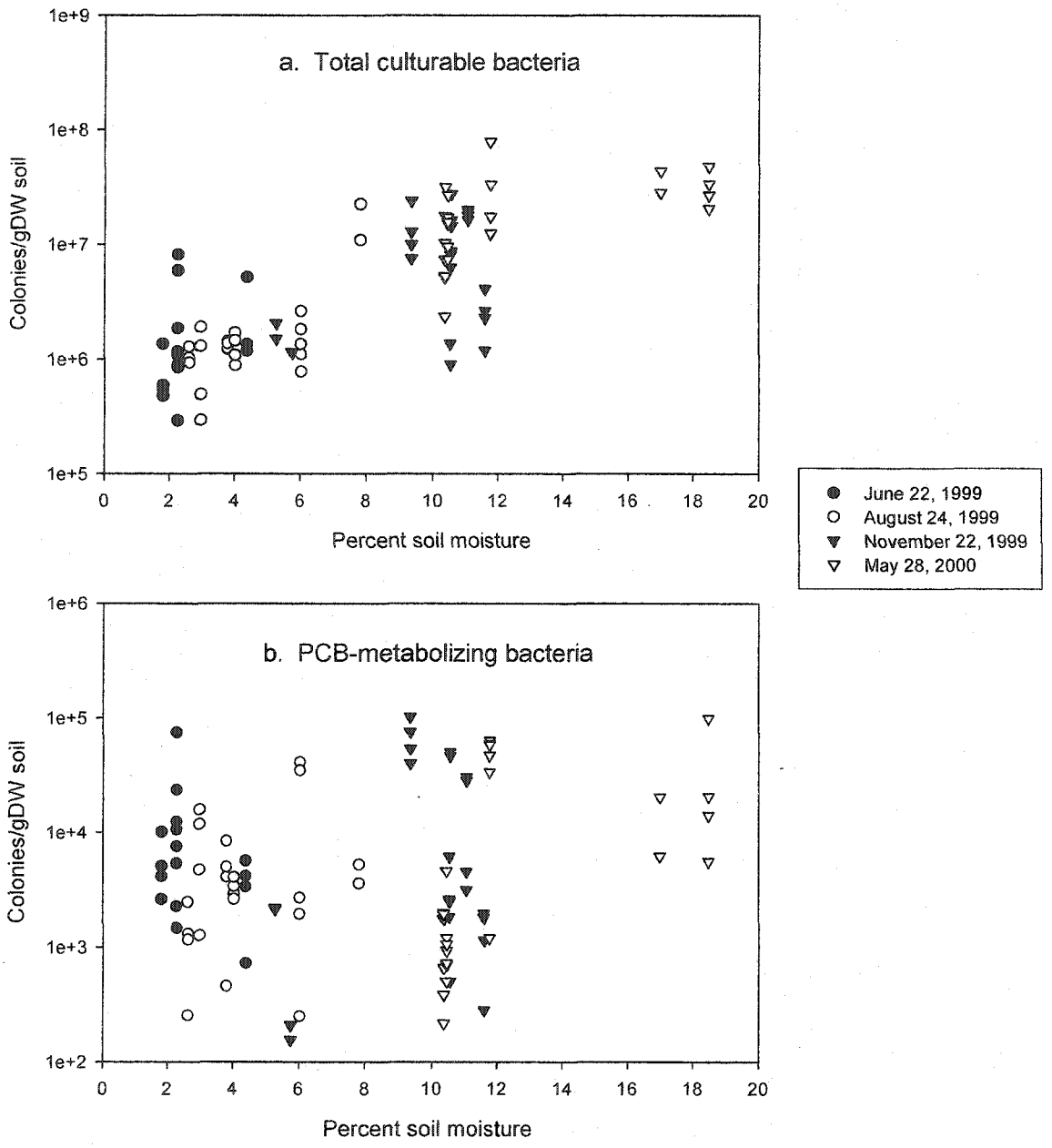

FIGURE 2. Relationship between soil moisture and numbers of a) total culturable bacteria and b) PCB-metabolizing bacteria (N=88).

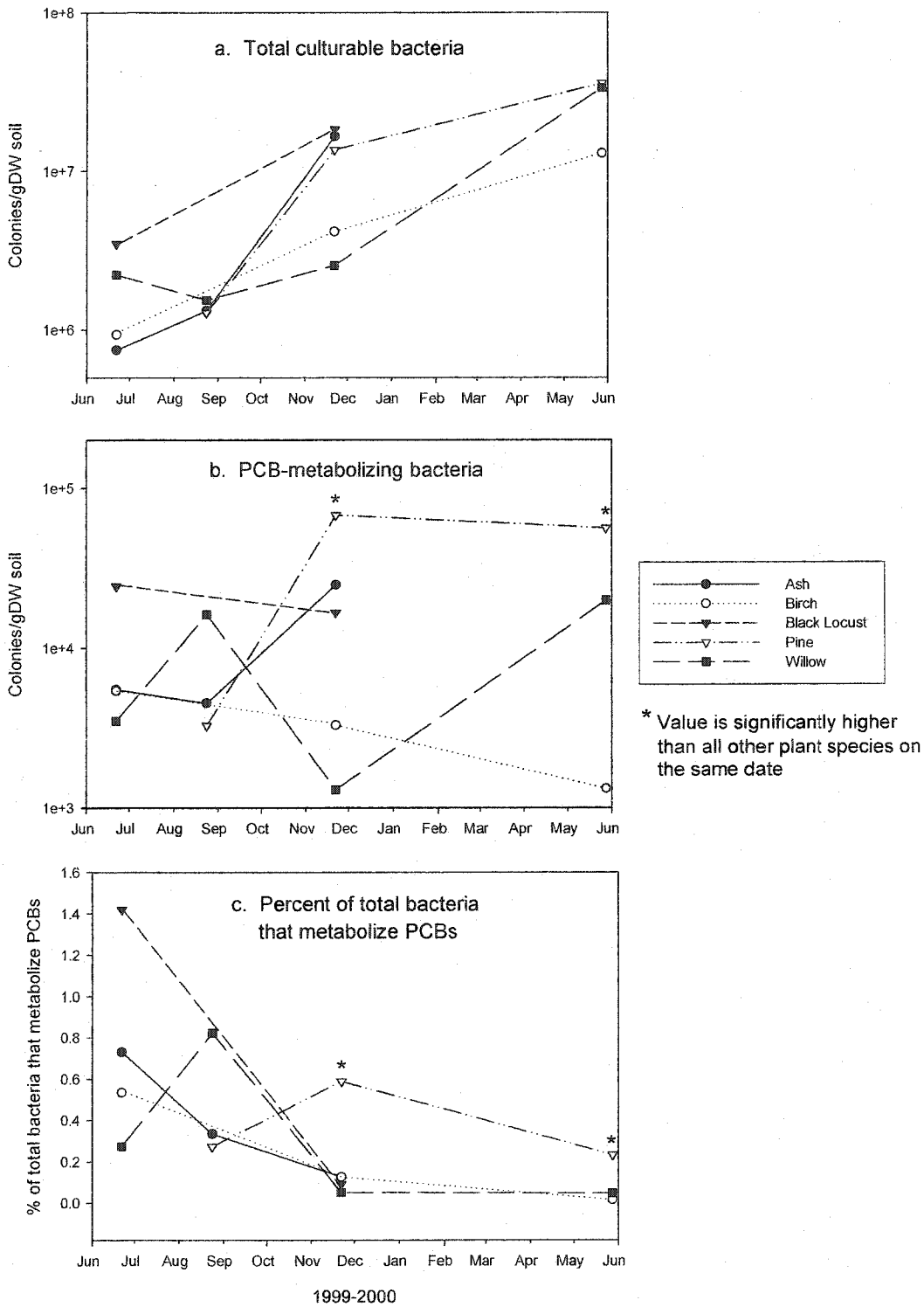

FIGURE 3. Seasonal trends in the mean numbers of a) total culturable bacteria b) PCB-metabolizers and c) the percentage of total bacteria that metabolize PCBs in the root zones of different plant species (20-40 cm depth) in 1999-2000.

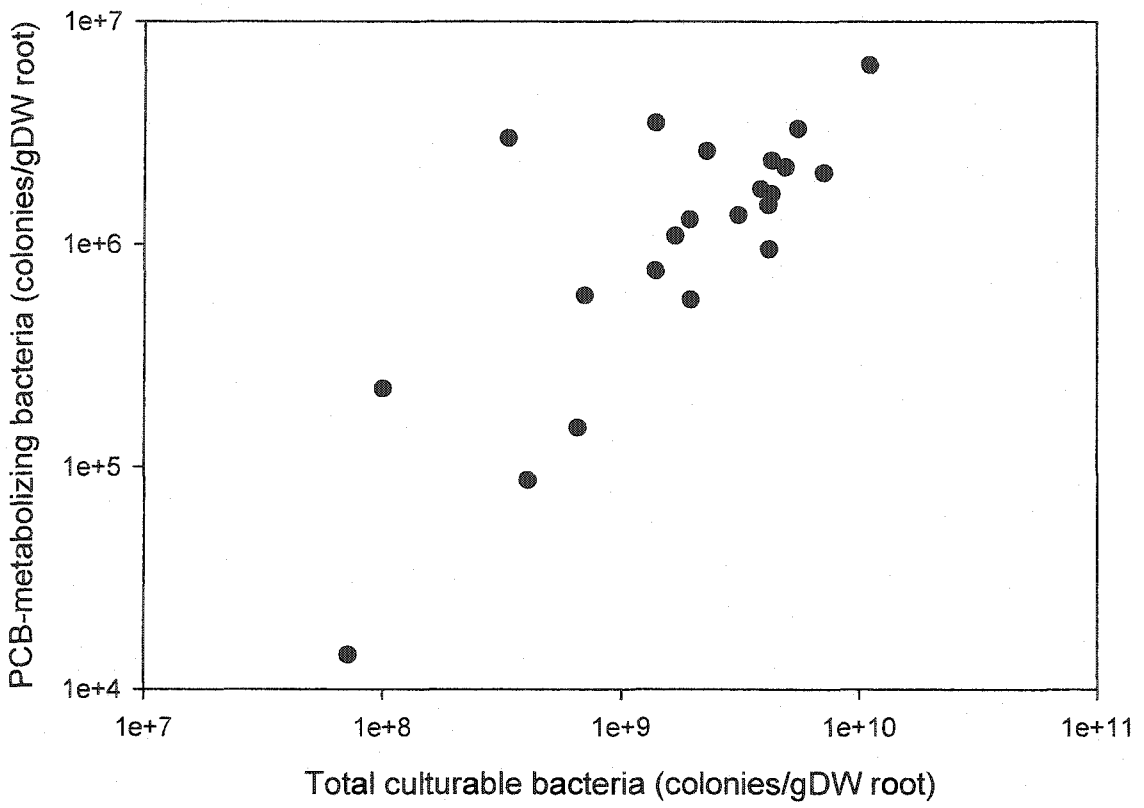

FIGURE 4. Relationship between numbers of total bacteria and PCB-metabolizers on the rhizoplane of all trees samples at 20-40 cm depth (N=23).

CHAPTER 3

Diversity and degradative abilities of PCB-degrading bacteria associated with plant roots *in situ* at a contaminated site

ABSTRACT

PCB-degrading bacteria associated with the roots of six different plant species growing naturally at a PCB-contaminated site in the Czech Republic were examined to determine their diversity, relative abundance and degradative abilities. PCB-degrading bacteria were cultured from root-zone soils and rhizoplanes of ash, birch, black locust, Austrian pine, goat willow and grasses using a direct agar plate method followed by screening for PCB metabolism using a clearing-zone clearing assay. Of the 163 PCB-metabolizing bacteria isolated from root-zone soils and 80 isolates from the rhizoplane, 93% and 100% of them were Gram-positive, respectively. Isolates were taxonomically identified by sequence analyses of 16S rDNA and comparison to the GenBank database. The majority of isolates were identified as members of the genus *Rhodococcus*, while *Arthrobacter oxydans, Bacillus sp., Microbacterium oxydans, Williamsia murale* and three different *Pseudomonas* species were also positively identified. One isolate could only be tentatively identified by its 96% 16S rDNA sequence homology to *Rhodanobacter lindanoclasticus*. Several isolates exhibited high PCB degradation rates and broad congener specificities when subjected to a PCB degradation assay. One isolate, *Rhodococcus* sp. 91B, exhibited the same degradative abilities as the outstanding PCB-degrader, *Burkholderia* sp. LB400. The results indicate that Gram-positive rhodococci are the most abundant taxon of PCB-degrading bacteria present in the root

zones and on the rhizoplane of plants growing *in situ* in contaminated soil and that they harbor outstanding degradative abilities. Thus, rhodococci have been identified as an important group of bacteria in the *in situ* rhizosphere bioremediation of PCB-contaminated soils.

INTRODUCTION

Polychlorinated biphenyls (PCBs) were extensively used by industry worldwide until their production and use was banned due to concerns about their persistence in the environment and toxicity towards animals. In efforts to remediate and restore contaminated soil ecosystems, the use of biodegradation would provide a less expensive and more permanent solution as compared to many conventional cleanup methods such as capping, specialized landfills and incineration. Understanding the diversity and degradative abilities of PCB-degrading bacteria *in situ* at PCB-contaminated sites is a means of evaluating the long-term degradative potential of indigenous microbial communities and of identifying how they may be manipulated to optimize their degradative activity.

Bacteria capable of degrading PCBs are widely distributed in the environment (13), and have been isolated from soils and sediments around the world. The majority of PCB-degraders isolated and studied to date have been Gram-negative, including members of the genera *Pseudomonas, Burkholderia, Alcaligenes, Acinetobacter, Ralstonia, Comamonas, Vibrio, Achromobacter, Agrobacterium, Enterobacter,* and *Flavobacterium* (3, 10, 21, 24, 29, 37, 46). There has been an increasing emphasis on the importance of Gram-positive PCB-degraders, especially *Rhodococcus* spp. (22, 28, 37, 41, 48), and

members of the genera *Arthrobacter, Corynebacterium, Bacillus* and *Paenibacillus* have also been isolated and studied (3, 22, 46).

Although considerable effort has been devoted to investigating the diversity of PCB-degrading bacteria, the distribution and relative abundance of different taxa has not been characterized for environmental samples collected from across a PCB-contaminated site. Of special interest are contaminated sites undergoing natural ecological recovery, as in a study of a PAH-contaminated site (34, 35) where invading plant species have colonized the site and modified the soil microclimate and chemistry by virtue of their physiological processes including evapotranspiration, root turnover and release of root chemicals. Since these processes vary among plant species, it follows that a range of unique root-soil habitats are created beneath a community of plants. In a concurrent study of a naturally-vegetated, PCB-contaminated site, some of these root-zone habitats were found to be more favorable for PCB-degrading organisms than others. Significantly higher numbers of PCB-degrading bacteria were present in the root zones of Austrian pine (*Pinus nigra*) and goat willow (*Salix caprea*) in comparison to other plant species or nonrooted soil, due to an enrichment effect in which a higher percentage of the total bacterial community beneath pine and willow was comprised of PCB-metabolizers (Chapter 2). Although this enrichment effect is noteworthy, the PCB-degradative potential of an indigenous community also depends on the composition of the community since it has been well-established that different PCB-degrading bacteria possess different degradative abilities including congener specificities and reaction rates (2, 3, 14). Thus, it is imperative that evaluation of the degradative potential of indigenous communities include documentation of taxa present, their abundance and degradative properties.

In the present study, the diversity and PCB-degradative abilities of the indigenous PCB-degrading bacteria present in the root zones of the six different plant species are investigated. To our knowledge, this is the first study performed *in situ* at a PCB-contaminated site that examines the PCB-degrading bacterial community associated with the root-zone soil or rhizoplane of plants.

MATERIALS AND METHODS

Site description

The study site was located on the grounds of the Colorlak paint production plant in Staré Město near Uherské Hradiště in south Moravia, Czech Republic. Empty barrels that had contained the commercial PCB mixture Delor 106, which is analogous to Aroclor 1260, were stored in this area from the 1950s to 1988, during which time inadvertent spillage of residual PCBs occurred. The fertility, pH and texture of soil at this site were described previously (Chapter 2).

The area was naturally vegetated with 25 different plant species, including five different tree species, which were growing in soil contaminated with PCBs ranging in concentration from 1-500 mg/kg. The major trees on the site were as follows: a 24-year-old Austrian pine (*Pinus nigra*), a 14-year old ash (*Fraxinus excelsior*), two weeping birch (*Betula pendula*) that were 11 and 13 years old growing adjacent to each other, and a 3-year old black locust (*Robinia pseudoacacia*). The ground was covered with a variety of grasses (*Agropyron repens, Agrostis alba (stolonifera), Calamogrostis epigeois, Lolium perenne)* and forbs (predominantly *Achillea millefolium*). The complete

plant species list and a map of the vegetation have been presented previously in Chapter 1 (25).

Sampling methods

Samples of soil and roots were collected from the root-zones of each different tree species and beneath the grass/forb mixture in 1999 on June 22, August 23-24 and November 21-22, and in 2000 on May 28. Soil was excavated at three depth increments, 0-20 cm, 20-40 cm and 40-60 cm. Soil from each depth increment was homogenized, approx. 50 g was placed in plastic bags for microbial analyses, and approx. 20 g was collected in aluminum foil for determination of PCB concentration. For rhizoplane samples, fine roots (approx. 1 mm dia.) were collected from each depth, loose soil was removed by shaking, and roots with tightly bound soil were stored in plastic bags. Shovels used to excavate were disinfected with ethanol between excavation sites. Samples were kept on ice during transport to the laboratory, and subsequently were stored at 4°C. For an uncontaminated control, samples were also collected from beneath a large birch tree growing elsewhere within the Colorlak factory.

Screening and isolation of PCB-metabolizing bacteria

PCB-metabolizing bacteria were cultured from soil and rhizoplane samples using direct plate count methods modified from Kastner *et al.* (20), with total numbers of replicates for each plant ranging from 30-61for root-zone soil and 10-30 for rhizoplane samples. For root-zone samples, one-gram aliquots of soil were placed in sterile, 50-ml

screw-cap plastic tubes with approximately 3 g of sterile glass beads (5 mm dia.) and 10 ml of 1% w/v sodium pyrophosphate ($Na_4P_2O_7$) solution. For rhizoplane samples, 0.1 g of fresh roots (approx. 1 mm dia.) was extracted in 10 ml solution in sterile, 15-ml screw-cap plastic tubes without glass beads. Tubes were shaken horizontally on a reciprocal platform shaker for 1 hour, and then were allowed to stand upright for 30 min. Serial dilutions were prepared in basal mineral liquid (2.13 g/L Na_2HPO_4, 1.3 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7\ H_20$) and 100 ul was spread-plated in duplicate onto agar plates. For selective growth of PCB-metabolizers, basal mineral agar was prepared using basal mineral liquid and 15 g/L Noble agar (Difco, Detroit, MI) with biphenyl provided as the sole carbon source by sprinkling a few crystals in the lid of the inverted Petri dish. Total culturable bacteria were grown on one-eighth strength plate count agar (2.94 g plate count agar and 13.12 g Bacto agar in 1L basal mineral liquid) in preparation for screening. The rationale for screening total culturable bacteria was to detect any PCB-metabolizing bacteria that needed additional cofactors that would preclude them from growing on minimal medium with biphenyl as the sole carbon source. Basal mineral plates were incubated for 3 weeks and $1/8^{th}$ plate count agar plates were incubated for one week at room temperature, and then all were subjected to a spray assay modified from Sylvestre (45) to screen for colonies that metabolize 4-bromobiphenyl, an inexpensive and readily available analogue of PCBs. A 5% w/v solution of 4-bromobiphenyl in ethyl ether was sprayed onto the agar surface using a TLC-plate sprayer to overlay the plate surface with a thin cloudy film. After additional incubation of 1-2 weeks, zones of clearing formed around colonies capable of metabolizing the compound. Morphologically different colonies that cleared 4-bromobiphenyl from each plant species

were isolated by repeated streaking onto $1/8^{th}$ strength plate count agar, and were again subjected to the spray assay to confirm successful isolation of PCB-metabolizers. Isolates were Gram-stained, and Gram-positive bacteria were also subjected to acid-fast staining. All isolates were then grouped based on colony and cell morphology. One to three representatives of each group were selected for taxonomic identification using 16S rDNA sequence analysis as described below.

Taxonomic identification

Isolates capable of PCB-metabolism were taxonomically identified by 16S rDNA sequence analysis. DNA was extracted from each isolate following growth on full-strength plate count agar using the DNA spin kit for soil (Bio101 Systems, Qbiogene, Carlsbad, CA), which involves cell lysis with a bead-beater followed by DNA adsorption to a matrix, washing and eluting on a spin filter. Although the kit is designed for soil DNA extraction, high-quality, PCR-ready genomic DNA was rapidly obtained from pure cultures using this system. 16S rDNA genes were then amplified by PCR using the primers 27F (5' AGA GTT TGA TCC TGG CTG AG) and 1522R (5' AAG GAG GTG ATC CA[AG] CCG CA) to generate a 1495-bp product (17). DNA extracts were PCR-amplified in duplicate with 50-ul reaction volumes containing 1μL template DNA, 4 μL MgCl2, 5 μL 10X PCR buffer, 1 μL dNTPs, 1 μL of each primer (100 ng/μL), 0.25 μL taq DNA polymerase (5U/ μL), 1.5 μL BSA, 5 μL betain and 30.25 μL H2O. Amplification was carried out in a Perkin-Elmer (Norwalk, Connecticut, USA) model 9700 thermal cycler using 30 cycles of denaturation at 94°C (1 min), annealing at 37°C (2 min) and extension at 72°C (2 min), followed by a final extension at 72°C for 10 min.

The presence and size of amplicons were confirmed by subjecting a 5 μL aliquot to agarose gel electrophoresis, and then the remaining PCR-product was purified with Wizard® PCR Preps (Promega, Madison, Wisconsin, USA) in accordance with the manufacturer's protocols in preparation for cycle-sequencing.

Cycle sequencing was carried out directly on the purified PCR product using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, California, USA) using the forward primers 27F (above), 530F (5' GTG CCA GCA GCC GCG G) and reverse primers 1522R (above) and either 1100R for Gram-positives (5' GGG TTG CGC TCG TTG) or 1069R for Gram-negatives (5' CCA ACA T[TC]T CAC A[AG]C ACG AG) (17). Cycle-sequencing reactions consisted of 3 μL 16s rDNA PCR product, 11 μL H_2O, 3 μL sequence dilution buffer, 1 μL each primer (10 ng/ul) and 2 μL cycle-sequence mix for a final volume of 20 μL. Cycle-sequencing was performed using 25 cycles of denaturation at 96°C (10 sec.), annealing at 50°C (5 sec) and extension at 60°C (4 min). The 20-μL cycle-sequencing products were purified using an ethanol precipitation method by adding them to 80 μL of a mixture of 3.0 μL 3M NaOAc pH 4.6, 62.5 μL 95% ethanol and 14.5 μL H2O, vortexing, allowing to stand for 20 min at room temperature and centrifuging for 20 min, followed by removing supernatants by shaking, allowing pellets to air-dry, then washing with 250 μL 70% ethanol followed by centrifugation for 15 min. Supernatants were again removed by shaking, and pellets were dried by vacuum centrifugation for 15 min. Purified sequencing reactions were run on an ABI Prism 377 automated sequencer (PE Biosystems, Foster City, California, USA). Electropherograms were assembled and edited with Sequencher 3.1 software (GeneCodes, Ann Arbor, Michigan, USA).

Contiguous 16s rDNA sequences were subjected to BLASTn searches of the GenBank database (1) to determine tentative taxonomic identities of the isolates. Homologies in 16S rDNA sequence above 97% are generally considered to be of the same bacterial species (43), thus isolates were positively identified based on nearest matches produced by BLASTn searches when homologies exceeded 97%.

To construct a phylogenetic tree of the PCB-degrading bacteria isolated, phylogenetic analyses were performed using PAUP* 4.b8 (44). Starting trees were obtained using random sequence addition, searched using equally weighted maximum parsimony (11) with TBR (tree bisection-reconnection) branch swapping and MulTrees and Steepest Descent not in effect. Gaps were inserted at positions where insertions-deletions occurred and these areas were treated as missing data in the nucleotide matrix (44).

PCB-degradation assay

The PCB degradation abilities of the isolates were testing following the method of Bedard *et al.* (3). Cells to be tested were grown in liquid PAS medium (3), except for isolates 46 and 70 that would not grow in PAS, which were instead grown on $1/8^{th}$ strength plate count broth (0.63 g/L tryptone, 0.31 g/L yeast extract, 0.13 g/L dextrose) in the presence of biphenyl. Live and heat-inactivated cells (heated at 70°C for 20 min) were assayed in duplicate for their ability to degrade congener mixtures 1B and 2B (3). After incubation by shaking upright on a rotary platform shaker at room temperature for 24 hours, PCBs were extracted using hexane with a small amount of anhydrous sodium sulfate by shaking horizontally for 20-30 min. Disappearance of PCBs was determined

by gas chromatography using a Varian 3300 gas chromatograph equipped with an electron capture detector and a glass column (6 ft [1.83 m] by 4 mm) packed with 1.5% SP-2250 and 1.95% SP-2401 on 100/120 Supelcoport (Supelco, Inc, Bellefonte, PA). Samples were run isothermally at 185°C, with injector and detector temperatures of 250°C and 300°C, respectively, using nitrogen as the carrier gas at a flow rate of approx. 60 ml/min. One microliter of each hexane extract was analyzed in duplicate, peaks were normalized to the internal standard peak (2, 4, 6, 2', 4'-pentachlorobiphenyl) and the readings were averaged and compared to the heat-inactivated controls to calculate percent disappearance of each congener.

RESULTS

Diversity of PCB-metabolizers

In total, 163 PCB-metabolizing bacteria were isolated from root-zone soil samples and 80 from the rhizoplanes associated with six different plant species. The majority of PCB-metabolizing bacteria isolated from both sample types were Gram-positive, representing 93% of the root-zone isolates and 100% of the rhizoplane isolates. All isolates were divided into 19 groups based on their colony and cell morphologies, which included 15 groups of Gram-positives and 4 groups of Gram-negatives. The majority of Gram-positives were acid-fast cocci in chains, and acid-fast cocci in clusters (isolate 22) and non acid-fast cocci in large clusters (isolates 45 and 212) were also isolated. The Gram-negative isolates were all very short rods occurring either in chains (isolate 24) or in pairs (isolates 52A, 70 and 118). One Gram-variable long rod was also isolated (isolate 63). Many isolates, especially the Gram-positives, released a bright yellow

compound during growth in the presence of biphenyl, presumably the biphenyl ring-cleavage product HOPDA (32).

Representatives of each morphological group were subjected to 16S rDNA sequencing, which successfully produced sequences ranging from 1371-1453 bp in length. Sequences were subjected to BLASTn searches of GenBank, and the nearest matches are presented in Table 1. All isolates shared 99-100% sequence homology with their nearest matches with the exception of isolate 70, which was only 96% homologous to its nearest match, *Rhodanobacter lindanoclasticus*. Among the Gram-positive isolates, seven different species of *Rhodococcus* were identified, as well as *Microbacterium oxydans*, *Arthrobacter oxydans*, a *Bacillus* sp. and *Williamsia murale* (Table 1). Three different Gram-negative isolates were identified as *Pseudomonas* spp. and one Gram-negative was tentatively identified as a relative of *Rhodanobacter lindanoclasticus.* Cell morphologies as well as Gram- and acid-fast staining characteristics of the isolates were consistent with known characteristics of each genus (16, 19, 30). *Williamsia murale* is a recently-described representative of a new genus and species of actinomycete (19), and *Rhodanobacter lindanoclasticus* was also first described recently as a new genus and species that belongs to the γ-Proteobacteria (30).

The association of different species of PCB-degrading bacteria with the root-zone soil and rhizoplane of different plant species is presented in Table 2. This table should be regarded as conservative, since certain bacteria may have been present beneath some plants but were not isolated and identified because of financial and time constraints. PCB-degrading rhodococci were ubiquitous, and were isolated from the root-zones of every plant investigated. *Rhodococcus wratislaviensis* represented the most frequently-

isolated PCB-metabolizing bacterium, and was the only species to be found in the root-zone of all plants. Gram-negative PCB-degraders were isolated from the root-zones of grass, locust, pine and willow. All PCB-degraders isolated from the rhizoplane samples were also found in root-zone soils.

A phylogenetic tree (Figure 2) was constructed based on the 16S rDNA sequences of each PCB-metabolizing bacterial species isolated from the site, including root-zone and rhizoplane organisms. The well-characterized PCB-degrading bacterium, *Burkholderia* sp. LB400 (46), is included in the tree for reference and as an outgroup. Numbers on the tree indicate branch lengths (number of bp difference), and reveal that some isolates with different nearest-match organisms on GenBank BLASTn searches (Table 1) are closely related to each other and are likely different strains of the same species, *i.e. Rhodococcus* spp. 35 and 44 and *Pseudomononas* spp. 118 and 24.

PCB-Degradative abilities of isolates

The PCB-degradative abilities of 12 different isolates and two well-characterized reference strains, *Burkholderia* sp. LB400 and *Rhodococcus* sp. RHA1, are reported in Figure 1. All isolates were capable of metabolizing some congeners with both the 2,3 and 3,4 sites open. Many of the isolates displayed 2,3-dioxygenase activity as evidenced by their metabolism of congeners with open 2,3 sites and inability to metabolize congeners with only the 3,4 sites open. One isolate, *Rhodococcus* sp. 91B, exhibited both 2,3 and 3,4-dioxygenase activities almost exactly like the reference strain, *Burkholderia* sp. LB400 (2). *Rhodococcus* sp. 108 was capable of metabolizing the highly recalcitrant congener 2,4,5,2',4',5'-chlorobiphenyl, in which both the 2,3 and 3,4 sites are blocked. This congener is only degraded by exceptional strains including LB400 (46), which has

been reported to catalyze 40-59% disappearance of this congener but in our hands degraded only up to 7%. Interestingly, isolates 48 and 59 exhibited different PCB degradative abilities, despite their being classified as the same *Rhodococcus* sp. by only having one base-pair difference (0.1%) in their 16S rDNA sequences (Table 1). Overall, the isolate with the broadest congener specificity and highest degradative rate was *Rhodococcus* sp. 91B, which surpassed the degradative properties of *Rhodococcus* sp. RHA1 and closely resembled those of LB400 towards the mixtures of PCBs examined.

The degradative capabilities of several isolates, including the outstanding degrader *Rhodococcus* sp. 91B, were reduced or completely lost following storage at -20°C in 16% glycerol or at 4°C on slants prepared from $1/8^{th}$ plate count agar with biphenyl provided, similar to the behavior reported previously for other PCB-degrading bacteria (28). Unfortunately in some cases, the activity loss occurred before PCB degradation assays could be conducted.

DISCUSSION

Fifteen different species of PCB-degrading bacteria were isolated and identified from the root-zones and rhizoplanes of six different plant species growing at a PCB-contaminated site. The isolates included members of the genera *Arthrobacter, Bacillus, Microbacterium, Pseudomonas, Rhodococcus* and *Williamsia*, and one isolate tentatively identified as a relative of *Rhodanobacter lindanoclasticus*. PCB degradation has previously been observed in species of *Arthrobacter* (13), *Bacillus* (18, 40, 42), *Pseudomonas* (6, 10, 13, 29, 37, 46) and *Rhodococcus* (including strains previously identified as *Corynebacterium* spp.) (6, 22, 37, 41, 46, 50) however to our knowledge;

this is the first report that species of *Microbacterium*, *Williamsia* or *Rhodanobacter* are capable of PCB metabolism. Among the microorganisms in GenBank that shared the greatest 16S rDNA sequence homology to our isolates, none were previously reported to degrade PCBs. However, isolates 25, 44, 58 and 26 exactly matched the much shorter, partial (933 bp) 16S rDNA sequence of *Rhodococcus* sp. RHA1, a well-characterized PCB-degrading bacterium (28, 41). Several closely matching organisms were capable of metabolizing other recalcitrant environmental contaminants. The *Rhodococcus* species that shared 100% homology with isolate 20 was a hexadecane-degrader isolated from oil-contaminated marine sediment (AY249053). Also, our isolate 91B exactly matched a nitrile-metabolizing *Rhodococcus* sp. (AF420413) isolated from cultivated soil (7), and isolate 70 shared 96% homology with *Rhodanobacter lindanoclasticus*, a γ-proteobacterium capable of using lindane as a sole carbon source (30).

The Gram-positive rhodococci were the most abundant culturable PCB-degrading bacteria throughout the site, representing 93% of root-zone isolates and 100% of rhizoplane isolates. These results indicate that the Gram-positive rhodococci are the most significant taxon of PCB-degraders present *in situ* in contaminated soil and in the root-zones of a variety of plants at this contaminated site. Although the diversity of PCB-degrading bacteria in contaminated soils has often been investigated, this is the first report of the relative abundance of different taxa in the environment. Our findings that Gram-positive PCB degraders are the most numerous is surprising considering that many studies of the diversity of PCB-degraders have focused on Gram-negative bacteria, which may be due to a bias imposed by the methodologies used to culture and isolate PCB-degraders. The majority of studies of the diversity and degradative abilities of PCB-

degrading bacteria in the environment have been performed by collecting composite samples from a site, creating enrichment cultures with biphenyl, and then isolating and characterizing members of the enriched community (6, 10, 29, 37, 41, 46), an approach which may result in an overemphasis on taxa that grow rapidly in liquid enrichment culture (*i.e.* Gram-negative pseudomonads) over other taxa (Gram-positives) that may be more prevalent in the environment. This approach has often yielded an abundance of Gram-negative PCB-degraders, however some Gram-positives have also been isolated in this way (6, 10, 37, 41, 46). The direct agar-plate method we employed was advantageous because it permitted the detection of different taxa of PCB-degraders regardless of their growth rates, while also providing indications of their relative abundance.

Several of the isolates exhibited outstanding PCB-degradative abilities relative to well-characterized reference strains. The isolate with the broadest congener specificity was *Rhodococcus* sp. 91B, which performed comparably to a reference strain considered one of the most outstanding PCB-degraders known, *Burkholderia* sp. LB400 (2). *Rhodococcus* sp. 108 had a narrower congener specificity, but degraded several congeners at higher rates than LB400 or 91B and was the only strain able to appreciably metabolize the highly recalcitrant congener 2,4,5,2',4',5-chlorobiphenyl. The two *Rhodococcus* isolates, 91B and 108, as well as *Pseudomonas* sp. 24 exceeded the degradative performance of the reference strain, *Rhodococcus* sp. RHA1 in our experiments. These results indicate that bacteria naturally present in contaminated soil and associated with the roots of various plant species are capable of degrading a wide range of PCB congeners.

In a concurrent study at this site, PCB-degraders were found to be enriched in the root-zones of pine and willow, resulting in significantly higher numbers of degraders than in the root-zones of other plant species or in nonrooted soil (Chapter 2). Rhodococci were found to be abundant in the root zones of each plant species examined at the site, including pine and willow (Table 2), indicating that they are a major component of the PCB-degrading population even when enriched beneath certain plants. Rhodococci are considered to be ubiquitous in many environments and are a significant part of the soil microbial community (49). Various species of *Rhodococcus* have previously been isolated from the rhizospheres of birch, alder and fescue (9), a variety of desert and crop plants (38) and rice in flooded paddies (47). The rhizoplanes of several plants including pea, Indian mustard (4), and papyrus (39) have also been reported to harbor rhodococci.

The enrichment of PCB-degrading bacteria, including the rhodococci, observed in the root zones of pine and willow at this site (Chapter 2) is proposed to be due to the release of substrates like phenolics, flavonoids and terpenoids from these plants since laboratory studies have shown that with these classes of plant compounds support the growth and degradative activity of PCB-degrading bacteria. Flavonoids and other plant phenolics released from plant roots have previously been demonstrated to support the growth and degradative activities of PCB-degrading bacteria in culture (8, 12, 26), including *Rhodococcus* sp. MB1 (previously identified as *Corynebacterium* sp. MB1) (8), and terpenoids have been shown to support the growth of PCB degraders as well (51). In two previous reports, Gram-positive rhodococci were found to be enriched in artificial soil microcosm systems in response to the addition of certain substrates. Rhodococci in PCB-contaminated sediment microcosms were enriched over the course of 6 months by

the addition of biphenyl (48), and in another microcosm study of soil spiked with PCBs, the addition of terpene-rich plant residues (orange peel, pine needles, etc.) enhanced numbers of PCB-degraders that were identified as members of the genera *Rhodococcus*, *Corynebacterium* and *Cellulomonas* (15).

Rhodococci have previously been recognized as a potentially valuable taxon for the biodegradation of a wide variety of environmental contaminants, including many simple hydrocarbons, chlorinated hydrocarbons, aromatic hydrocarbons and nitroaromatics, as well as PCBs (5, 49). In addition to their broad catabolic abilities, rhodococci have a number of other traits making them attractive for bioremediation. Rhodococci are hydrophobic because of aliphatic chains of mycolic acids in the cell wall, which may assist in the degradation of hydrophobic pollutants by allowing cells to adhere to oil/water interfaces (31). Some rhodococci also produce surfactants that facilitate uptake of hydrophobic contaminants (5, 23), a step in biodegradation that is often rate-limiting. In the environment, rhodococci are autochthonous and generally exhibit a K-type strategy, characterized by slow growth but great persistence in the environment, including under conditions of starvation (49). Rhodococci, unlike pseudomonads and other Gram-negative bacteria, also do not seem to exhibit catabolite repression in the biodegradation of aromatics (49), which would make them advantageous in rhizoremediation where an array of root-released substrates would also be present.

Examination of root-zone samples from this PCB-contaminated site showed that the rhodococci were the most prominent group of PCB-degrading bacteria beneath all of the species examined. Thus, introduction and cultivation of a dense stand of selected tree species at a contaminated site may create a widely distributed, favorable habitat for a

community of PCB-degrading bacteria dominated by rhodococci. Fundamental to the concept of plant-driven rhizoremediation is the knowledge that root systems of perennial plants continuously explore new regions of the soil (33, 36) and release organic chemicals through root turnover that function as substrates for degradative bacteria (26), potentially enriching the microbial community with organisms that degrade organic soil pollutants. Recognizing the prominence of rhodococci in the root zones of the plants growing at this PCB-contaminated site provides incentive to examine more closely the interactions between chemicals released by selected tree species (*i.e. Pinus nigra* and *Salix caprea*) and the associated microbial community.

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TABLE 1. Results of GenBank BLAST searches of 16S rDNA sequences obtained from PCB-degrading bacterial isolates.

Isolate	No. of bp sequenced	Nearest GenBank match		No. of bp different	% Similarity
		Species name	Accession no.		
Gram-negative					
24	1453	*Pseudomonas* sp.	AY263468	3	99.8
118	1431	*Pseudomonas* sp.	AY263482	5	99.7
52A	1387	*Pseudomonas* sp.	AF500621	2	99.9
70	1441	*Rhodanobacter lindanoclasticus*	AF039167	57	96.0
Gram-positive					
22	1375	*Arthrobacter oxydans*	X83408	3	99.8
63	1444	*Bacillus* sp.	AY131222	1	99.9
46, 212	1435	*Microbacterium oxydans*	Y17227	7	99.5
211, 100, 108	1394	*Rhodococcus erythreus*	X789289	0-7	99.5-100
48, 59	1446	*Rhodococcus wratislaviensis*	Z37138	0-1	99.9-100
35	1371	*Rhodococcus* sp.	AF420421	5	99.9
44, 58, 26, 204	1391	*Rhodococcus* sp.	X80616	0-4	99.5-100
20	1426	*Rhodococcus* sp.	AY249053	0	100.0
91b	1404	*Rhodococcus* sp.	AF420413	0	100.0
16	1408	*Rhodococcus* sp.	AB023374	13	99.1
45	1443	*Williamsia murale*	Y17384	14	99.0

TABLE 2. The presence of PCB-degrading bacteria in the root zones and on the rhizoplanes of different plant species growing in PCB contaminated soil.

Identity of PCB-degrading isolates	Isolate on phylogenetic tree	Isolated from: Ash	Birch	Grass	Locust	Pine	Willow
Gram (-)							
Pseudomonas sp.	24				RZ		RZ
Pseudomonas sp.	52A			RZ			RZ
Pseudomonas sp.	118					RZ	
Rhodanobacter sp.	70						RZ
Gram (+)							
Arthrobacter sp.	22				RZ		
Bacillus sp.	63			RZ			
Microbacterium oxydans	46	RZ	RZ, RP	RP		RP	
Rhodococcus erythreus	211	RZ (RP)	(RZ, RP)	(RZ, RP)	RZ, RP		
Rhodococcus wratislaviensis	48	RZ	RZ	RZ	RZ	RZ	RZ
Rhodococcus sp.	35					RZ	
Rhodococcus sp.	44	RZ, RP	(RP)	(RP)	(RZ)		RZ
Rhodococcus sp.	20	(RZ)			RZ		(RZ)
Rhodococcus sp.	91B		RZ	RZ			RZ
Rhodococcus sp.	16	RZ					
Williamsia murale	45	RZ					

Key: RZ = isolated from the root-zone soil, RP = isolated from the rhizoplane. Parentheses denote locations from which a bacterium was isolated that was tentatively identified based on morphological similarity to isolates that had been positively identified by 16S rDNA sequence analysis.

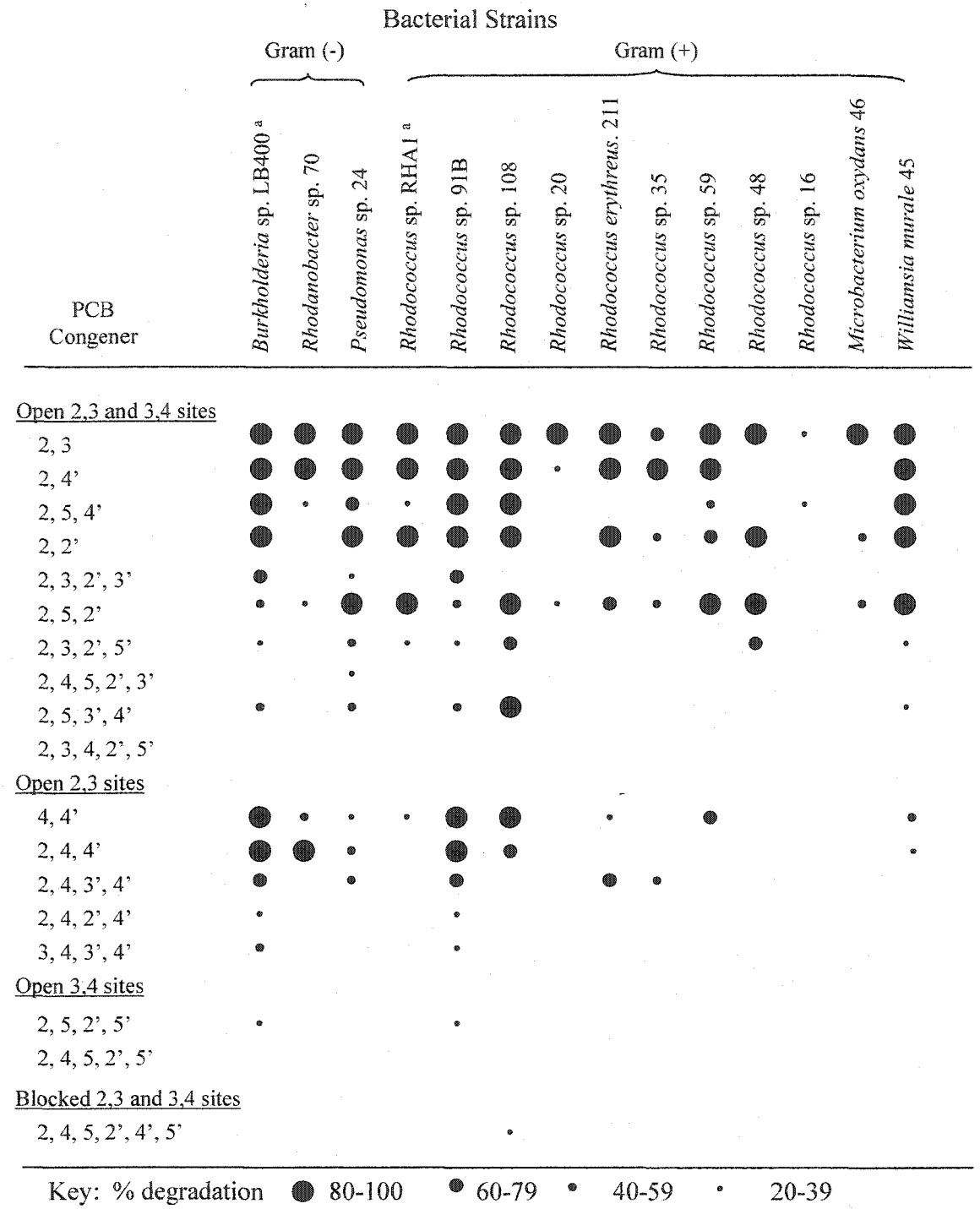

FIGURE 1. PCB degradative abilities of bacteria isolated from the root zone or rhizoplane of plants growing in PCB-contaminated soil. Reference strains *Burkholderia* sp. LB400 and *Rhodococcus* sp. RHA1 are included for comparison.

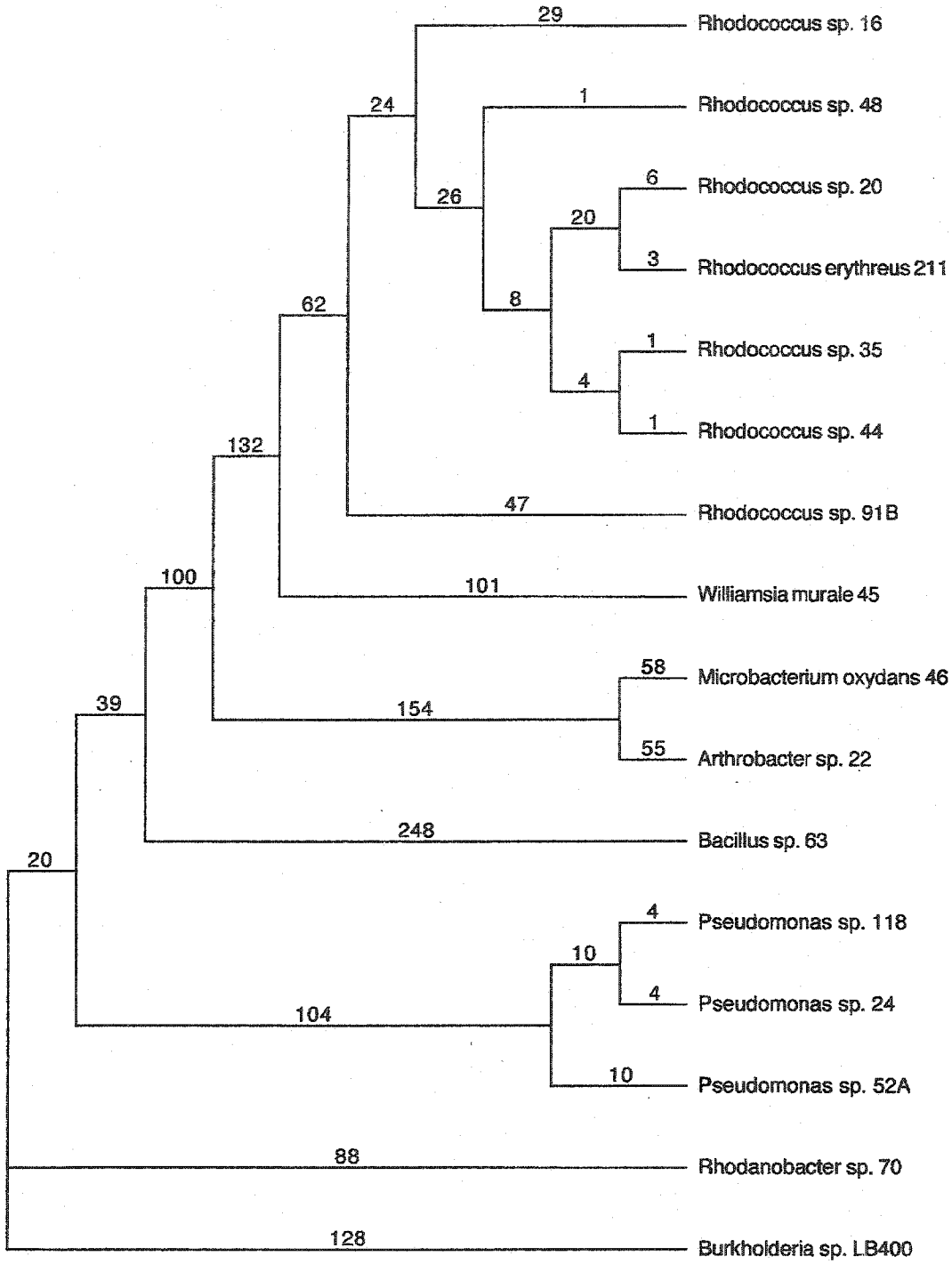

FIGURE 2. Phylogenetic tree of PCB-degrading bacterial isolates based on 16S rDNA sequences. Numbers on the lines indicate branch lengths (number of base-pair differences). The reference strain *Burkholderia* sp. LB400 is included for comparison.