Phytoremediation of pentachlorophenol by crested wheatgrass (Agropyron cristatum L.)
by Erica Kathleen Miller

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Soils
Montana State University

Abstract:
Pentachlorophenol (PCP), a potent biocide used mainly for wood preservation, occurs widely as a soil and water contaminant due to point source pollution and leaching from treated lumber (United States Environmental Protection Agency, 1998). It is estimated that PCP contaminates 700 sites across the United States (Mueller et at. 1989), presenting site owners with substantial cleanup costs. Phytoremediation, or the use of plants to ameliorate soil and water contamination, is an emerging and potentially cost-saving technology (Cunningham et al. 1996). Previous research showed that the presence of 'Hycrest' crested wheatgrass (CWG) (Agropyron cristatum [L.] Gaertn. X A. desertorum [Fischer ex Link] Schultes) accelerated degradation of PCP in contaminated soil (Ferro et al. 1994) and that sensitive plants were able to grow in contaminated soil after seed inoculation with a PCP-degrading Pseudomonas sp. (Pfender 1996). In my work, greenhouse studies using Pine Tree Timber (PTT) site soil contaminated with 30 mg kg^-1 PCP showed that soil planted with CWG contained less PCP than unplanted soil 13 weeks after seeding. 'Hycrest' CWG seedlings inoculated with PTT soil-derived inoculum grew in sterile sand containing 1.5- to 3-fold higher PCP concentrations than uninoculated sterile seedlings. 14C-labeled PCP was used to examine 14C distribution in sealed flow-through systems in sterile, inoculated or autoclaved inoculum-treated CWG seedlings plus unplanted controls. Systems treated with PTT soil inoculum, both planted and unplanted, mineralized the greatest amount of PCP to 14CO2, while all sterile treatments had similar, low levels of mineralization. Further studies on the PTT soil inoculum revealed that the number of PCP-degrading organisms increased 100-fold in the presence of CWG root exudates. These results support the hypothesis that indigenous soil microorganisms can mineralize PCP and are stimulated in the CWG rhizosphere.

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Master of Science

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MONTANA STATE UNIVERSITY—BOZEMAN
Bozeman, Montana

October 1999

APPROVAL

of a thesis submitted by

Erica Kathleen Miller

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ACKNOWLEDGMENTS

I sincerely thank my advisor, Dr. Bill Dyer, for allowing me the freedom to explore my own interests and supporting me in those endeavors. I am grateful to my committee members, Dr. Bill Inskeep, Dr. Tim McDermott and Dr. Warren Jones, for their direction and assistance. In addition, I am grateful to the Center for Biofilm Engineering for their endorsement of my research. I also sincerely thank the United States Environmental Protection Agency for their support of my research through the Science to Achieve Results (STAR) Graduate Fellowship Program.

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ABSTRACT

Pentachlorophenol (PCP), a potent biocide used mainly for wood preservation, occurs widely as a soil and water contaminant due to point source pollution and leaching from treated lumber (United States Environmental Protection Agency, 1998). It is estimated that PCP contaminates 700 sites across the United States (Mueller et al. 1989), presenting site owners with substantial cleanup costs. Phytoremediation, or the use of plants to ameliorate soil and water contamination, is an emerging and potentially cost-saving technology (Cunningham et al. 1996). Previous research showed that the presence of 'Hycrest' crested wheatgrass (CWG) (*Agropyron cristatum* [L.] Gaertn. X *A. desertorum* [Fischer ex Link] Schultes) accelerated degradation of PCP in contaminated soil (Ferro et al. 1994) and that sensitive plants were able to grow in contaminated soil after seed inoculation with a PCP-degrading *Pseudomonas* sp. (Pfender 1996). In my work, greenhouse studies using Pine Tree Timber (PTT) site soil contaminated with 30 mg kg^{-1} PCP showed that soil planted with CWG contained less PCP than unplanted soil 13 weeks after seeding. 'Hycrest' CWG seedlings inoculated with PTT soil-derived inoculum grew in sterile sand containing 1.5- to 3-fold higher PCP concentrations than uninoculated sterile seedlings. ^{14}C-labeled PCP was used to examine ^{14}C distribution in sealed flow-through systems in sterile, inoculated or autoclaved inoculum-treated CWG seedlings plus unplanted controls. Systems treated with PTT soil inoculum, both planted and unplanted, mineralized the greatest amount of PCP to $^{14}CO_2$, while all sterile treatments had similar, low levels of mineralization. Further studies on the PTT soil inoculum revealed that the number of PCP-degrading organisms increased 100-fold in the presence of CWG root exudates. These results support the hypothesis that indigenous soil microorganisms can mineralize PCP and are stimulated in the CWG rhizosphere.

CHAPTER 1

LITERATURE REVIEW

Introduction

Since the dawn of the Industrial Revolution, vast quantities of carbon-based contaminants have been released into our environment. Pentachlorophenol (PCP), a priority pollutant designated by the United States Environmental Protection Agency (U.S. EPA) (Keith and Telliard 1979) and one of the most widely used biocides contaminates an estimated 700 sites across the United States (Mueller et. al 1989). Today, the only registered use of PCP in the United States is as a 3% to 5% solution to preserve wood products, such as fence posts, utility poles, and cross arms (Kroschwitz and Howe-Grant 1993). Prior to a 1987 ban on over-the-counter uses, PCP was used as a bean seed treating agent, a post- and pre-emergence herbicide, a preservative for glues, starches, paints and photographic papers, an antimicrobial agent in pulp and paper processing, a mold inhibitor in finished yarn, cloth and leather (U.S. EPA 1999) and as an additive in food wrapping paper (Crosby 1981).

The majority of soil and water contamination by PCP occurs as a result of spillage and drippage of treatment solution during wood treatment, and from leaking storage tanks. The U.S. EPA reports that between 1987 and 1993, 45,360 kg of PCP were released into the environment (U.S. EPA 1999). A draft report by the Montana Department of Agriculture (Rise, unpublished) showed that PCP was detected in 15 of 1,812 Montana ground water samples since monitoring began in 1984. Six of the 15

samples exceeded the maximum contaminant level for PCP determined by the EPA, which is 0.001 mg/L or 1 ppb.

Currently, soils contaminated with organic xenobiotics such as PCP are excavated and incinerated, landfilled, or landfarmed, or may be treated by thermal desorption, soil washing or stabilized by addition of cement (Cunningham et al. 1995; Cunningham et al. 1996). All of these techniques require massive soil excavation and manipulation, which are very costly. Landfill space is quickly diminishing (McBean and Rovers 1999) and transport, incineration, and chemical or heat stripping costs can be exorbitant (Cunningham et al. 1996). Owners of PCP-contaminated sites are often not directly responsible for the contamination since it may have occurred decades ago (Reynolds, personal communication). Phytoremediation offers an attractive alternative for several reasons: 1) if the contamination is shallow, plants can be seeded directly for *in situ* treatment resulting in considerable cost savings; 2) plants stabilize contaminated soil by reducing wind and water erosion and reduce leaching through transpiration; and 3) a planted site is more aesthetically pleasing to the public than methods that purposely defeat plant establishment such as landfarming (Cunningham et al. 1996; Cunningham and Berti 1993).

PCP Soil Chemistry

It is important to understand the chemistry of PCP (Table 1) to predict how it will interact with soils, plants and microorganisms in the environment. The lower log K_{ow} value that Crosby (1981) reported likely results from analysis of the pentachlorophenate

anion instead of the protonated form, which solely depends on solution pH. Chemicals with log K_{ow} values less than 0.5 are considered water-soluble and those greater than 3.0 are considered hydrophobic (Schnoor et al. 1995). Therefore, even if PCP is predominantly in its anionic form (pH > 5), it is still moderately hydrophobic (U.S. EPA 1999). Despite its hydrophobicity, however, PCP can be very mobile in alkaline and porous mineral soils, especially when soil water percolation is significant (Chrisodoulatos and Mohiuddin 1996; Christodoulatos et al. 1994; Rise, personal communication).

Table 1. Physical Properties of Pentachlorophenol as Presented by Three Authors.

	U.S. EPA (1999)	Schellenberg, et al. (1984)	Crosby (1981)
log K_{ow}	5.12	5.24	2.15
log K_{oc}	3.48-3.60	4.52	--
pK_a	4.74	5.25	4.70
Solubility (30°C)	0.02 g/L	--	0.02 g/L
Vapor Pressure (20-25 °C)	0.00011 mm Hg	--	0.00017 mm Hg

PCP sorption to soil increases: 1) as soil organic matter (SOM) increases, due to partitioning (Schellenberg et al. 1984); 2) as soil pH decreases, due to protonation of the pentachlorophenate anion resulting in reduced solubility (Bellin et al. 1990); and 3) as soil surface area increases, due to an increased number of sorption sites (Christodoulatos et al. 1994). Sorption also depends on the presence of cosolvents, such as petroleum hydrocarbons, in the soil solution. Such cosolvents can greatly increase the leaching potential of PCP through the soil profile (Lee et al. 1990). The Fruendlich model, a nonlinear partition model, best describes PCP sorption to soil, implying that PCP sorbs to various sites with varying degrees of affinity (Christodoulatos et al. 1994; Bellin et al. 1990). PCP becomes increasingly irreversibly bound to the soil as it ages, while the

amount of transiently bound PCP decreases as degradation or covalent modifications take place (Alexander 1999). Only labile PCP is considered available for biodegradation, thus low pH soils may exhibit lower rates of PCP degradation (Edgehill 1996; Bellin et al. 1990; Lafrance et al. 1994; Brown et al. 1986). Kuwatsuka and Igarashi (1975) showed that PCP degradation was positively correlated with SOM content for ten soils, indicating that both microbial activity and SOM are critical for PCP degradation. SOM may act to reduce PCP toxicity by reducing its bioavailability, as several studies have shown that the presence of non-substrate sorptive material can enhance PCP degradation in liquid culture (Apajalahti and Salkinoja-Salonen 1984; Barbeau et al. 1997).

PCP Toxicity

PCP's biocidal properties are a result of its ionizable hydroxyl group and its hydrophobic chlorinated aromatic ring. PCP was found to uncouple oxidative phosphorylation at low concentrations (3 to 8 μM) and inhibit electron transfer at higher concentrations (500 μM), as shown in isolated potato (*Solanum tuberosum* L.) mitochondria (Ravanel and Tissut 1986). The pentachlorophenate anion, which predominates at pH > 5, efficiently shuttles H^+ ions across the mitochondrial membrane, uncoupling oxidative phosphorylation. The five substituted chlorine atoms decrease the pK_a of the hydroxyl group relative to lesser chlorinated phenols (pK_a of PCP = 4.74, pK_a of phenol = 9.1) and increase the hydrophobicity of the molecule (log K_{ow} of PCP = 5.19, log K_{ow} of phenol = 1.49). In potato, PCP inhibited electron transport near the quinone pool and in complex II of the electron transport chain (Ravanel and Tissut 1986). The

authors suggested that plants might resist PCP toxicity by rapid detoxification, binding of PCP by plant structural tissue, or by limiting uptake of PCP, especially into sensitive areas such as the mitochondria and chloroplast.

PCP has demonstrated toxicity to reproductive systems in mink and sheep (Beard et. al 1997; Rawlings et. al 1998). Mink fed 1 mg PCP per kg body weight per day experienced decreased fertility, and ewes fed 2 mg kg^{-1} PCP twice a week for 43 days showed an increased incidence of oviductal intraepithelial cysts. In addition, the oral lethal dose (LD_{50}) to 50% of male and female rats, in mg kg^{-1} body weight, is 146 and 175, respectively (Gaines 1969), as compared to phenol which has an LD_{50} (orally in rats) of 530 mg kg^{-1} body weight (Deichmann and Witherup 1944). Clearly, soil and water contamination by PCP presents a serious health hazard in our environment.

Octachloro-, heptachloro- and hexachloro-*p*-dibenzodioxins and -dibenzofurans are often present as trace impurities in technical grade PCP (Crosby 1981), which are themselves highly toxic. The LD_{50} of the most toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), in male and female rats is 0.022 and 0.045 mg kg^{-1} body weight, respectively (Schwetz et al. 1973). While not a focus of this thesis, it is important to note the occurrence of these highly toxic and recalcitrant compounds along with PCP and its various hydrocarbon carriers at contaminated sites.

Microbial Degradation of PCP

Fungal Degradation of PCP

Some species of white-rot fungi are known to degrade lignin, the major complex polyphenolic component of wood (Okeke et al. 1997). Therefore, it seems reasonable that several species are also able to mineralize the related phenolic compound PCP. The lignin-degrading fungi *Phanerochaete chrysosporium*, *Phanerochaete sordida*, and *Trametes hirsuta* mineralized over 50% of the PCP in soil at a wood preserving facility in Mississippi by 56 days after inoculation (Lamar and Evans 1993). *P. sordida*, inoculated as fungal biomass on a standard grain-sawdust substrate at 10% (w/w, dry basis) and amended with 2.5% wood chips, decreased soil PCP concentration by 89% after 56 days. Furthermore, the fungi *Lentinula edodes* ("shiitake" mushrooms) and *Trametes versicolor* decreased PCP concentration to <1% and 4% of the initial concentration after 10 weeks and 42 days, respectively, when inoculated into sterilized soil (Okeke et al. 1997; Toumela et al. 1999). Despite these positive results, white-rot fungi have not gained widespread acceptance as a bioremediation alternative, most likely due to their inability to compete with indigenous organisms, the relatively large amount of incorporated fungal biomass required for efficient PCP removal and the formation of chloroanisoles by fungal metabolism (Toumela et al. 1999).

Many organisms such as *Phanerochaete* sp. rapidly detoxify their environment through PCP methylation to create pentachloroanisole (PCA), the volatile methyl ether of PCP (Lamar and Dietrich 1990; Toumela et al. 1999). PCA is a "dead end" metabolite of PCP, since it is believed that PCA must first be converted back to PCP before it can be

mineralized (Boyd et al. 1989) and production of PCA transfers the pollution from the soil to the atmosphere. Such pollution is limited by laws such as the Clean Air Act (U.S EPA 1998).

Bacterial Degradation of PCP

Watanabe (1973) isolated a PCP-degrading organism most likely of the genus *Pseudomonas* from a Japanese paddy soil where PCP was used as a herbicide for many years. Suzuki (1977) isolated another *Pseudomonas* sp. from paddy soil that mineralized 50% of PCP (45 mg L^{-1}) in the growth medium in 1 hour. The author reported trace amounts of tetrachlorohydroquinone (TCHQ) and tetrachlorocatechol (TCC) metabolites resulting from bacterial catabolism, indicating that both aerobic and anaerobic degradation may have been occurring. Stanlake and Finn (1982) isolated several strains of PCP-degrading *Arthrobacter* spp. and studied their growth kinetics on various carbon substrates. Two of the five cultures required yeast extract and one required biotin for growth on simple substrates, indicating that they required cofactors or vitamins to metabolize even simple carbon compounds. In addition, prior exposure of a soil to PCP was not necessarily required for PCP-degrading organisms to be present (Stanlake and Finn 1982).

One especially well-characterized microorganism was at first thought to be a *Flavobacterium* species, but has since been reclassified as *Sphingomonas chlorophenolica* (strain ATCC 39723). Isolated by Saber and Crawford (1985), this organism's PCP degradation pathway is relatively well described (Figure 1). A PCP 4-monooxygenase (PcpB) hydroxylates PCP at the *para* position producing 2,3,5,6-

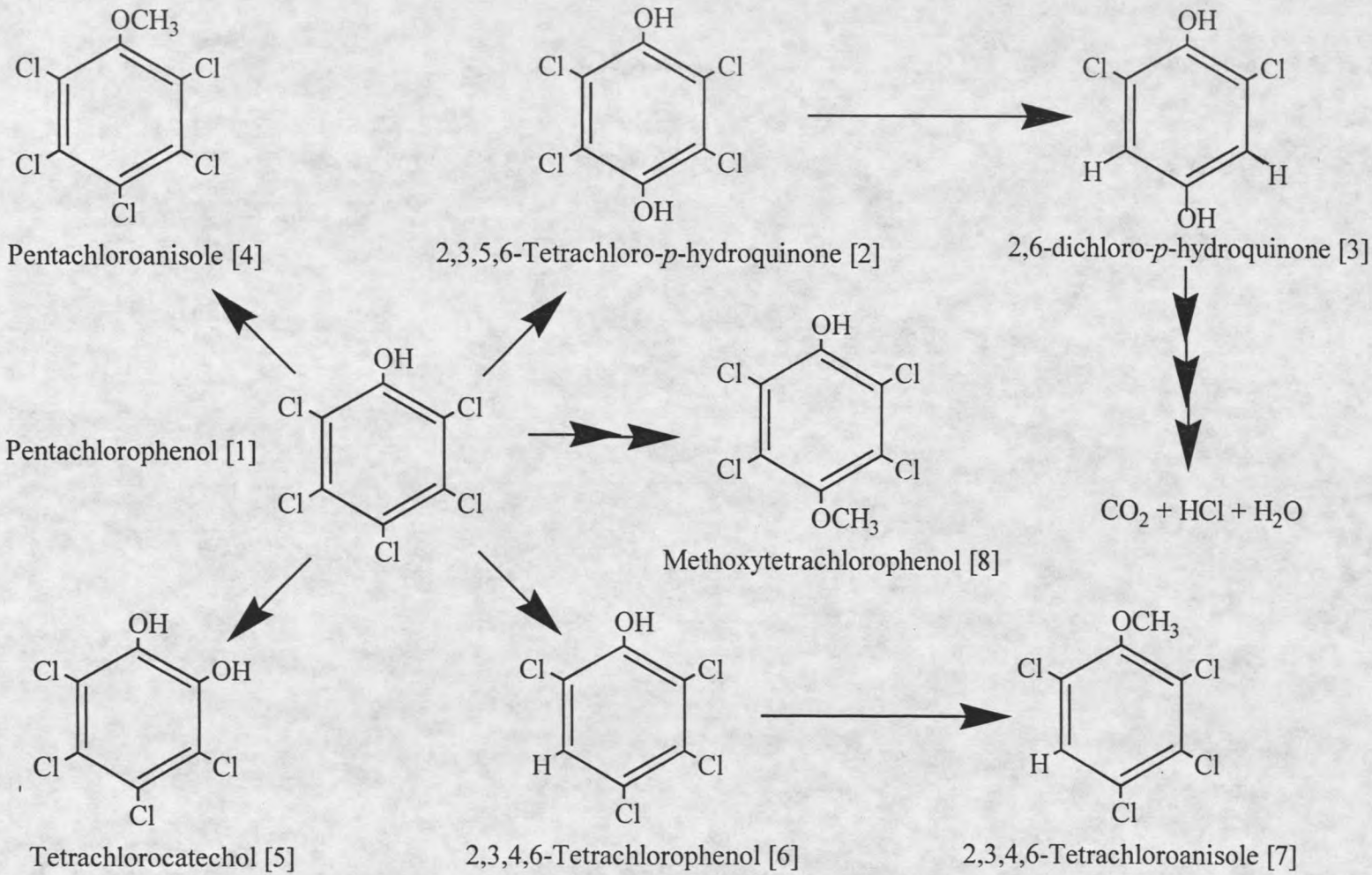

Figure 1. Possible Metabolic Pathways for PCP in *Sphingomonas chlorophenolica* (strain ATCC 39723) and Other Microorganisms. Numbers in brackets are used to refer to metabolites in the text.

tetrachloro-*p*-hydroquinone [2] (Xun and Orser 1991*b*; Xun et al. 1992; Orser et al 1993*b*), then a reductive dehalogenase (PcpC) dechlorinates the molecule twice more yielding 2,6-dichloro-*p*-hydroquinone [3] (Lee and Xun 1997). A third enzyme (PcpA) implicated in the PCP catabolic pathway likely continues PCP mineralization by attacking 2,6-dichloro-*p*-hydroquinone, possibly yielding a ring fission product (Chanama and Crawford 1997). Genes for these three enzymes have been cloned (Xun and Orser 1991*a*; Orser et al. 1993*a*; Orser et al. 1993*b*), allowing Saboo and Gealt (1998) to use DNA hybridization techniques to test for the presence of PCP degraders in contaminated soil. DNA from two bacterial isolates capable of growth in the presence of PCP hybridized to *pcpB* probe sequences, and yet the organisms did not degrade PCP. It thus appears that nucleic acid hybridization and/or growth in the presence of PCP are insufficient evidence to confirm PCP-degrading capabilities (Saboo and Gealt 1998).

PCP degradation has been shown to occur faster in anaerobic soils than aerobic soils (Kuwatsuka and Igarashi 1975; Reddy and Sethunathan 1983); however, no PCP-degrading anaerobic organisms had been isolated by 1988 (Häggblom, et al. 1988). Several mixed cultures that anaerobically degrade PCP were found to reductively dechlorinate PCP at the *ortho* position as the first or second metabolic step (Stuart and Woods 1998; Larsen et al. 1991). In contrast, aerobic degradation of PCP generally proceeds via hydrolytic *para* dechlorination, forming the metabolite *p*-tetrachlorohydroquinone [2], as seen in *Rodococcus chlorophenolicus* (Apajalahti and Salkinoja-Salonen 1987), *Arthrobacter* sp. strain ATCC 33790 (Schenk et al. 1989), and *Flavobacterium* (*Sphingomonas*) sp. strain ATCC 39723 (Steiert and Crawford 1986).

Several aerobic mixed cultures that degrade PCP have been studied. Microbial biofilms on the surface of rocks collected from a man-made channel of the Mississippi River degraded PCP as well as *Sphingomonas chlorophenolica* strain ATCC 39723 for most levels of PCP tested (Brown et al. 1986). PCP degradation rates by the microbial consortium were 10- to 100-fold lower in the absence of rocks, indicating greater stability of the consortium as a biofilm attached to rock surfaces. Karamanev and Samson (1998) observed that a mixed culture of PCP-degraders was much less affected by environmental stresses, such as temperature or pH variations, when present as a biofilm in an immobilized soil bioreactor, as compared to planktonic cultures. Another study reported a mixed culture that degraded PCP contained three predominant strains: *Flavobacterium gleum*, *Agrobacterium radiobacter* and a *Pseudomonas* sp. (Yu and Ward 1996). The individual organisms degraded PCP as pure cultures, but the original mixed culture and a mixture of the three pure cultures exhibited the highest rates of complete degradation. Tetrachlorophenol [6] was observed when the strains were grown separately on PCP, but metabolites were absent in cultures containing all three organisms.

Phytoremediation

The term "phytoremediation" unites two languages: the Greek, phyto-, meaning "plant" and Latin, –remedi(um), meaning "to heal". Taken together, the term phytoremediation can be literally defined as "the use of plants to heal". More generally, the term is used to describe the use of plants to decontaminate polluted soil or water. Using plants to remove harmful chemicals from soil and water is not a new idea, although

phytoremediation has only recently emerged as a scientific discipline. In Europe, industrial wastewater has been treated using reed beds since the 1960s (Kadlec 1999), and farmers have used crop plants to ameliorate relatively small pesticide spills (Cunningham et al. 1996).

Plants are capable of soil and water decontamination by two primary mechanisms: phytodecontamination and phytostabilization (Cunningham et al. 1995). Phytodecontamination mechanisms remove the contaminant from the soil, while phytostabilization reduces the toxicity and mobility of the soil contaminant by sequestration or precipitation in the soil matrix. Phytodecontamination can be further subdivided into phytoextraction, rhizofiltration, phytovolatilization and rhizostimulation. Phytoextraction removes pollutants, such as the heavy metals Co, Cu, Cr, Pb, Ni, Zn and Mn, from soil by hyperaccumulation of the metal in plant tissue (defined as greater than 1,000 μg metal/g dry plant matter or 10,000 μg/g for Mn and Zn) (Kumar et al. 1995; Baker and Brooks 1989). Similarly, rhizofiltration describes the process by which plant roots, especially from *Brassica* spp. and sunflower (*Helianthus annuus* L.), absorb heavy metals, radionuclides, or other contaminants from polluted water (Dushenkov et al. 1995; Cooney 1996). Phytovolatilization describes transpiration of a volatile contaminant from the soil to the atmosphere as in the cases of trichloroethylene (TCE) transpiration by hybrid poplar trees (*Populus* spp.) (Newman et al. 1997) and mercury transformation and volatilization by genetically engineered *Arabidopsis thaliana* (Rugh et al. 1996). Poplar trees and axenic cell cultures derived from *Populus trichocarpa* x *P. deltoides* were found to partially metabolize, or phytodegrade, TCE to trichloroethanol, trichloroacetic acid and

carbon dioxide in the absence of soil and microorganisms (Newman et al. 1997). However, studies on whole plant metabolism are often unable to distinguish between bacterial- and plant-mediated xenobiotic transformation (Ferro et al. 1994; Casterline et al. 1985; Weiss et al. 1982) because it is extremely difficult to grow and study whole plants in a sterile manner due to the intimate associations between microorganisms and plants. Plants can also decontaminate soils by a process called rhizostimulation in which bacteria in the rhizosphere, or root-soil interface, are stimulated by exudates and organic material from roots, thus enhancing mineralization of the pollutant (Anderson et al. 1993; Cunningham et al. 1996).

Limited reports on the mechanisms of phytodegradation of organic contaminants other than agricultural pesticides are found in the literature. Herbicide metabolism by plants has been studied in detail, and several general mechanisms of transformation are known to occur such as oxidation-reduction, hydrolysis, conjugation, acylation, alkylation, cyclization, ring cleavage and binding of herbicide residues (Hatzios and Penner 1982). The majority of these reactions are carried out by constitutively-expressed plant enzymes. In addition, mixed-function oxidases, especially cytochrome P-450s, may be induced upon exposure to a herbicide to catalyze hydroxylation and oxidative dealkylation, deamination, and dehalogenation reactions. Cytochrome P-450 systems principally bind lipophilic compounds and usually require a reduced cofactor, such as NADH or NADPH, (as a source of electrons to reduce O_2 to H_2O) to oxidize a herbicide by incorporating singlet oxygen from O_2. For example, ring hydroxylation of 2,4-D occurs via 2,4-D *p*-hydroxylase, as found in peas and cucumbers (Hatzios and Penner

1982). Free radicals, such as superoxide ($O_2^{\bullet-}$) and the hydroxyl radical ($OH^{\bullet}$), formed during oxidation-reduction reactions have been implicated in the activation of herbicides such as paraquat (Youngman and Dodge 1979). Hydroxylation does not require cofactors but is stimulated by divalent cations and generally occurs on side-chain esters and amide bonds of herbicide molecules (Hatzios and Penner 1982). Conjugation to glutathione, glucosides and amino acids occurs widely in plants as a defense against pathogens and herbicide toxicity (Hatzios and Penner 1982). Reduction, cyclization reactions, or ring cleavage are not common mechanisms of xenobiotic transformation in plants. Finally, herbicides may be "bound" in plants as a final means of storing the compound. Herbicides and/or herbicide metabolites have been shown to be bound to globulin proteins, lignins, tannins, pectins, starch and cellulose, and the binding mechanisms are proposed to mimic incorporation of naturally-occurring plant components into these macromolecules (Hatzios and Penner 1982).

The Rhizosphere

The rhizosphere supports 5 to 20 times more microorganisms than nonrhizosphere soil on average, and up to 100 times as many microorganisms may proliferate in the rhizosphere as compared to bulk soil (Cunningham et al. 1996; Miller et al. 1989). Plant roots are known to exude simple carbon compounds such as carbohydrates, organic acids, amino acids, aliphatic and aromatic substances, and growth factors, such as B-vitamins and *p*-amino benzoic acid, as well as complex mucilages, which are produced to facilitate root growth through soil (Curl and Truelove 1986; Anderson et al. 1993). Carbohydrates appear to make up the majority of root exudate (Lynch 1982; Barber and Gunn 1974),

which has an overall C:N ratio of 30:1 based on amino acid content, as compared to the microbial biomass C:N ratio of 10:1 (Lynch 1982). Therefore, microbial proliferation caused by root exudates has the potential to deplete the rhizosphere of nitrogen. Root border cells (BRDs), which are respiring cells actively released by growing roots into soil, have been identified as another mechanism by which plants may influence the rhizosphere (Hawes and Brigham 1992). Total carbon released to the rhizosphere, including root exudates and BRDs, can reach approximately 20% of total plant dry mass, thus creating a highly metabolically active zone of microorganisms around roots (Rovira 1979). The presence of microorganisms appears to stimulate root exudation, since axenic plants exude only about half the amount of carbon as plants in contact with microorganisms (Prikryl and Vancura 1980; Rovira 1979). Additionally, bacteria have been shown to produce substances with phytohormone activity such as auxins, gibberellins, cytokinins and ethylene (Lynch 1982). The presence of such plant growth-promoting bacteria may thus enhance plant survival under stressful conditions. In addition, Prikryl and Vancura (1980) found that levels of root exudates released into the growth medium were positively correlated with root growth, and Barber and Gunn (1974) found that root exudation of amino acids and carbohydrates increased when plants were grown in 1- and 3-mm glass beads compared to hydroponic cultures. The glass beads caused the seminal roots of barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) to be shorter and bear more lateral roots, but total root dry mass was not different from roots grown hydroponically. Thus, physical factors can significantly influence root morphology and the amount of root exudation.

Bacterial colonization of root surfaces has been shown to be non-uniform, with distinct colonies near root cell junctions (Lynch 1982; Chin-A-Woeng et al. 1997) or in indented areas of the epidermal surface (Chin-A-Woeng et al. 1997). Further, different bacterial species apparently have different capacities to adhere to roots (Lynch 1982) or to proliferate in the rhizosphere (Miller et al. 1989). *Pseudomonas* spp. are typical rhizosphere inhabitants, whereas *Bacillus* spp. are widely distributed throughout the soil and are not sensitive to the rhizosphere influence (Miller et al. 1989). The highest concentrations of bacterial biomass were suggested by Lynch (1982) and found by Chin-A-Woeng et al. (1997) to be around older roots of tomato (*Lycopersicon esculentum* Mill. cv. Carmello). In contrast, Norton and Firestone (1991) observed the greatest microbial biomass and the most active bacteria adjacent to young root tips of ponderosa pine (*Pinus ponderosa* Laws.) seedlings. A significant proportion of the bacteria colonizing subterranean clover (*Trifolium subterraneum* L.) and Wimmera ryegrass (*Lolium rigidum* Gaud.) were of the genera *Arthrobacter*, and the majority of isolates from the rhizosphere of these plants were found to be gram-negative (Sperber and Rovira 1959). In another study, bacteria capable of colonizing maize roots were all gram-negative (Scher et al. 1984). Watanabe (1977) reported a positive correlation between increased numbers of PCP-tolerant and gram-negative bacteria, and 40 PCP-degrading isolates from three different contaminated sites were all found to be gram-negative bacteria (Saber and Crawford 1985). These findings suggesting that the rhizosphere may provide a suitable niche for PCP-degrading bacteria.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used to visually analyze rhizospheres of field-grown plants. Elliot et al. (1984) used SEM to determine that, even though the number of root-colonizing microorganisms was greater on wheat roots grown in untilled compared to tilled soil, bacterial presence in the rhizosphere did not affect final wheat yield. TEM was used to examine the endorhizosphere, or the root epidermis-cortex zone, of wheat (*Triticum aestivum*) where bacteria were seen penetrating lysed root cells (Lynch 1982).

Bacterial populations in soil can be negatively affected by the presence of pollutants, and roots may offer a more hospitable environment under such stressful conditions (Metzger et al. 1986; Alvey and Crowley 1996). For instance, bacterial numbers were highest on plant roots grown in retorted shale, a highly alkaline and saline byproduct of oil shale processing, as compared to bacterial numbers on plant roots grown in control soil or in retorted shale without plants (Metzger et al. 1986). The authors surmised that increased root exudation may have been a result of plant stress in response to adverse soil conditions, which in turn enhanced bacterial numbers. In another study, the long-term survival of an atrazine-mineralizing bacterial consortium was enhanced in the rhizosphere of corn as compared to unplanted soil (Alvey and Crowley 1996). Corn seedlings enhanced the formation of the metabolite hydroxyatrazine but had no effect on atrazine mineralization, indicating that the microorganisms responsible for atrazine mineralization are not selectively stimulated by the presence of corn roots.

Phytoremediation of Organic Contaminants

Dissipation of soil contaminants can be enhanced by the presence of plants and associated rhizosphere microorganisms. Reddy and Sethunathan (1983) found greater mineralization of ^{14}C-parathion in soil planted with 25-day old rice seedlings under flooded and nonflooded conditions compared to unplanted controls after 15 days. Flooded and nonflooded planted treatments released 22.6% and 9.2% of the applied radioactivity as $^{14}CO_2$, respectively, while less than 5.5% of the applied radioactivity was released as $^{14}CO_2$ in unplanted treatments. Sandmann and Loos (1984) compared numbers of 2,4-D-degrading microorganisms in rhizosphere and nonrhizosphere soil. They showed that soil from African clover (*Trifolium africanum* L.) and sugarcane (*Saccharum officinarum* L.) rhizospheres stimulated 2,4-D degradation approximately 29-fold and 7- to 261-fold, respectively, compared to nonrhizosphere soil. In another study, degradation rates of four recalcitrant polycyclic aromatic hydrocarbons (PAHs) were significantly increased in soil planted with a mixture of eight prairie grasses as compared to unplanted soil after 219 days of incubation (Aprill and Sims 1990). Walton and Anderson (1990) reported that rhizosphere soil slurries had a faster rate of ^{14}C-TCE mineralization than nonrhizosphere soil after 3 days of incubation, and Anderson and Walton (1995) found that ^{14}C-TCE mineralization occurred faster in soil planted with each of four different plant species compared to nonvegetated controls after 3 weeks. All of these studies provide evidence of plant-enhanced biodegradation, yet none explicitly examined the specific plant or microorganism responsible for the transformations or the fundamental mechanisms of accelerated degradation.

Enzymes released from plant roots could be a mechanism by which plants intensify xenobiotic degradation. For example, *in vivo* studies of tomato and water hyacinth [*Eichhornia crassipes* (C. Mart) Solms-Laub.] showed that guaiacol was precipitated as tetraguaiacol on root surfaces, most likely as a result of polymerization by root surface peroxidases (Adler et al. 1994). In the same study, horseradish peroxidase removed phenol, 2-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol from solution *in vitro*, most likely by polymerization and precipitation of polyaromatic products (Klibanov et al. 1983).

Microorganisms capable of using a xenobiotic compound as a sole source of carbon are termed catabolizers, while microorganisms that incidentally degrade a contaminant but grow on typical carbon compounds are called cometabolizers (Haby and Crowley 1996). Cometabolizing organisms may therefore be stimulated in the carbon-enriched rhizosphere, causing increased rates of contaminant disappearance. Haby and Crowley (1996) found that 3-chlorobenzoate (3-CB) was initially degraded more rapidly in rhizosphere soil slurries compared to nonrhizosphere slurries. They also showed similar levels of enhanced degradation of 3-CB in nonrhizosphere soil after the addition of glucose, mannitol or benzoate, suggesting that simple carbon compounds from roots might be the stimulating factor needed by indigenous cometabolizers.

Burken and Schnoor (1996) treated sand (0.1% organic matter) and silt loam soil (2.5% organic matter) with ^{14}C-atrazine together with either acetate (control), an equivalent amount of carbon as poplar cutting root exudate, or dry, pulverized poplar cutting roots (sand treatment only). Atrazine mineralization was monitored weekly over

7 months, and in both sand and soil atrazine mineralization was significantly greater in the root exudate treatments compared to the acetate controls. The pulverized root treatment (in sand only) showed by far the greatest amount of atrazine mineralization, and it was also the treatment with the greatest amount of organic carbon. Additionally, atrazine mineralization was about 10-fold higher in the silt loam than in the sand treatments. The authors suggested that increased organic matter in soil might enhance microbial degradative pathways (Burken and Schnoor 1996).

Another proposed mechanism by which plants enhance pollutant disappearance is by the selective stimulation of rhizosphere inhabitants that specifically degrade a certain pollutant. Sandmann and Loos (1984) found an extremely high R:S ratio of 2,4-D degraders (number of 2,4-D-degraders in rhizosphere soil/number of 2,4-D-degraders in nonrhizosphere soil) in the rhizosphere of sugarcane. The authors hypothesized that the large stimulatory effect of sugarcane on the number of 2,4-D-degrading organisms may have resulted from the selective enrichment of aromatic compound-metabolizing microorganisms by phenolics released by sugarcane roots. Indeed, other authors (Haby and Crowely 1996) argue that the high R:S ratio of 2,4-D-degraders found in the sugarcane rhizosphere is far higher than would be expected from the general microbial population increase due to the rhizosphere effect alone. An additional study found that *l*-carvone, a nonpolar component of spearmint (*Mentha spicata* L.) extracts, induced polychlorinated biphenyl (PCB) degradation by *Arthrobacter* sp. strain B1B (Gilbert and Crowley 1997). *l*-Carvone proved to be toxic to this strain at concentrations greater than 500 mg L^{-1} and was not utilized as a growth substrate. The compound did, however,

trigger the bacteria to cometabolize 26 of the 38 PCBs tested. Furthermore, other plant components structurally similar to *l*-carvone such as *p*-cymene and isoprene induced PCB degradation in strain B1B significantly more than biphenyl, which has been traditionally used to stimulate PCB cometabolism. The authors suggested that these natural plant products, which are less toxic than biphenyl, may be effective in stimulating PCB degradation in soil, and plants that exude these compounds could eventually be used for *in-situ* bioremediation of PCB-contaminated soils (Gilbert and Crowley 1997).

Phytoremediation and Plant Metabolism of PCP

Soil contaminated with PCP may be a likely candidate for phytoremediation since the log K_{ow} of the pentachlorophenate anion (3.25) would allow root uptake, xylem translocation and metabolism in shoots, roots and rhizosphere (Cunningham and Berti 1993). Haque et al. (1978) found that rice shoots contained about 3% of the 23 kg ha^{-1} ^{14}C-PCP applied after one week, of which 50% was unextractable. Ninety percent of the remaining radioactivity was intact PCP, 9% was unidentified conjugates, and 1% was a tetrachlorophenol. Weiss et al. (1982) found 7.5% and 5.2% of the applied ^{14}C-PCP (23 kg ha^{-1}) in rice straw and roots, respectively by 4 months after treatment. Most of the radioactivity in tissue samples was unextractable, while dechlorinated phenols, anisoles and methoxylated derivatives were identified as metabolites in the extractable portion. These results suggest that PCP uptake by rice plants occurs quickly (because by 1 week after PCP treatment, rice shoots contained half of what rice shoots contained 4 months later) whereas PCP transformations occur more slowly (as demonstrated by the high percentage of untransformed PCP in rice shoots). PCP uptake may be limited by reduced

bioavailability over time due to PCP sequestration by SOM. Neither study was able to determine if the PCP metabolites were of plant or bacterial origin.

Plant cell culture studies have been used to elucidate the nature of plant-derived metabolites of PCP. The earliest study examined cell suspension cultures of soybean and wheat dosed with 1 mg L^{-1} PCP and incubated for 48 hr (Scheel et al. 1984). Autoclaved cell suspensions contained only unchanged ^{14}C-PCP, but wheat and soybean cell cultures transformed 89% and 73% of the added ^{14}C-PCP, respectively. In both cultures, polar metabolites represented 50 to 60% of the radioactivity while non-polar metabolites constituted less than 0.2% of the applied radioactivity. The remaining radioactivity was sequestered in the insoluble fraction (37.6% in wheat and 11.3% in soybean cultures) or released into the growth medium (4.4% in wheat and 24.5% in soybean cultures). At least 5% of the radioactivity associated with the insoluble fraction in wheat cell cultures had been copolymerized into lignin. Further studies identified the PCP conjugates PCP-βD-glucoside and PCP-(*O*-malonyl)-βD-glucoside in the polar fraction from both wheat and soybean cell cultures (Schmitt et al. 1985). In a related wheat cell culture study, 90% of the radioactivity in the glucosidase-HCl digested polar fraction (which represents sugar conjugates of PCP or metabolites) was found to be TCC ([5] in Figure 1), while only about 5% was identified as TCHQ [2] and PCP [1] (Schäfer and Sandermann 1988). TCC, TCHQ and PCP were found in the Björkman lignin fraction, representing 31% of the total applied radioactivity. Again, TCC represented the largest fraction at 70% while TCHQ and PCP represented 5% and 25%, respectively. The authors concluded that TCC was readily incorporated into lignin, while PCP was not an efficient lignin precursor.

Moreover, it is thought that PCP dechlorination in plant cells occurs through involvement of radical species and activated oxygen, such as occurs in paraquat metabolism in plants (Youngman and Dodge 1979), rather than by a specific enzyme reaction, such as in cytochrome P450-mediated pesticide metabolism (Schäfer and Sandermann 1988). It is notable that TCC was not detected in soybean cell cultures, which implies that soybean cells have an entirely different mechanism of PCP metabolism. With respect to phytoremediation, this means that potential plant species must be studied individually to understand plant metabolism of the target xenobiotic.

Langebartels and Harms (1985) repeated PCP metabolism experiments in soybean, wheat and lupine (*Lupinus polyphyllus* LINDL.) cell cultures as well as in aseptically-grown wheat plants. The major portion of nonextractable radioactivity from wheat plants and wheat cell cultures was associated with the protein, lignin, hemicellulose and pectin components of cell wall fractions. The authors suggested that polar conjugates of PCP or its metabolites are incorporated into cell wall fractions by several mechanisms. Additionally, considerable amounts of PCP or its metabolites were released from cell wall fractions by enzymatic action, indicating a potential hazard to herbivores consuming plants grown in PCP-contaminated soil (Langebartels and Harms 1985).

Carrots (*Daucus sativa* L.) and barley were grown in 1 or 4 mg kg^{-1} ^{14}C-PCP-spiked soil under controlled laboratory conditions and in outdoor lysimeters (Scheunert et al. 1986). Less than 2% of the radioactivity was recovered in the volatile organic carbon trap, and radioactivity recovered as $^{14}CO_2$ ranged from 5% in the 4 mg kg^{-1} barley

treatment to 39% in the 1 mg kg^{-1} carrot treatment after 7- and 21-day laboratory experiments, respectively. Eighty-seven percent of the 1 mg kg^{-1} applied ^{14}C-PCP was unextractably bound to the soil after one growing season in the outdoor lysimeter experiment and thus considered unavailable for plant uptake. Scheunert et al. (1986) concluded that short-term laboratory experiments could not be extrapolated to predict long-term behavior of PCP in the environment.

Spinach and soybean plants were analyzed for plant-derived PCP metabolites after being grown in sterile soil containing 10 mg kg^{-1} PCP for 64 and 90 days, respectively (Casterline et al. 1985). Soybean shoots contained 0.7% of the applied PCP, while their roots contained 2.1%. Soybean roots bioconcentrated PCP, and contained the highest concentration at 39 μg g^{-1} PCP, and stems, leaves, pods, and seeds contained 9, 2.4, 0.49 and 0.04 μg g^{-1} PCP, respectively. Soybean roots exhibited a bioconcentration factor (BCF) of 3.9, where BCF is defined as [μg g^{-1} PCP in plant tissue] / [μg g^{-1} PCP in soil initially] (Pfender 1996) and indicates the relative partitioning of a chemical from the environment into plant tissue. Small quantities of the metabolites methoxytetrachlorophenol (MTCP) ([8] in Figure 1), 2,3,4,6-tetrachlorophenol (TCP) [6], 2,3,4,6-tetrachloroanisole (TCA) [7], and pentachloroanisole (PCA) [4] were found in the nonpolar fraction, which represented 91% of total PCP absorbed by the whole plant. The aqueous fraction, representing 9% of absorbed PCP, contained trace amounts of TCP and MTCP as glucoside conjugates. Up to 95% of the 10 mg kg^{-1} PCP applied to the sterile soil disappeared after 90 days in the soybean treatment. Unfortunately, there were no unplanted controls, so abiotic or microbial transformation of PCP could not be

determined. In general, the same unconjugated metabolites were found in spinach plants, although conjugated PCP and its metabolites were present in much lesser amounts. The authors also noted that 2,3,4,6-TCP ([6] in Figure 1) is a product of PCP photodegradation and that the majority of this compound was found in the leaves of both soybean and spinach plants, implying photolytic-assisted transformation (Casterline et al. 1985).

Ferro et al. (1994) examined PCP degradation in soil planted with 'Hycrest' crested wheatgrass (CWG) (*Agropyron desertorum* [Fischer ex Link] Schultes) versus unplanted soil and found that the presence of CWG accelerated PCP degradation. After 155 days, 74% of the added radioactivity remained in the soil of the unplanted treatment, while only 31% remained in the planted soil. CWG shoots accumulated 15% of the applied radioactivity, and the roots contained 21%. About 1.5% of the total radioactivity was captured in volatile organic traps, in close agreement with the results of Scheunert et al. (1986). Most notably, 22.1% and 6.3% of applied radioactivity were recovered as $^{14}CO_2$ in planted and unplanted treatments, respectively, as a result of a 3.5-fold faster rate of mineralization in planted treatments after a 25-day lag phase. The authors proposed that the lag time reflected the time needed for germination and rhizosphere establishment. They further suggested that increased mineralization was due to microbial catabolism in planted treatments via increased organic matter from root exudation or stimulation of organisms capable of mineralizing PCP in the rhizosphere (Ferro et al. 1994).

In another study, Proso millet (*Panicum miliaceum* L.), either inoculated with the PCP-degrading *Pseudomonas* strain SR3 or left uninoculated, was grown in soil spiked

with 175 mg kg^{-1} PCP (Pfender, 1996). After 4 weeks, uninoculated plants had accumulated PCP (BCF = 13) in their roots and grew poorly. In contrast, heights of inoculated plants were similar to plants grown in uncontaminated soil, and their roots contained only 16 mg kg^{-1} PCP (BCF = 0.09). PCP concentrations in soil were reduced to 5 and 3 mg kg^{-1} in the inoculated planted treatment and in the inoculated unplanted control, respectively. The author speculated that enhanced growth of inoculated plants in PCP-contaminated soil was likely due to rapid PCP mineralization in the rhizosphere. Pfender (1996) suggested that PCP chemistry is favorable for mineralization in the rhizosphere since it is taken up by roots slowly, increasing its residence time in the rhizosphere.

The preceding information on PCP degradation by plants suggests that: 1) Only about 1-2% of PCP is lost from soil by volatilization, regardless of initial concentration or treatment; 2) All plant species studied take up PCP into their roots and translocate it to their shoots, and the amount absorbed depends on soil PCP concentration, plant species, growth conditions, soil type, and presence or absence of microorganisms; 3) Plants are capable of limited PCP dechlorination and hydroxylation, usually followed by glucoside conjugation of PCP and PCP metabolites; 4) Significant amounts of PCP and its metabolites are bound to insoluble and cell wall fractions; 5) PCP and its metabolites appear to have limited mobility in root and shoot tissues; 6) PCP mineralization by plants has not been conclusively demonstrated.

Thesis Objectives

The long-term goal of phytoremediation research needs to address the basic mechanisms by which plants ameliorate xenobiotic toxicity in the rhizosphere and how to control and maintain specific microbes in the rhizosphere (U.S. Department of Energy [U.S. DOE], 1994). In addition, the U.S. DOE (1994) recommended further investigation of the effects of soil properties on mineralization of organic contaminants. The research reported here attempts to elucidate the mechanism by which CWG accelerates PCP degradation in soil. Since plants and bacteria interact intimately in the rhizosphere, an immediate question arises about the relative roles of the plant versus bacteria in phytoremediation. I hypothesized that indigenous rhizosphere microorganisms are responsible for PCP mineralization and that CWG accelerates the degradation of PCP in soil by preferentially stimulating microorganisms capable of PCP mineralization. Chapter 2 shows results from studies on the disappearance of PCP from CWG planted and unplanted PCP-contaminated soil and on the response of sterile versus inoculated CWG seedlings to increasing PCP levels. Chapter 3 quantifies mineralization of ^{14}C-PCP in the presence of sterile versus inoculated CWG seedlings. Chapter 4 describes PCP-degrader enumeration techniques and the response of PCP degraders to the presence of CWG root exudate. Finally, Chapter 5 summarizes the findings of this research.

CHAPTER 2

AXENIC AND INOCULATED CRESTED WHEATGRASS RESPONSE TO PCP

Introduction

Ferro et al. (1994) showed that the presence of 'Hycrest' CWG accelerated degradation of 100 mg kg^{-1} PCP in soil that had not previously been exposed to PCP. The authors speculated that the greater metabolic activity of rhizosphere microorganisms may have caused accelerated PCP degradation as compared to unplanted soil. If such accelerated degradation is to be helpful for *in situ* bioremediation, then it must occur in various soils and under different environmental conditions. In order to confirm the usefulness of CWG for phytoremediation, greenhouse studies were performed using PCP-contaminated soil planted with CWG seed or left unplanted.

Assuming such accelerated degradation does occur under various environmental conditions, determining the organisms responsible for that degradation would further our understanding of the rhizostimulation mechanism of phytoremediation. Once the mechanism is clearly defined, conditions most conducive to PCP biodegradation can be explored. Pfender (1996) demonstrated that a PCP-degrading *Pseudomonas* inoculated onto millet seed enabled the plant to survive in soil containing 175 mg kg^{-1} PCP. It appears that *Pseudomonas* strain SR3 was able to significantly reduce the amount of PCP absorbed by the millet because of rapid PCP degradation, since inoculated plants grew as well as controls while uninoculated seedlings grew very little or died before the end of

the experiment. Therefore, I speculated that compounds released into the rhizosphere by 'Hycrest' CWG caused PCP-degrading microorganisms to proliferate in the rhizosphere.

A PCP dose response experiment was conducted on inoculated and axenic (microbe-free) 'Hycrest' CWG seedlings to determine the extent to which microorganisms enhanced plant survival in the presence of PCP. CWG plants may encourage proliferation of rhizosphere microbial populations capable of transforming PCP, thereby enhancing plant survival in the presence of PCP. I hypothesized that CWG plants require the presence of rhizosphere microorganisms capable of degrading PCP in order to survive in soil or sand contaminated with PCP.

A PCP dose response experiment was conducted on axenic CWG seedlings and seedlings inoculated with one of four mixed microbial cultures derived from plant roots and rhizosphere soil from a PCP-contaminated site. Control seedlings were inoculated with the known PCP-degrading *Sphingomonas chlorophenolica* strain ATCC 39723 (Saber and Crawford 1985) in order to determine the relationship between PCP degradation in liquid culture and protection of CWG seedlings from PCP toxicity.

Materials and Methods

Soil and Inoculum Sources

Two local PCP-contaminated sites were selected for soil, plant and seed sources in the summer of 1996 after receiving permission from the Montana Department of Environmental Quality (MDEQ). The Davis Post Yard (DPY) in Willow Creek, MT was a small fence post treatment operation for a local sheep rancher about 20 years ago. Posts

were soaked in a below-ground concrete vat containing a PCP-creosote mixture and dried about 20 meters away. The soil directly adjacent to the dip vat was assayed for polychlorinated-*p*-dibenzodioxins/polychlorinated-dibenzofurans by Core Lab (Indianapolis, IN) according to the current Statement of Work DFLM01.1 (Modified 8280) by low resolution GC/high resolution MS and found to contain 69 $\mu g\ kg^{-1}$ (ppb) equivalents of the most toxic dioxin congener, 2,3,7,8-TCDD. In addition, HPLC analysis (described below) found 16,000 $mg\ kg^{-1}$ PCP in the same soil. This area was devoid of vegetation except for one CWG plant. Seed was collected from that plant and nearby CWG plants and stored at 4°C.

The Pine Tree Timber (PTT) site in Belgrade, MT was a commercial wood post treatment facility with several owners before it ceased operation in the 1980s (RAM Environmental 1998). The treatment vat was below ground and covered by a shed to which a pulley system was attached for handing the posts. Despite meticulous design by the original owner, the system was never fully completed and the overflow pipe from the vat to the outside ended abruptly in the soil. The majority of soil contamination on the site resulted from this unregulated overflow waste, and additional contamination resulted from solution drippage at the front of the treatment shed. In March 1994, contaminated soil was excavated from the area around the overflow pipe and transferred onto plastic tarps to prevent further contaminant leaching. The excavated soil was segregated into three piles, which contained high, medium and low levels of PCP contamination. A composite soil sample from all three piles was found to contain 64 $mg\ kg^{-1}$ PCP and about 3,000 to 5,000 $mg\ kg^{-1}$ total petroleum hydrocarbons in 1993 by the MDEQ. The

soil piles containing high- and medium-levels of PCP served as a source of PCP-contaminated soil and PCP-degrading microbial consortia. CWG was found growing in the most highly contaminated soil at the shed front, and additional seed samples were gathered from this site.

Enrichment for PCP Degradation in Mixed Cultures

Dr. Richard Veeh (Center for Biofilm Engineering, Montana State University) prepared enrichment cultures using the following methods. Four mixed cultures were derived from roots and rhizosphere soil of plants growing in PCP-contaminated soil at the PTT site. Roots of CWG and downy bromegrass (*Bromus tectorum* L.) were harvested in early April and were thoroughly rinsed of soil particles in 10 mM phosphate buffer (pH 7.4) and dried. Roots were homogenized in 10 ml of the same buffer for 2 minutes using a tissue homogenizing probe, then sonicated for 5 minutes at room temperature. The homogenate was used as the root-derived inoculum. Five g of soil rinsed from the roots were shaken by hand for 5 minutes and sonicated for 5 minutes in 50 ml phosphate buffer. The resulting soil slurry was used as the rhizosphere soil-derived inoculum.

Four ml of root- and rhizosphere soil-derived inocula were used to inoculate Erlenmeyer flasks containing 95 ml sterile PCP degrader medium (g per liter: K_2HPO_4, 1.73; KH_2PO_4, 0.68; $(NH_4)_2SO_4$, 0.83; $MgSO_4 \cdot 7H_2O$, 0.10; PCP [1% in 0.2 M NaOH] 0.05; and 1% 100X micronutrient solution containing in g per 100 ml: $FeSO_4 \cdot 7H_2O$, 0.05; $MnSO4 \cdot H_2O$, 0.03; $Ca(NO_3)_2 \cdot 4H_2O$, 0.23; Na_2EDTA, 0.02). Additionally, cultures were amended with 0.05 g L^{-1} glucose and 0.05 g L^{-1} glutamate. After 50 days, PCP degradation was determined by GC analysis as follows: 10 ml of culture were acidified

with 2 drops of concentrated H_2SO_4 and extracted into 2 ml methyl tert-butyl ether (MTBE). Two µl of the MTBE were injected in a Hewlett-Packard 5890 GC equipped with a Supelco PTE-5 QTM capillary column (15 m x 0.53 mm) using splitless injection with a split flow of 50 ml min^{-1}, make-up flow of 25 ml min^{-1} and a column flow of 5 ml min^{-1} at 18 kPa and 50°C. The initial temperature was held at 70°C for 2 minutes and increased by 15°C min^{-1} to a final temperature of 200°C, which was held for 3 minutes. The injector and FID detector were set at 250°C and 300°C, respectively. When the PCP concentration was below 1 mg L^{-1}, 10 ml of each culture were used to inoculate 90 ml of fresh PCP degrader medium over 3 culture cycles. The four mixed cultures that showed PCP-degrading ability were labeled as HPK4, HPK5, SF and HP.

PCP-degrading cultures were maintained by subculturing 1 ml of the turbid culture into 50 ml PCP degrader medium (which contained 0.05 g L^{-1} PCP and 0.05 g L^{-1} benzoic acid unless otherwise specified) every 2 or 3 weeks. The week in which the cultures were not transferred, 0.05 g L^{-1} PCP and benzoic acid were added to each culture. The HPK4 and SF cultures were combined 1:1 (vol.:vol.) for use in a PCP dose response experiment after 5 months of culturing. Inoculated roots from seedlings surviving 5 mg kg^{-1} PCP in this dose response were rinsed of sand in sterile deionized distilled water and spread onto a plate of 0.1x(2xYT) + 1 g L^{-1} glucose medium containing in g per L: tryptone, 1.6; yeast extract, 1; NaCl, 0.5; (+) D-glucose, 1; and 0.8% Difco (Detroit, MI) agar. The plate was incubated at room temperature, and after 21 days the bacteria surrounding the roots were scraped off the plate and inoculated into

50 ml PCP degrader medium. This culture was labeled BFR (Bacteria From Roots) and treated as the other mixed cultures.

Sphingomonas chlorophenolica (strain ATCC 39723; Chanama and Crawford 1997), a known PCP-degrader, was used as a PCP-degrading positive control in mixed culture experiments.

Pure Culture Isolation

The mixed cultures HPK4/SF, HPK5, HP and BFR were serially diluted 10-fold and streaked onto PCP degrader medium plates containing 50 mg L^{-1} PCP, 50 mg L^{-1} benzoic acid and 1.5 % agar and incubated at 25°C. Individual bacterial colonies were isolated based on differences in colony morphology and restreaked onto the same medium. Three colonies from these isolation plates were used as replicates to inoculate 10 ml of PCP degrader medium. Additionally, 10 ml of PCP degrader medium were inoculated with 10 µl of the HPK4/SF, HP, HPK5, or BFR cultures and all cultures were incubated with shaking at 240 rpm and 25°C for 18 days. There were three replicate tubes for each inoculum source, and PCP remaining after 18 days was quantified in filtered (0.22 µm polysulfone syringe filter [Whatman Inc., Clifton, NJ]) culture fluids using a Hitachi Model U-2000 spectrophotometer set at 317 nm.

Greenhouse Study

The purpose of this study was to replicate the results of Ferro et al. (1994). PTT soil, a Beaverton sandy loam (loamy-skeletal, mixed Typic Argiboroll; Table 2), was sifted to less than 2 mm immediately after collection and stored at -20°C for 12 months

and then at room temperature for 8 months. For comparison, Table 2 also presents properties of the sand (plus growth medium) used in experiments described below. At the beginning of each experiment, a sample of the PTT soil was thoroughly mixed and three subsamples analyzed for PCP. CWG seed was collected from the DPY site in the fall of 1996, and 'Hycrest' CWG seed was obtained from the Montana State University Seed Laboratory, Bozeman, MT.

Table 2. Characteristics of PTT Soil Used in the Greenhouse Experiment and Sterile Sand (plus Hoagland's Growth Medium) Used in PCP Dose Response and ^{14}C-PCP Experiments.

	% Sand*	% Silt	% Clay	CEC meq/ 100 g	EC mmhos/ cm	NO3- N mg/kg	Olsen P mg/kg	K mg/kg	pH	% OM
PTT -soil	55	32	13	16.4	0.19	40.0	8.7	282	7.4	2.92
Sand	100	--	--	0.3	0.09	22.9	1.7	46	6.4	<0.05

*Texture determined by Modified Day Mechanical Analysis

Ten 'Hycrest' CWG or DPY CWG seeds were planted in white plastic growth tubes ('conetainers', 4 cm x 21 cm) containing PTT soil contaminated with 31.1 mg kg^{-1} or 18.5 mg kg^{-1} PCP in the first or second experiment, respectively. It should be noted that the PTT soil used contained appreciable levels of other organic contaminants such as diesel range organics, but only PCP concentration was monitored since it is the primary biocidal agent in wood preservation solutions. Seeds were covered with 0.5 cm of uncontaminated PTT soil, which was also added to unplanted conetainers. There were four and five replications per treatment in the first and second experiments, respectively. Planted tubes were arranged in a completely randomized design in the greenhouse under 15-hr days (average 400 μmol m^{-2} sec^{-1} under full sun conditions) at 28°C. The soil was

watered every other day with approximately 5 ml of water for about 5 weeks, and then planted and unplanted treatments received 10 ml and 5 ml, respectively. There were 1 to 14 seedlings per tube. The number of seedlings per tube was not standardized, following the protocol reported by Ferro et al. (1994). Two subsamples of soil from each replicate were analyzed for PCP concentration after 13 weeks and a third subsample weighed, dried at 55°C for 2 days, and weighed again for soil moisture determinations.

PCP analysis was carried out using organic extraction and high-pressure liquid chromatography (HPLC) (Doughten 1997). Approximately 10 g of anhydrous Na_2SO_4 and 10 mg of $Na_2S_2O_4$ were mixed with moist soil (4 to 6 g) in a 20-ml glass scintillation vial. Acetone:hexane (1:1, vol.:vol.) (9.75 ml) and concentrated H_2SO_4 (0.25 ml) were added and the vial was capped, Parafilmed, and shaken horizontally (200 cpm) overnight. Soil particles were allowed to settle and a 1-ml aliquot of the supernatant transferred to a 1.5-ml centrifuge tube and dried under N_2. The residue was resuspended in 250 µl 0.05 N NaOH, gently vortexed for 4 h, and filtered through a 0.22 µm polysulfone syringe filter (Whatman Inc., Clifton, NJ). The filtrate was combined with an equal volume of 80% HPLC-grade acetonitrile and 20% sterile 0.2% H_3PO_4. HPLC was carried out using a reverse phase, C-18, 10 µm Econosil column (Alltech Associates, Inc., Deerfield, IL) with an isocratic mobile phase of 80% HPLC-grade acetonitrile: 20% sterile 0.2% H_3PO_4 at a flow rate of 1 ml min^{-1}. Peak detection and integration were conducted at 214.8 nm using a Waters photodiode array detector and Millenium software (Waters Corp., Milford, MA). Calibration curves were obtained from 0.1, 1, 10, and 50 mg L^{-1} standards of 2,4,6-trichlorophenol (R_f 4.35 min), PCP (R_f 6.3 min) and pentachloroanisole (R_f 12.5

min). The standard 2,4,6-tribromophenol (Aldrich Chemical Co., Milwaukee, WI) was used as a surrogate (85.5% recovery) and as an internal standard (102% recovery). Concentrations are expressed as mg analyte per kg dry soil.

Seed Sterilization

Seed coats of Hycrest CWG seeds were removed by hand, and the caryopses were surface sterilized in 2 ml 0.1% $HgCl_2$ for 90 seconds in a sterile 50-ml Falcon tube. Caryopses were vortexed in 50 ml sterile deionized distilled water for four rinses of 1, 2, 5, and 10 minutes. Caryopses were then vortexed in sterile 0.2 M KNO_3 for 5 min and soaked for 5 min, and the procedure was repeated. Caryopses were germinated embryo-side up on plates containing, in g per L: tryptone, 1.6; yeast extract, 1; NaCl, 0.5; (+) D-glucose, 1; and 1.2% Difco (Detroit, MI) agar for 14-20 days at 4°C then for 3 days at 24°C/18°C day/night under 15 hours of daylight. Germinated seedlings and agar plates were observed for microbial growth and any seedlings with bacterial or fungal contamination were discarded. Germination plates were maintained in the incubator after seedlings were used in an experiment in order to continue monitoring for contamination. The percentage of apparent contaminated seedlings ranged from 0-10%.

PCP Dose Response Experiments

'Hycrest' CWG seedlings were grown in sterile sand containing 0 to 100 mg kg^{-1} PCP to compare the tolerance of axenic and inoculated plants to PCP. Four polycarbonate plant tissue culture boxes (Magenta boxes or GA-7 Vessels; Sigma, St. Louis, MO) were stacked to make a self-contained growth environment called a

BOCSystem (Biological Organism Closed System, Figure 2), modeled after the growth box unit of McDermott and Kahn (1992). The bottom box was intact and contained 150 ml 0.75x Hoagland's No. 2 basal salt medium (pH 4.7; Sigma). An 8-mm hole was drilled in the bottom center of the second box, through which a Whatman 3 MM filter paper wick was placed. Two more Magenta boxes with bottoms removed were stacked on top of the second box, and the polypropylene lid was fitted with a 0.2 μm polypropylene filter. The BOCSystems were autoclaved for 60 min at 121°C prior to use. Sand (Table 2; industrial quartz, 75% retained on 40 mesh or coarser, Unimin Corp., Emmett, ID) was sterilized by autoclaving 3 times for 90 min at 121°C on 3 consecutive days. Sterile sand (200 g) was treated with 5 ml of acetone spiked with PCP stock solution and an additional 5 ml acetone rinse. Stock solutions were made fresh for each experiment by dissolving PCP (99+% purity, Sigma) in absolute ethanol. The sand was stirred vigorously until dry using aseptic techniques. The spiked sand was then poured over the wick into the second Magenta box and two to eight axenic, 0.5- to 5-cm tall 'Hycrest' CWG seedlings were aseptically transplanted into the sand. The BOCSystems were then assembled, the three joints taped with colored paper tape (Fisher Scientific, Pittsburgh, PA), aluminum foil wrapped around the rooting environment portion of the BOCSystem, and transferred to a growth chamber. Plants were cultured under a 15-hr photoperiod (86 μmol m^{-2} s^{-1}), 24°C/18°C day/night. Plant heights and shoot dry mass after drying at 55°C for 48 hours were measured 8 weeks after transplanting unless stated otherwise.

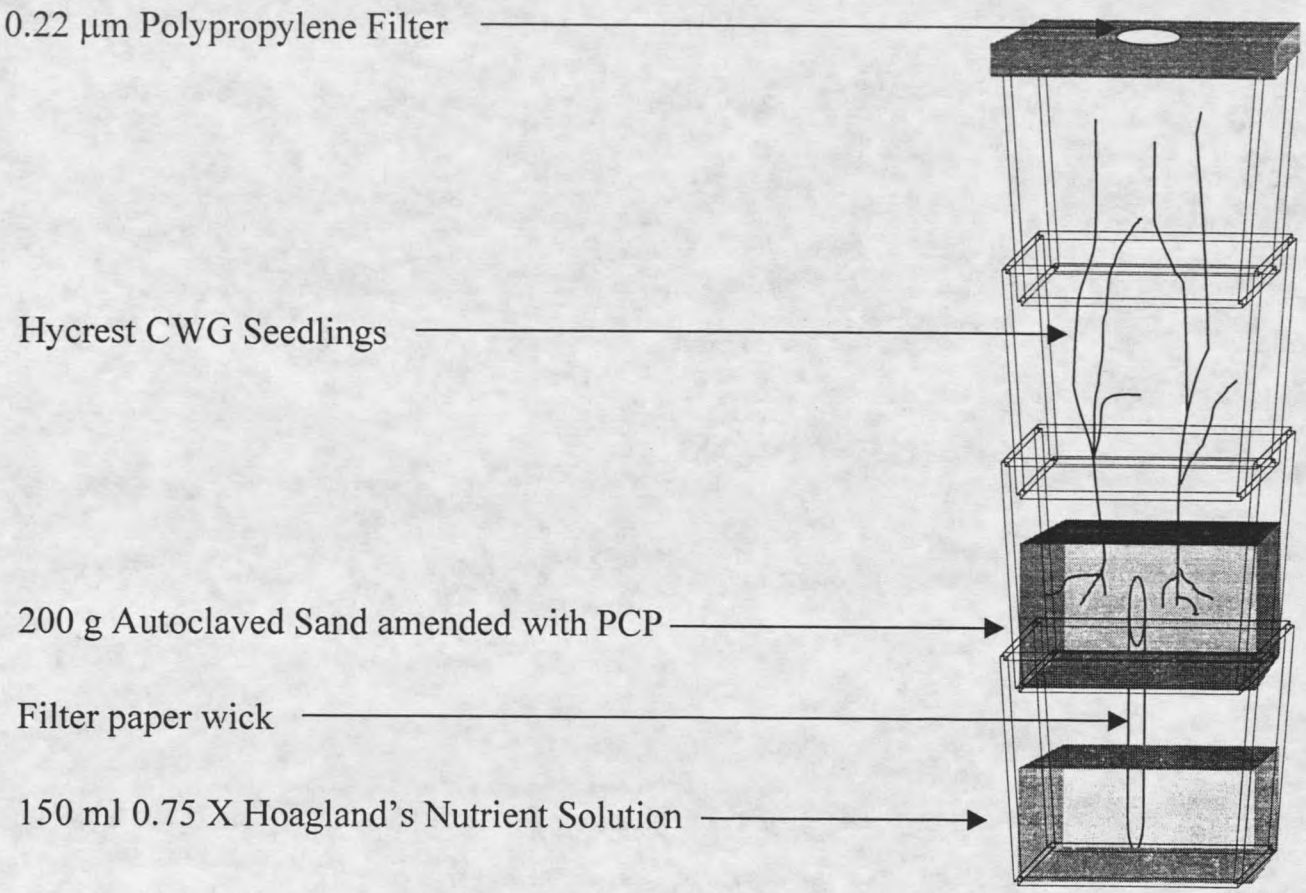

Figure 2. Biological Organism Closed System (BOCSystem) used for PCP Dose Response Experiments.

Sterile seedlings were inoculated by submerging the roots for 30 seconds in 5 ml of a liquid culture of either HPK4/SF, or the supernatant of a PTT soil suspension. HPK4/SF was found to degrade 50 mg L^{-1} PCP in one week at the start of the first dose response experiment, and most probable number (MPN) estimates (see Chapter 4) were used to enumerate PCP degraders at the beginning of each experiment in which PTT soil extract was used as inoculum. Briefly, the MPN was determined by preparing five 5-ml cultures of 10^{-2} to 10^{-5} dilutions of the inoculum in PCP MPN medium (containing 50 mg L^{-1} PCP; see Chapter 4), and a 10^{-2} dilution of autoclaved inoculum and uninoculated PCP MPN medium served as controls. After 2 weeks, an aliquot was filtered (0.22 μm polysulfone syringe filters, Whatman Inc.) and PCP concentration quantified on the spectrophotometer at 317 nm. Dilution tubes were rated positive for PCP degradation if PCP concentrations were less than 20 mg L^{-1}.

Sterile seedlings were transplanted into sand containing 0, 1, or 5 mg kg^{-1} PCP, while HPK4/SF-inoculated seedlings were exposed to 0, 1, 5, 10, or 15 mg kg^{-1} PCP. There were 6 to 8 seedlings per treatment with two replications and one replication of the sterile control. Controls included 0 mg kg^{-1} PCP treatments, with one acetone control replicate for both sterile and inoculated CWG treatments and one replicate without acetone in the inoculated CWG treatment only. There was also one replicate of an autoclaved inoculum-treated CWG control at 5 mg kg^{-1} PCP. Shoot height, fresh weight, and dry weight were measured 7 weeks after transplanting. Contamination of sterile treatments was checked by placing sand, Hoagland's solution and roots on 0.1x(2xYT) + 1 g L^{-1} glucose medium containing 1% agar in 100 x 15 mm petri dishes. Low levels of a

green-colored fungus were observed from approximately 70% of low-dose PCP (< 5 mg kg^{-1}) sterile treatments. However, the unidentified fungus: 1) did not adversely affect seedling growth, as it was also present in the no-PCP controls which grew well and 2) was shown to have no ability to degrade PCP in separate liquid culture experiments (data not shown).

The HPK4/SF culture lost its ability to degrade PCP after 10 months of subculturing and no longer enhanced CWG growth in the presence of PCP. Therefore, a PCP-degrading microbial consortium was prepared from PTT soil used in a 36-week greenhouse experiment and stored at 4°C for up to 3 months. Twenty g moist PTT soil were shaken for 2 hours in 100 ml 2.5 mM KH_2PO_4 (pH 7.2) in 250-ml polypropylene screw-cap centrifuge bottles. After centrifugation at 1,000 x G for 10 min, the supernatants were used immediately as inoculum or stored at 4°C and used within 24 hours. Spectrophotometer data showed negligible amounts of PCP (< 1 mg L^{-1}) in the inoculum.

Sterile seedlings, obtained as described before, were transplanted into 0, 1, 2, 3, 4, or 5 mg kg^{-1} PCP while seedlings inoculated with PTT soil extract were exposed to 0, 2, 4, 6, 8, 10, or 12 mg kg^{-1} PCP. A control treatment of autoclaved inoculum-treated seedlings grown in 4 mg kg^{-1} PCP was also included, since both inoculated and sterile seedlings were dosed with 4 mg kg^{-1} PCP. There were five seedlings per replicate and three replicates per treatment. The dose response using PTT soil extract as inoculum was repeated once, with sterile seedlings grown in 0, 2, 3, 4, 5, or 8 mg kg^{-1} PCP and inoculated seedlings grown in 0, 2, 3, 4, 5, 6, 8, 10, or 12 mg kg^{-1} PCP. Autoclaved

inoculum-treated seedlings were grown in 5 mg kg^{-1} PCP as a sterile inoculum control. Again there were five seedlings per replicate and three replicates per treatment. The five shoots from each replicate were pooled and their dry mass measured 8 weeks after transplanting.

Mixed Culture Screen for Optimum Plant Protection against PCP

To determine if the mixed cultures could protect CWG seedlings against PCP toxicity, the HP, HPK4/SF, BFR, and HPK5 mixed cultures and the *Sphingomonas chlorophenolica* culture were used to inoculate axenic CWG seedlings as described above. Six inoculated seedlings were transplanted into sand containing 5 or 10 mg kg^{-1} PCP. There were two replicates of each treatment and an uninoculated control, and plant heights were measured 6 weeks after transplanting. The experiment was conducted once.

Results and Discussion

Greenhouse Study

Initial soil PCP concentrations were 31.1 and 18.5 mg kg^{-1} in the first and second experiments, respectively (Table 3). After 13 weeks in the greenhouse, planted treatments contained significantly less PCP than the unplanted treatments in the first experiment as determined by 5% LSD pairwise comparison. The second experiment showed no differences among treatments, although all treatments showed a significant (5% LSD) decrease in PCP concentration after 13 weeks. There were no apparent differences between the 'Hycrest' or DPY CWG plants at harvest, and fibrous roots extended throughout the soil in all planted replicates in both experiments. Therefore,

plants from DPY seeds do not appear to have any genetic or endophyte-conferred abilities for PCP degradation. The soil used in the second experiment most likely had a lower initial PCP concentration because the soil had been stored near field capacity and at room temperature (due to lack of cold storage space) for approximately 15 months prior to the experiment. Such optimum conditions would likely enhance microbial degradation of labile PCP. Additionally, the amount of PCP in the soil was solely from field contamination—no PCP was added in the laboratory. Such PCP contamination has been in this soil for at least 10 years, thus the effects of aging and sequestration would be expected to render a significant portion of the PCP unavailable for biodegradation (Alexander 1999). These circumstances may have increased the proportion of unavailable to bioavailable PCP, which would then cause desorption of sequestered PCP to become the rate-limiting step in degradation.

Table 3. PCP Levels in Planted versus Unplanted PTT Soil from Greenhouse Experiments as Determined by HPLC.

Treatment	Average mg kg^{-1} PCP ± Standard Error	% PCP Reduction
	Experiment #1	
Initial soil PCP concentration	31.1 ± 1.8	--
'Hycrest' CWG	21.6 ± 1.3	30.5%
DPY CWG	21.5 ± 2.1	30.9%
Unplanted	27.5 ± 2.0	11.6%
	Experiment #2	
Initial soil PCP concentration	18.5 ± 1.6	--
'Hycrest' CWG	15.4 ± 0.8	16.8%
DPY CWG	15.4 ± 0.7	16.8%
Unplanted	15.0 ± 0.6	18.9%

Organic contaminants that have been present in soils for long periods of time usually show "hockey stick-shaped" kinetics of degradation (Alexander 1999). The

contaminant is initially degraded at a fairly rapid rate and the rate then slows considerably until degradation essentially ceases, most likely because the remaining concentration of the contaminant is unavailable for bioremediation. The mechanisms of contaminant aging are not well understood, although there are two hypotheses: 1) the contaminants diffuse into hydrophobic, tortuous soil pores that are smaller than microbes, and/or 2) organic contaminants incorporate into soil organic matter and move into the interior by solid-state diffusion (Alexander 1999). The amount of a pollutant that is sequestered by a soil depends on the environment, the pollutant, and the amount of time the two are in contact. Therefore, the results of the experimental repeat shown here may reflect additional PCP aging and subsequent lack of bioavailability in PTT soil.

PCP Dose Response Experiments

PCP dose response experiments were conducted to compare the highest levels of PCP tolerated by axenic and inoculated 'Hycrest' CWG seedlings. Two preliminary dose response experiments were conducted to determine the maximum PCP dose that sterile CWG seedlings could tolerate. Axenically grown Hycrest CWG seedlings (1-2 cm tall) were transplanted into sand containing 0.001, 0.01, 0.1, 1, 5, 10, 25, 50 or 100 mg kg^{-1} PCP sand. There were five seedlings per replicate and one replicate per treatment. Observations from these experiments showed that sterile Hycrest CWG seedlings tolerated less than 5 mg kg^{-1} PCP.

Figure 3 shows the response of HPK4/SF-inoculated CWG seedlings to PCP concentration as compared to sterile CWG seedlings. Sterile seedling heights were greater than inoculated seedling heights at 1 mg kg^{-1} PCP, but sterile seedlings perished

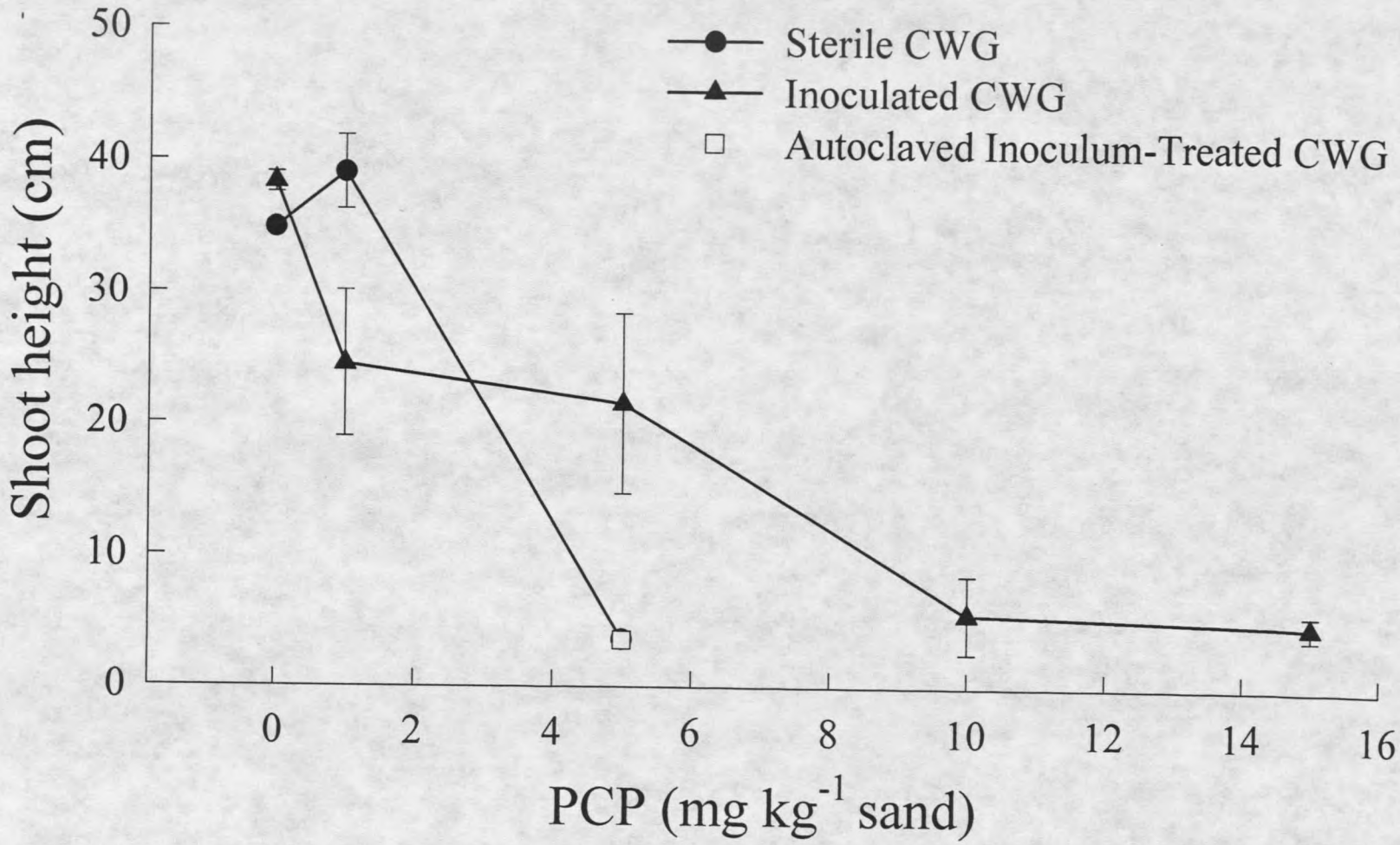

Figure 3. PCP Dose Response of CWG in the Presence and Absence of HPK4/SF Inoculum. Vertical bars represent standard errors of the means.

at 5 mg kg^{-1} PCP. In contrast, inoculated seedlings grew well at 5 mg kg^{-1} PCP and even showed slight growth at 10 and 15 mg kg^{-1} PCP. At low PCP doses, sterile seedlings acquired more shoot height than inoculated seedlings, but this was not the case in uncontaminated controls (0 mg kg^{-1} PCP). It is possible that inoculated CWG seedlings lost a substantial amount of carbon to the rhizosphere in the presence of both microorganisms and low levels of PCP. The autoclaved-inoculum treated CWG at 5 mg kg^{-1} PCP grew as poorly as sterile CWG at the same dose. Overall, the presence of HPK4/SF inoculum enhanced 'Hycrest' CWG growth and survival in the presence of PCP greater than 5 mg kg^{-1}. These results agree with those of Pfender (1996) who showed that the PCP-degrading *Pseudomonas* strain SR3 significantly enhanced millet growth and survival in the presence of PCP. Pfender's (1996) observations are also consistent with the fact that *Pseudomonas* spp. are typical rhizosphere inhabitants (Miller et al. 1989) and are likely able to compete with other microorganisms and establish in the rhizosphere.

In a repetition of the dose response experiment using HPK4/SF as inoculum, enhanced plant growth was not observed in any inoculated treatments (data not shown). The loss of plant protection may have been due to the loss of PCP degraders specifically adapted to the rhizosphere after extended culturing. Additionally, it appears that microbes protect the plant at least partially through rapid PCP degradation in the rhizosphere, since a decrease of PCP degradative activity of HPK4/SF (data not shown) coincided with its inability to provide CWG seedling protection from PCP toxicity. To

overcome this problem, a microbial consortium freshly extracted from PTT soil was used as inoculum in subsequent experiments.

The two PTT inoculum dose response experiments were combined and are presented in Figure 4. The data points represent the means of three or six replicates. Inoculated seedlings accumulated more shoot mass than sterile seedlings from 4 to 8 mg kg^{-1} PCP. Inoculated seedlings also showed growth at 10 and 12 mg kg^{-1} PCP, while sterile seedlings showed no growth at PCP concentrations greater than 4 mg kg^{-1}. Thus, both dose response experiments showed the same trend: sterile seedlings grew more than inoculated seedlings at the lowest PCP doses, while inoculated seedlings accumulated more shoot mass at higher PCP doses. These finding support the hypothesis that CWG plants require the presence of rhizosphere microorganisms capable of transforming PCP in order to survive in PCP-contaminated sand. The first dose response experiment with HPK4/SF as inoculum (Figure 3) showed that a PCP-degrading consortium increased CWG tolerance of PCP by 10-fold. The PTT soil inoculum used in the second and third dose response experiments increased CWG tolerance to PCP only about 1.5- to 3-fold. Since the PTT soil inoculum was not enriched for PCP degraders beyond the PCP contamination in the soil (about 40 mg kg^{-1} PCP), such inoculum might be expected to contain fewer PCP degrading microorganisms. These findings also support the hypothesis that PCP degradation by microorganisms is an important component in the overall mechanism of accelerated PCP degradation in the CWG rhizosphere.

MPN experiments (see Chapter 4) showed that approximately 165 and 245 PCP degraders were added to the 200 g sand in each BOCSystem in the second and third dose

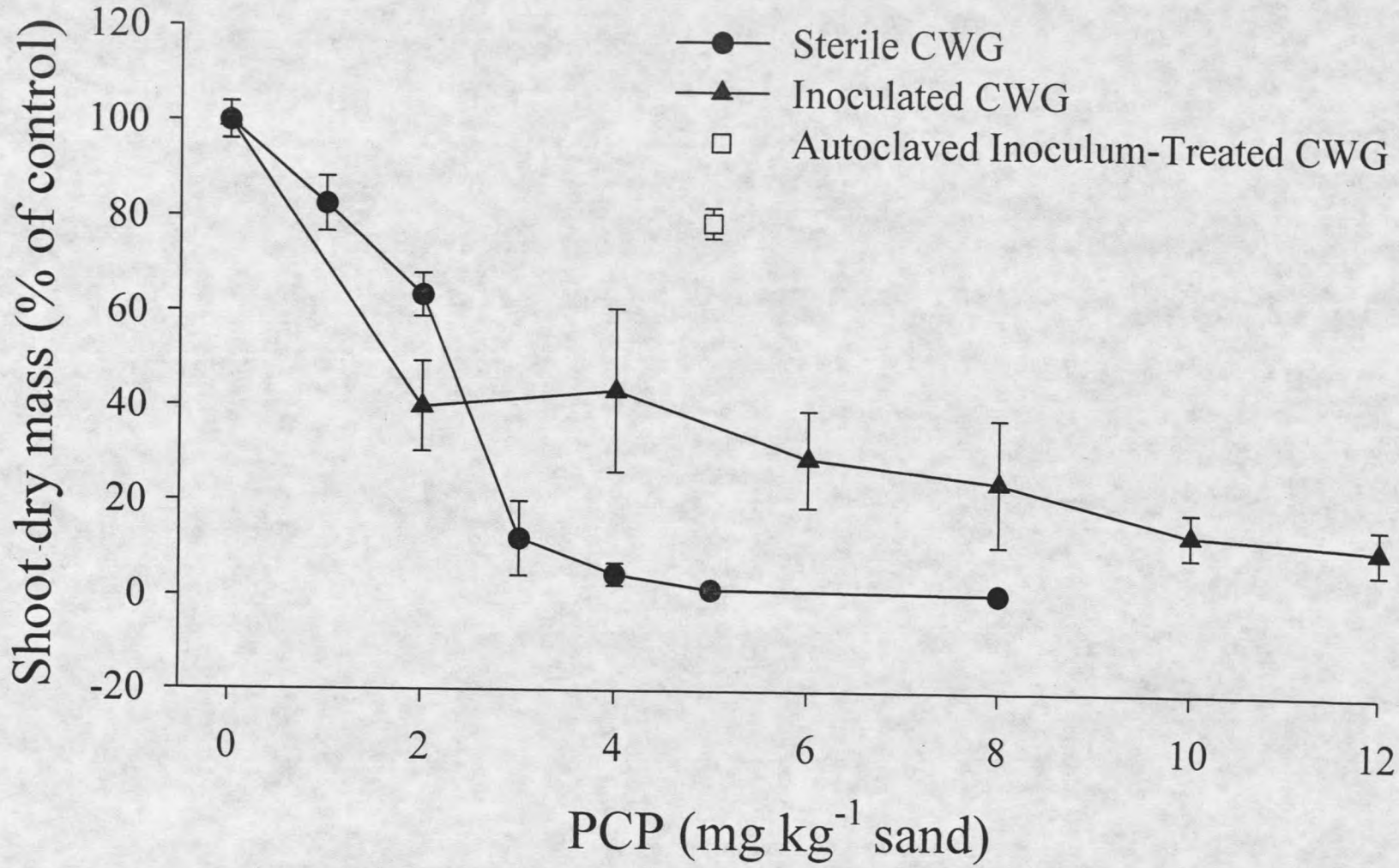

Figure 4. PCP Dose Response of CWG in the Presence and Absence of PTT Inoculum. Vertical bars represent standard errors of the means.

response experiments (in which PTT inoculum was used), respectively. PCP degraders were not enumerated in the first dose response experiment in which HPK4/SF was used as inoculum.

Autoclaved inoculum-treated CWG seedlings grown in 4 mg kg^{-1} PCP acquired slightly more shoot mass than sterile seedlings at 4 mg kg^{-1} PCP (data not shown), but the large amount of shoot mass accumulated by the autoclaved inoculum treatment at 5 mg kg^{-1} PCP in the PTT inoculum dose response experiment was unexpected (Figure 4). This result might be due to compounds from cell lysates in the autoclaved inoculum acting as plant growth hormones or cell membrane fragments associating with PCP, reducing PCP toxicity and making PCP less available for root uptake. Additionally, humics in the inoculum may have been altered by autoclaving, giving rise to large organic molecules that may have sequestered PCP in solution.

Since a 1:1 (vol.:vol.) mixture of two PCP-degrading consortia (HPK4 and SF) increased CWG tolerance to PCP in the first dose response experiment, the other PCP-degrading mixed cultures or the PCP-degrading *Sphingomonas chlorophenolica* (ATCC 39723) might provide greater protection to CWG seedlings. Thus, before the HPK4/SF culture lost its ability to degrade PCP in liquid culture, a PCP dose response experiment was conducted in which the four mixed cultures and *Sphingomonas chlorophenolica* were used as CWG root inocula.

PCP Degradation by Soil Bacteria and Levels of Plant Protection

Table 4 shows the PCP concentrations remaining after 18 days in cultures initially containing 50 mg L^{-1} PCP and inoculated with one of four mixed cultures or one of ten

Table 4. PCP (50 mg L^{-1}) Degradation by Mixed Cultures and Isolates after 18 Days; and Heights of Inoculated CWG Plants Grown in the Presence of PCP for 6 Weeks.

Mixed Culture or Isolate	Final PCP Concentration in Liquid Culture (mg L^{-1}) ± Standard Error	Average Plant Height (cm)	
		5 mg kg^{-1} PCP	10 mg kg^{-1} PCP
ATCC 39723	2.5 ± 0.1	7.7	5.4
HPK5	2.6 ± 0.1	5.6	5.7
HP	3.0 ± 0.2	13.7*	5.6
BFR	23.5 ± 2.7	6.1	6.1
HPK4/SF	29.5 ± 1.4	14.7*	8.7
Uninoculated Control	43.6 ± 1.0	6.1	5.9
Isolate 6, from BFR	44.0 ± 2.7		
Isolate 1, from HP	44.6 ± 1.4		
Isolate 10, from HPK5	45.3 ± 1.2		
Isolate 3, from BFR	46.6 ± 2.1		
Isolate 9, from HP	47.6 ± 2.8		
Isolate 2, from BFR	47.7 ± 0.4		
Isolate 5, from HPK5	48.4 ± 2.3		
Isolate 7, from BFR	49.3 ± 1.7		
Isolate 8, from HPK4/SF	51.4 ± 1.5		

* Significantly greater than other treatments at 5 mg kg^{-1} PCP by the Student-Newman-Keuls method of pairwise multiple comparison ($P < 0.05$).

bacterial isolates. The *Sphingomonas chlorophenolica* positive control degraded PCP to a final concentration of 2.5 mg L^{-1} PCP, while 43.6 mg L^{-1} PCP was detected in the uninoculated controls. Of the mixed cultures, HP and HPK5 degraded PCP to 3.0 and 2.6 mg L^{-1}, respectively, while HPK4/SF and BFR degraded PCP to 29.5 and 23.5 mg L^{-1}, respectively after 18 days. Of the mixed cultures, HPK4/SF had the highest level of PCP remaining in liquid culture after 18 days, but CWG seedlings inoculated with HPK4/SF showed the greatest amount of growth in the presence of 5 mg kg^{-1} PCP. PCP degradation was not observed in any of the ten bacterial isolates obtained from the mixed cultures. This result may indicate that the mixed cultures either contained PCP-degrading bacteria that were not culturable in PCP degrader medium or could not utilize PCP as a

sole carbon and energy source. PCP degradation in the mixed cultures may proceed by cometabolism, by sequential metabolism by several members of the consortium, or may require additional vitamins or cofactors. HPLC analyses of mixed culture supernatants never showed any potential metabolite peaks; however, metabolites might have been consumed as rapidly as they were produced. Isolated cultures were not analyzed for PCP metabolites.

PTT-derived mixed cultures HPK4/SF and HP conferred significantly greater growth to CWG seedlings at 5 mg kg^{-1} PCP than the other treatments, while HPK5 and BFR cultures provided no protection compared to sterile controls. *Sphingomonas chlorophenolica* (strain ATCC 39723), a known sediment-derived PCP-degrader, also did not provide significant protection against 5 mg kg^{-1} PCP. These results show that PCP degradation in liquid culture by a pure strain or consortia is not predictive of the organisms' ability to protect CWG plants from the toxic effects of PCP. A consortium that degrades PCP in liquid culture yet does not protect CWG seedlings may not be suited to compete in the CWG rhizosphere. In contrast, a consortium that does not degrade PCP well in liquid culture may multiply and utilize PCP as a substrate in the CWG rhizosphere very effectively, possibly due to the availability of a necessary cofactor or vitamin in CWG root exudate. Isolation of contaminant-degrading organisms often fails due to the lack of required cofactors, vitamins or essential amino acids in culture media (Alexander 1999). In order for a consortium or any microorganism to be effective *in situ*, it must successfully compete for nutrients, avoid predation, and tolerate the competitive and rigorous environment of a rhizosphere in contaminated soil (Alexander 1999).

It appears that when bacteria capable of PCP degradation were present in the rhizosphere, they either significantly reduced the amount of PCP absorbed by the plant or altered the plant's response to PCP. Do the microorganisms degrade PCP so that the plant root does not encounter the pollutant, or do the microorganisms stimulate the plant's own defenses against PCP? Perhaps the microorganisms bind PCP, rendering it less bioavailable to the plant, or the microbes cause the plant roots to exude PCP-binding compounds. To investigate some of these possibilities, ^{14}C-PCP experiments were carried out to better understand PCP transformation in the 'Hycrest' CWG rhizosphere.

CHAPTER 3

^{14}C-PCP METABOLISM IN INOCULATED AND AXENIC CRESTED WHEATGRASS SEEDLINGS

Introduction

Experiments discussed in the preceding chapter showed that sterile CWG seedlings were more susceptible to the toxic effects of PCP than CWG seedlings inoculated with microorganisms extracted from PCP-contaminated soil. While these results suggest that microorganisms are protecting the seedlings from PCP toxicity, they provide no information on the extent of PCP disappearance or how PCP is distributed throughout the plant-microbe-sand system. Many plants are known to metabolize herbicides that are structurally similar to PCP (Hatzios and Penner 1982), and several genera of microorganisms are known to degrade PCP (Steiert and Crawford 1985; Chaudhry and Chapalamadugu 1991). This chapter presents experiments used to assess the relative contributions of plants and indigenous soil microbes to PCP mineralization in order to better understand PCP degradation in the CWG rhizosphere. Inoculated and axenic CWG seedlings in BOCSystems were exposed to ^{14}C-labeled PCP and the distribution of radioactivity determined after 6 weeks. The results of this study showed that indigenous soil microorganisms were capable of PCP mineralization whereas axenic CWG seedlings were not.

Materials and Methods

The BOCSystems described in Chapter 2 were modified to allow continuous air flow by fitting them with an inlet air port in the top box and an outlet air port at the level of the sand surface (Figure 5) in order to quantify ^{14}C-PCP mineralization rates. Ten g quartz sand were autoclaved and treated with 2 mg kg^{-1} (297,098 dpm and 272,227 dpm in the first and second experiments, respectively) ^{14}C-PCP as described in Chapter 2. A portion of the treated sand (9.5 g) was poured into a sterile 15-ml polypropylene Falcon tube (Fisher Scientific, Pittsburgh, PA), which had its bottom removed and a rolled Whatman 3 MM filter paper wick inserted. The tubes were placed into the second Magenta box so that the wicks were partially submerged in the 0.75x Hoagland's growth medium. Axenic CWG seedlings were treated by submerging their roots in either 250 µl PTT inoculum (PTT soil extract as described in Chapter 2), autoclaved inoculum or sterile 2.5 mM phosphate buffer (sterile control) and transplanted into the sand. The remaining inoculum or buffer was dripped onto the roots, which were then covered with 0.5 g PCP-treated sand. Phosphate buffer (750 µl [first experiment] or 1 ml [second experiment]) was added to the sand to aid in seedling establishment. There were 2 CWG seedlings per BOCSystem. Unplanted treatments were prepared in the same manner. The BOCSystems were assembled, sealed with 100% silconized acrylic, and connected to a filtered (0.22 µm) air supply at 250 ml min^{-1}. Air leaving the BOCSystems was passed through individual traps containing 100 ml 0.5 N NaOH to trap mineralized ^{14}C-PCP as $^{14}CO_2$. A 5-ml aliquot of the NaOH was sampled every 7 days and analyzed for radioactivity using liquid scintillation counting (LSC). Volumes of the CO_2 traps were

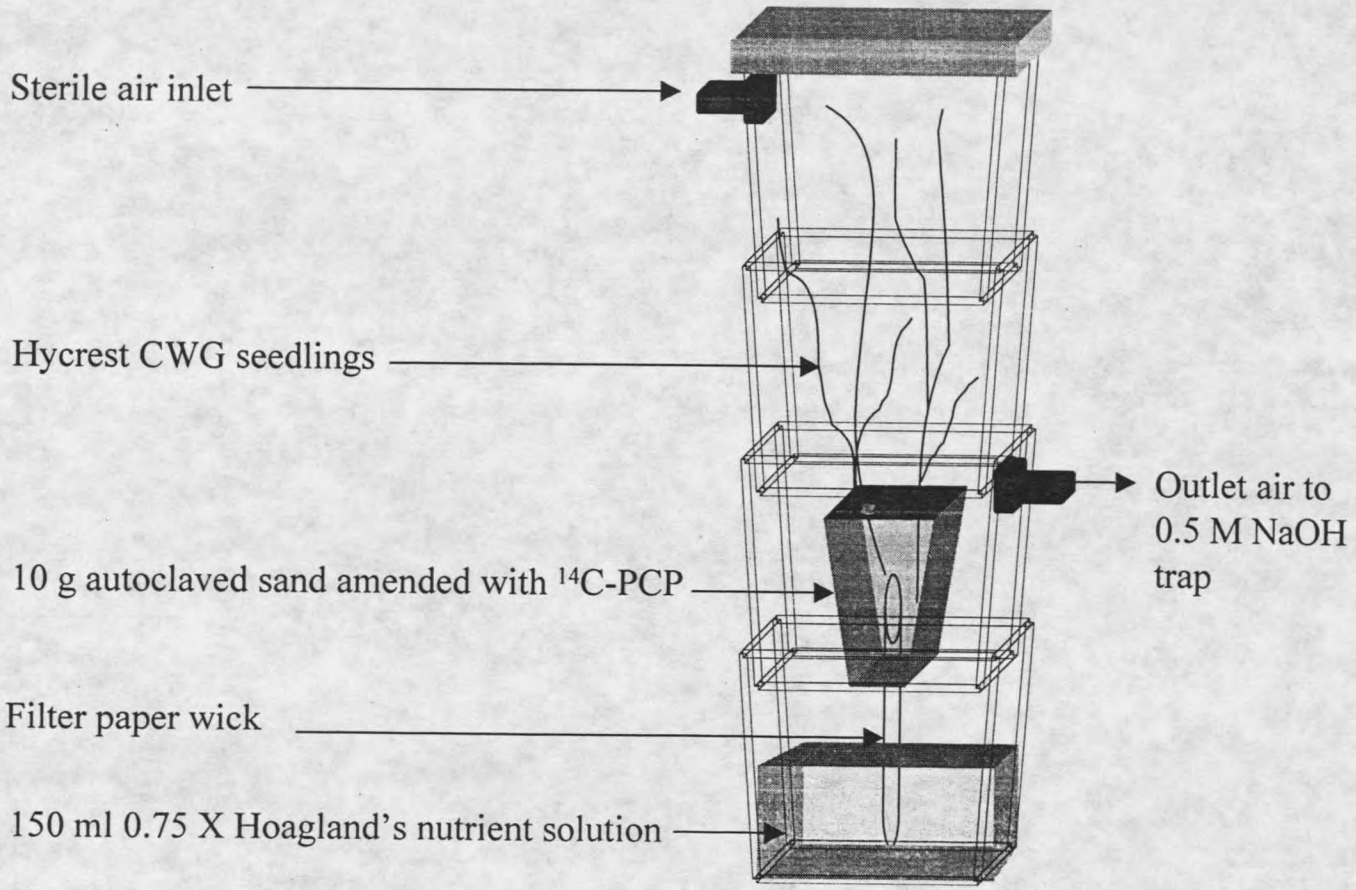

Figure 5. Biological Organism Closed System (BOCSystem) Modified for Continuous Air Flow used for ^{14}C-PCP Metabolism Experiments.

measured at each sampling time to normalize the data. Radioactivity in the NaOH was assumed to be from $^{14}CO_2$, and no attempt was made to trap volatile organic ^{14}C since similar studies found less than 2% of applied ^{14}C-PCP in the volatile organic fraction (Ferro et. al 1994; Scheunert et al. 1986). The experiment was conducted for 42 days under a 15-hr photoperiod (86 $\mu mol\ s^{-1}\ m^{-2}$) and a 24°C/16°C day/night temperature regime.

After 42 days, shoots were excised at the sand surface, roots were gently rinsed of sand with deionized distilled water, and the sand and wick were set aside to air dry. Fresh and dry weights were determined for shoots and roots from each BOCSystem, the plant tissue was combusted in a biological sample oxidizer (Model OX500, R.J. Harvey, NJ), and the resulting $^{14}CO_2$ trapped in basic scintillation cocktail. Air-dried sand and wicks were gently separated by hand and the sand was weighed, stirred and one (first experiment) or two (second experiment) 1-g subsamples were oxidized. The dried wicks were weighed, and twenty 3 mm x 4.5 mm subsamples from each wick were pooled and oxidized. Final volumes of the growth medium were determined in each BOCSystem and 5-ml subsamples taken for radioactivity determination using LSC.

There were six treatments (sterile, inoculum and autoclaved inoculum in the presence and absence of CWG) with three replicates, plus one replicate each of no-^{14}C-PCP sterile, inoculated and autoclaved inoculum-treated CWG controls (which were combined for the "Control" data) for a total of 21 BOCSystems. The experiment was repeated once. One way analysis of variance was conducted on the amounts of radioactivity in roots and shoots in the second experiment and a 5% LSD pairwise

multiple comparison test was used to determine differences among treatments. Kruskal-Wallis one way analysis of variance on ranks was conducted on the $^{14}CO_2$ data and the Student-Newman-Keuls pairwise multiple comparison test was used to determine differences among treatments.

Results and Discussion

Table 5 shows the distribution of radioactivity in the different compartments of the plant-microbe-sand system and the percent of radioactivity recovered from each treatment. Overall, both planted and unplanted inoculated treatments caused greater mineralization of ^{14}C-PCP than sterile treatments. BOCSystems containing inoculated plants mineralized 18.3% of ^{14}C-PCP while BOCSystems with axenic plants only converted 5.3% of ^{14}C-PCP to $^{14}CO_2$. Inoculated (unplanted) sand mineralized 20.9% of ^{14}C-PCP versus 4.4% mineralization in sterile sand. Thus, inoculated treatments showed significantly greater ($P < 0.05$) mineralization than sterile treatments. The sterile, unplanted treatment represents abiotic PCP behavior and distribution within the BOCSystem. The amounts of $^{14}CO_2$ evolved from sterile CWG (5.3%) and autoclaved inoculum (4.8% and 4.9%, unplanted and planted, respectively) treatments were not significantly different from the sterile control (4.4%), indicating that microorganisms, but not plants, were capable of PCP mineralization.

The two experimental repeats are presented separately in Table 5 for four reasons. First, the majority of CWG seedlings in all treatments expired before the end of the first experiment, whereas seedlings were still growing well by the end of the second

Table 5. ^{14}C Recovered from the Modified BOCSystem Compartments Expressed as Percent of ^{14}C Initially Applied ± the Standard Error of the Mean of Three Replicates in the Individual Experiments and Six Replicates in the Combined* Data.

Treatment	$^{14}CO_2$	Growth Medium	Sand	Wick	Shoots	Roots	Total Recovered
			Experiment #1				
Control (No ^{14}C-PCP)	0	0	0	0	0	0	0
Inoculated	19.6 ± 0.6	0.3 ± 0.2	20.7 ± 4.6	3.4 ± 0.0	--	--	44.0 ± 4.8
Inoculated + CWG	17.1 ±0.6	0.2 ± 0.1	14.9 ± 2.3	3.4 ± 0.0	2.6 ± 1.3	14.1 ± 0.8	52.3 ± 1.7
Sterile	4.5 ± 1.1	0.6 ± 0.1	13.5 ± 2.1	3.4 ± 0.0	--	--	21.9 ± 2.6
Sterile CWG	4.5 ± 0.1	0.7 ± 0.5	10.1 ± 1.3	3.4 ± 0.0	4.9 ± 0.4	19.6 ± 0.8	43.2 ± 2.0
Sterile Inoculum	5.1 ± 0.9	1.0 ± 0.2	14.4 ± 3.4	3.4 ± 0.0	--	--	23.8 ± 3.9
Sterile Inoculum + CWG	4.7 ± 0.2	0.4 ± 0.1	10.0 ± 0.2	3.4 ± 0.0	4.2 ± 1.4	15.8 ± 3.0	38.4 ± 4.3
			Experiment #2				
Control (No ^{14}C-PCP)	0	0	0	0	0	0	0
Inoculated	22.1 ± 4.4	0.3 ± 0.1	32.7 ± 9.4	6.4 ± 1.1	--	--	52.9 ± 11.3
Inoculated + CWG	19.4 ± 1.7	0.1 ± 0.1	20.4 ± 5.3	3.5 ± 0.6	3.9 ± 1.3	15.1 ± 2.2	62.9 ± 7.7
Sterile	4.3 ± 0.6	1.1 ± 0.1	9.5 ± 0.5	2.4 ± 0.5	--	--	17.7 ± 0.3
Sterile CWG	6.1 ± 0.2	0.1 ± 0.1	10.3 ± 0.9	2.4 ± 0.5	10.5 ± 1.0	30.2 ± 3.8	61.5 ± 1.3
Sterile Inoculum	4.6 ± 0.9	0.2 ± 0.1	12.3 ± 1.8	2.7 ± 0.3	--	--	21.9 ± 3.2
Sterile Inoculum + CWG	5.0 ± 0.8	0.2 ± 0.2	7.9 ± 1.3	1.9 ± 0.4	10.5 ± 1.6	29.6 ± 2.0	56.9 ± 3.1
			Experiments #1 and #2 Combined*				
Control (No ^{14}C-PCP)	0	0	0	0	0	0	0
Inoculated	20.9 ± 2.1	0.3 ± 0.1	26.7 ± 5.4	4.9 ± 0.8	--	--	48.5 ± 5.8
Inoculated + CWG	18.3 ± 1.0	0.2 ± 0.0	17.7 ± 2.9	3.4 ± 0.3	3.3 ± 0.9	14.6 ± 1.1	57.6 ± 4.2
Sterile	4.4 ± 0.5	0.8 ± 0.2	11.5 ± 1.3	2.9 ± 0.3	--	--	19.8 ± 1.5
Sterile CWG	5.3 ± 0.4	0.4 ± 0.2	10.2 ± 0.7	2.9 ± 0.3	7.7 ± 1.3	24.9 ± 2.9	52.4 ± 4.2
Sterile Inoculum	4.8 ± 0.6	0.6 ± 0.2	13.3 ± 1.8	3.0 ± 0.2	--	--	22.9 ± 2.3
Sterile Inoculum + CWG	4.9 ± 0.4	0.3 ± 0.1	9.0 ± 0.7	2.6 ± 0.4	7.4 ± 1.7	22.7 ± 3.5	47.7 ± 4.7

* Data combined for qualitative purposes only.

experiment. This is reflected by the fact that twice the amount of radioactivity in the roots and shoots of sterile and sterile inoculum CWG seedlings was recovered in the second experiment as compared to the first experiment. The increased seedling survival rate in the second experiment is probably due to better root establishment because of an additional 250 μl of 2.5 mM phosphate buffer added to the sand just after transplanting. Second, root radioactivity and $^{14}CO_2$ data from the two experiments could not be combined because of unequal variances and non-normal distribution, respectively. Third, radioactivity amounts in the wicks were estimated as the average radioactivity in 5 representative wick samples in the first experiment whereas the radioactivity of each BOCSystem wick was measured in the second. Fourth, since there were two subsamples of sand analyzed in the second experiment versus only one subsample in the first experiment, I believe that sand data from the second experiment are more reliable. Even with these differences between experiments, both showed the same relative levels of $^{14}CO_2$ production among the different treatments, suggesting that data from the two experiments should provide an accurate representation of treatment effects on $^{14}CO_2$ production.

PCP is readily incorporated into organic matter (Christodoulatos et al. 1994; Bellin et al. 1990; Bengtsson et al. 1993) such as microbial biomass and plant roots by sorption or direct uptake. Planted treatments had the greatest amount of biomass and the highest amounts of radioactivity recovered (Table 5). The amount of radioactivity recovered from the sterile treatments was low, and was lowest in the sterile unplanted treatment, which contained no organic matter other than the wick (cellulose fibers) and

the Falcon tube (polypropylene). Although the Falcon tubes were not analyzed for PCP, it is likely that PCP sorbed to the plastic, as polypropylene is the most hydrophobic component in the BOCSystem and PCP has been found to readily sorb to organic polymers such as polyacrylamide (Bengtsson et al. 1993). Amounts of radioactivity recovered from the sand were significantly greater in the inoculated (unplanted) treatment and than the sterile treatments in the second experiment (Table 5). I speculate that some of the radioactivity recovered from the sand in the inoculated treatments was derived from ^{14}C incorporated into microbial biomass. Microorganisms also colonized the wicks in the inoculated treatments, especially in the unplanted, inoculated treatments, which might explain the greater radioactivity in these treatments. Only a small amount of radioactivity was found in the growth medium from all treatments (Table 5), which was most likely a result of a small amount of treated sand falling into the growth medium chamber during set-up, but could also be due to $^{14}CO_2$.

Figure 6 shows the amounts of $^{14}CO_2$ evolved over time expressed as percentages of the applied radioactivity. Sterile treatments showed a low but steady release of $^{14}CO_2$ over 42 days, while the inoculated treatments exhibited greater $^{14}CO_2$ production by 7 days after transplanting as compared to the sterile treatments. The highest rates of $^{14}CO_2$ evolution in the inoculated treatments occurred between 7 and 14 days after transplanting (1.0% and 0.96% of applied radioactivity released as $^{14}CO_2$ per day in inoculated and inoculated + CWG treatments, respectively). These results may be explained by an initial acclimation period (about 7 days) followed by a high rate of $^{14}CO_2$ evolution which progressively decreased due to reduced concentrations of bioavailable PCP.

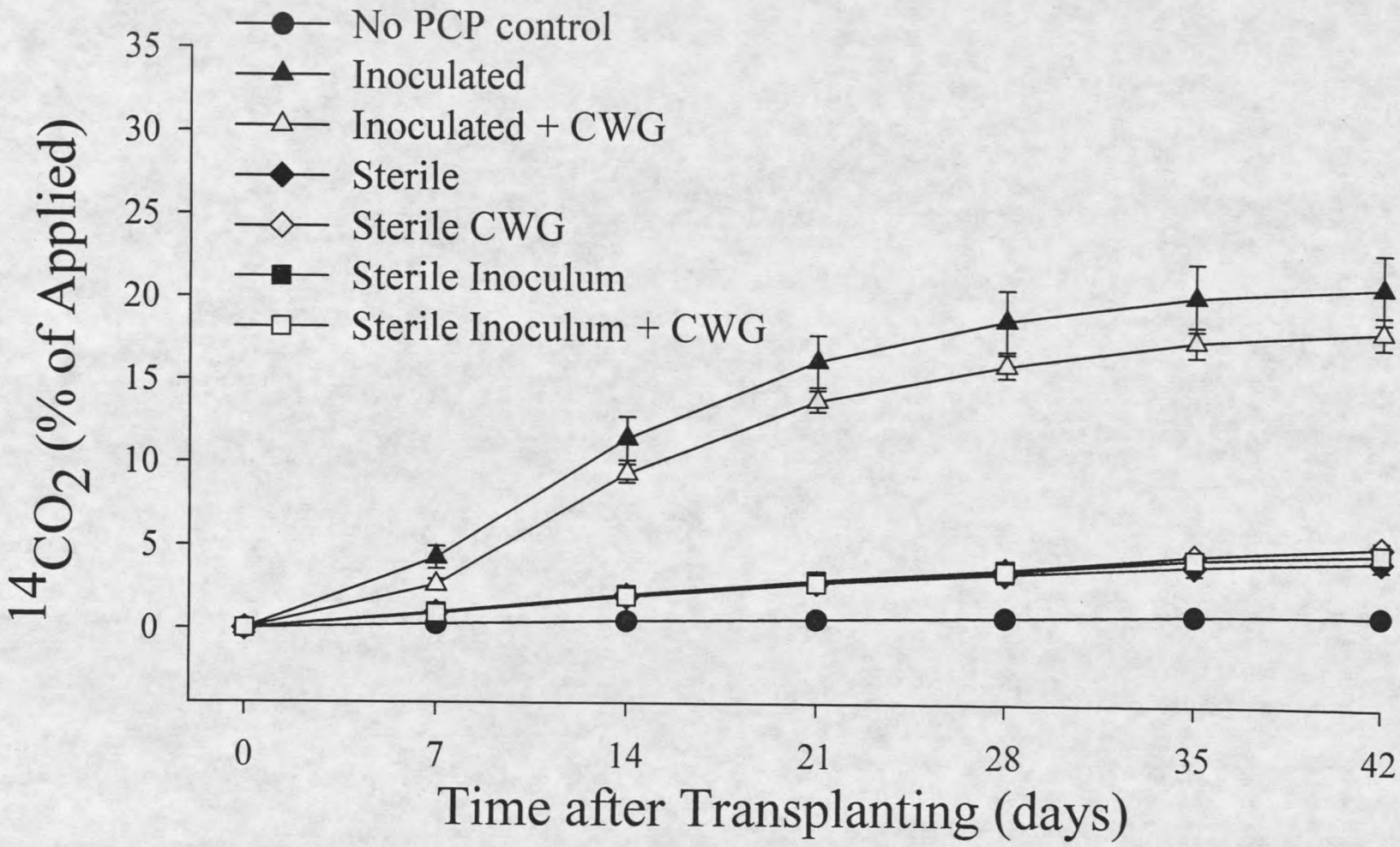

Figure 6. ^{14}C-PCP Mineralization in the Modified BOCSystems. Vertical bars represent standard errors of the means.

One of the hypotheses of this thesis is that CWG plants accelerate the degradation of PCP in soil. The results in Figure 6 showing that $^{14}CO_2$ production was not significantly different in inoculated and inoculated + CWG treatments do not support this hypothesis. The BOCSystems used for these experiments were designed to represent a simplified ecosystem to examine the individual contributions of plants and microbes to PCP mineralization without the potentially confounding aspects of whole soil, such as contamination in sterile treatments. In contrast to the results obtained here, Ferro et al. (1994) reported that planted treatments mineralized 22% of applied radioactivity as compared to unplanted (unsterile) controls, which mineralized 6.3% of applied radioactivity. I suspect that my results were different from those in whole soils because of lower levels of CWG root exudation in the BOCSystems and the low concentration of PCP used in the BOCSystem, as explained below. Root exudation is strongly affected by light intensity (Ferro et al. 1994), particle size of growth medium (Barber and Gunn 1974), growth and health of the plant (Barber and Gunn 1974; Metzger et al. 1986), and presence or absence of microorganisms (Prikryl and Vancura 1980; Rovira 1979). The light intensity in my experiments was only 86 $\mu mol\ m^{-2}\ s^{-1}$ during a 15-hour photoperiod as compared to 350 $\mu mol\ m^{-2}\ s^{-1}$ during a 16-hour photoperiod reported by Ferro et al. (1994). Those authors point out that higher radiation and longer photoperiod significantly increase root exudation, thus lower photosynthetic activity and root exudation might be expected of the BOCSystem CWG. Sand is coarser than the sandy loam soil used by Ferro et al. (1994), which might cause less root branching and less root exudation from CWG plants grown in sand (Barber and Gunn 1974). Finally, the

numbers of microorganisms inoculated into BOCSystems is only a small fraction (0.5%, according to buffer dilution of PTT soil) of the number of microorganisms found in the same volume of whole soil. Therefore, such low numbers of microorganisms introduced into BOCSystems might be expected to induce less root exudation in the BOCSystem CWG as compared to CWG seedlings grown in whole soil. Thus, conditions under which CWG seedlings were grown in my experiments may have altered the composition or amount of root exudates, and hence limited their stimulatory effect on PCP degradation. Another reason my results do not agree with those of Ferro et al. (1994) might be due to the lower PCP concentration used in my experiments (2 mg kg^{-1} compared to 100 mg kg^{-1} used in Ferro et al.'s [1994] experiment). Results from my experiments on PCP disappearance in PTT soil suggest that at lower PCP concentrations there are no differences among planted and unplanted treatments, although differences were observed at higher PCP concentrations (Chapter 2).

$^{14}CO_2$ production in the inoculated treatments asymptotically approached 25% of the applied radioactivity (Figure 6). In contrast, Saber and Crawford (1985) reported that five PCP-acclimated pure cultures released 73% to 83% of applied ^{14}C-PCP as $^{14}CO_2$ in liquid medium after 2 to 4 days. The lower rate of PCP mineralization in my experiments could be due to incorporation of radioactivity into microbial biomass and sorption to the plastic tubes and filter paper wicks (Bellin et al. 1990; Bengtsson et al 1993). Bengtsson et al. (1993) observed linear distribution coefficients (D) of 11,000 L kg^{-1}, 860 L kg^{-1} and 3 L kg^{-1} for PCP sorption onto bacteria, polymethylacrylate and kaolinite clay, respectively, at pH 6.5 (where $D = C_s/C$; C_s is the concentration of PCP associated with

the solid phase in mol kg^{-1} and C is the concentration of PCP in solution in mol L^{-1}). Because bacteria and hydrophobic polymers sorb PCP much more strongly than clays, which are typically negatively charged and hydrophilic (like sand), PCP may have been partitioned among the BOCSystem components and thus less bioavailable than in a well-mixed liquid culture.

Figure 7 provides a graphical representation of the radioactivity recovered from shoots and roots in the second experiment.

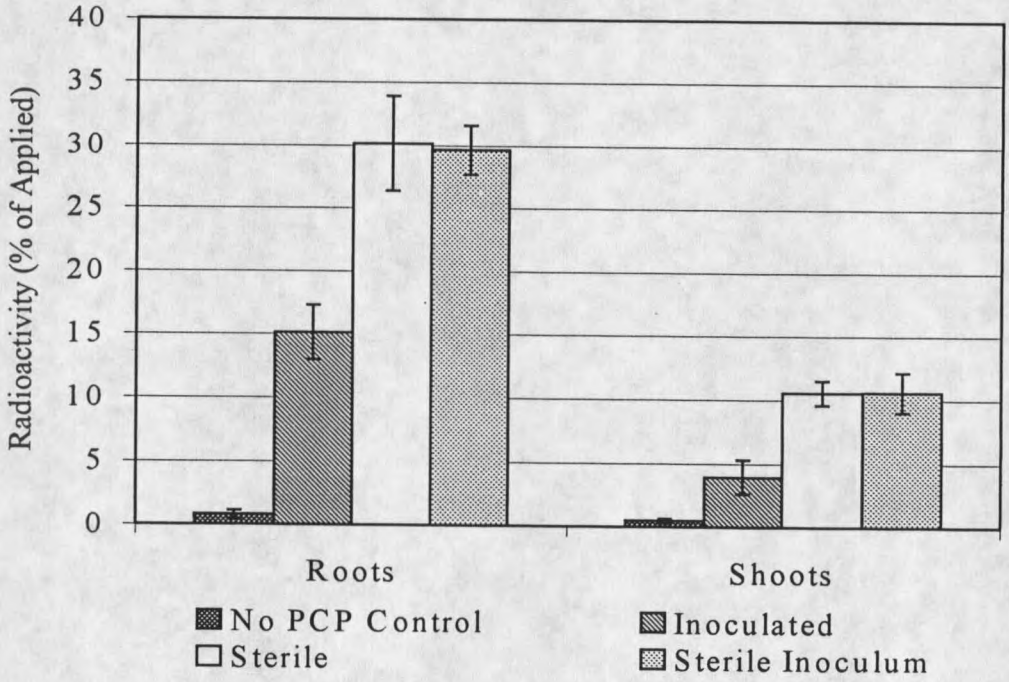

Figure 7. Radioactivity Recovered from CWG Roots and Shoots in ^{14}C-PCP Experiment #2 Shown as Percentage of Applied Radiation. Vertical bars represent standard errors of the means.

Inoculated seedlings contained 49% less radioactivity in their roots and 63% less radioactivity in their shoots as compared to sterile and sterile inoculum-treated CWG

seedlings. At the end of the second experiment, CWG seedlings from all treatments were similar in size, with inoculated and sterile inoculum-treated seedling shoots averaging 0.09 g dry mass, sterile seedling shoots averaging 0.12 g and control seedling shoots averaging 0.16 g. Control seedling roots averaged 0.14 g dry mass, and inoculated, sterile, and sterile inoculum-treated seedling roots averaged 0.12 g, 0.09 g, and 0.05 g dry mass, respectively. Thus, the level of PCP used in this experiment allowed all seedlings to grow so that uptake of radioactivity could be accurately compared among treatments. Sterile and sterile inoculum-treated roots and shoots contained 30% and 10.5% of the applied radioactivity, respectively, and inoculated seedlings contained 15.1% and 3.9% of the applied radioactivity in their roots and shoots, respectively. These results suggest that microorganisms in the CWG rhizosphere reduced the amount of PCP taken up and translocated by CWG seedlings. Rhizosphere microorganisms capable of rapid PCP mineralization might intercept a significant amount of PCP before it reaches the plant root, thus reducing plant uptake of PCP.

The use of ^{14}C-labeled PCP in the CWG-microbe-sand system showed that microorganisms are responsible for PCP mineralization and that sterile CWG plants are incapable of mineralizing PCP under the conditions described here. However, my first planted and unplanted PTT soil experiment (Chapter 2) and Ferro et al.'s (1994) experiments suggest CWG plants play a role in PCP degradation. Did CWG root exudates stimulate rhizosphere microbes beyond the simple effect of growth-linked metabolism of PCP? The next chapter explores this question using CWG root exudates and liquid cultures of PTT soil extract.

CHAPTER 4

ENUMERATION OF PCP-DEGRADING MICROORGANISMS FROM CONTAMINATED SOIL

Introduction

Experiments discussed in the previous chapters suggest that indigenous soil microorganisms are primarily responsible for PCP mineralization in the CWG rhizosphere. It would be useful to know the number of microorganisms capable of PCP transformation per gram of soil to establish repeatability, for future comparison with other contaminated soils, and to better understand the CWG rhizosphere system. The most probable number (MPN) method of microbial enumeration was conducted on freshly collected PTT soil before the soil was used as a source of inoculum in Chapter 2 and 3 experiments. The number of microorganisms from PTT soil added to BOCSystems was also estimated at the beginning of each dose response and ^{14}C-PCP experiment by the MPN method.

The MPN approach is used to enumerate slow-growing bacteria or bacteria for which there is no selective medium available (Cochran 1950). The method requires an observable parameter that can be rated as positive or negative for microbial activity, such as turbidity from growth, nitrate reduction, or colorimetric change due to metabolic activity (Alexander 1982; Cochran 1950). The culture to be enumerated is diluted, usually by a series of ten-fold dilutions, until it is suspected that the organisms of interest have been diluted to extinction. Three to ten replicate cultures of each dilution are made,

and after incubation, the cultures are rated as positive or negative for microbial presence according to the chosen and relevant parameter. The MPN is determined from tabulated values according to the highest dilution for which all tubes are rated positive for microbial presence and the number of positive tubes in the next highest two dilutions (Alexander 1982). It should be noted that the MPN method assumes that: 1) the microbes are randomly distributed throughout the original culture and, 2) a culture containing at least one relevant organism will be rated as positive. If the second assumption is not met, that is, if more than one organism or a longer incubation is required for a positive rating, then the MPN will underestimate the number of microorganisms present in the culture (Cochran 1950). Thus the MPN can be considered a conservative estimate and is best used when compared to other MPN estimates obtained in the same fashion.

Several studies have used the MPN method to track changes in the numbers of PCP-degrading microorganisms (Sato 1997; Sato and Lee 1996; Watanabe 1977) or to determine the number of 2,4-D degraders (Sandmann and Loos 1984) in soil. Sandmann and Loos (1984) used a bromocresol purple colorimetric assay to determine the MPN of 2,4-D degraders in rhizosphere and nonrhizosphere soil. Watanabe (1977), Sato (1997) and Sato and Lee (1996) measured the disappearance of 10 mg L^{-1} PCP from dilution cultures (which also contained 0.05-0.1 g L^{-1} yeast extract) after 4 or 5 weeks of incubation. The MPN method used in the present study estimated PCP-degrading microbial numbers in PTT soil based on the disappearance of 50 mg L^{-1} PCP from culture medium after 2 weeks of incubation; therefore, the MPN estimates in this study are more conservative than in previously published studies.

While it appears that the microbial component of the CWG-microbe system is responsible for PCP mineralization, the CWG must provide a stimulus to microorganisms in the rhizosphere in order to explain enhanced PCP degradation in the rhizosphere as compared to unplanted soil (Ferro et al. 1994). Because plants interact with bacteria through root exudates (Curl and Truelove 1986), I hypothesized that CWG root exudates would selectively encourage the proliferation of microorganisms capable of transforming PCP. To test this idea, an MPN experiment was conducted on PTT soil cultures in the presence and absence of CWG root exudates.

Materials and Methods

MPN of PCP-Degraders in PTT Soil

A preliminary MPN experiment was conducted on freshly collected PCP-contaminated PTT soil. PTT soil inocula were prepared as described in Chapter 2 and Mueller et al. (1991) with the exception that 10 g of moist soil were extracted in 50 ml 2.5 mM phosphate buffer (pH 7.0) by shaking at 250 cpm for 2 hours. The supernatant was used to make 10^{-2} to 10^{-8} dilutions in 2.5 mM phosphate buffer. One-half ml of these dilutions was inoculated into tubes containing 4.5 ml of PCP MPN medium (Saber and Crawford 1985; Watanabe 1977) (in g per L: $NaNO_3$, 0.5; K_2HPO_4, 0.65; KH_2PO_4, 0.17; $MgSO_4 \circ 7H_2O$, 0.2; yeast extract, 0.1; PCP as Na^+ PCP^- in 0.2 M NaOH, 0.05; final pH ~7.3) to make 10^{-2} to 10^{-9} culture dilutions. Sterile inoculum was used directly to inoculate a 10^{-2} dilution, and 2.5 mM phosphate buffer was used for an uninoculated control. Cultures were incubated at 30°C at 250 rpm for 2 weeks, after which they were

filtered through a 0.22 μm polyethersulfone syringe filter (Millipore) and the filtrate analyzed for PCP concentration spectrophotometrically at 317 nm. Tubes initially contained 45 mg L^{-1} PCP and were rated positive if after two weeks they contained less than 25 mg L^{-1} PCP, the level chosen as described by Alexander (1982) and Cochran (1950). There were five replicate tubes per dilution.

MPN Estimates on Inoculum Used in CWG Experiments

The numbers of PCP-degrading microorganisms inoculated into each BOCSystem at the beginning of the PCP dose response and ^{14}C-PCP experiments were determined as described above, except using 20 g of moist PTT soil extracted into 100 ml of 2.5 mM phosphate buffer. Two replicate extractions were made each time and the supernatants combined for a total of approximately 200 ml inoculum for use in the experiment and for MPN estimation. Additionally, initial PCP concentration in the MPN dilution tubes was 50 mg L^{-1}. The incubation and rating criteria remained the same as before.

DPY CWG Root Exudate Collection

CWG root exudates were collected for use in MPN experiments with the PTT soil inoculum to better understand the role of CWG in accelerated PCP degradation. Root exudate collection followed the methods of Graham et al. (1981). CWG plants were grown in the greenhouse for one year from seed collected from the PCP-contaminated DPY site. Plants were grown in a greenhouse soil mix composed of equal parts Bozeman silt loam soil, washed concrete sand, and Canadian sphagnum peat moss. Two CWG plants were removed from their pots and the roots rinsed thoroughly under cool tap water.

The root mats with the shoots attached were soaked in sterile, aerated 0.5 mM $CaCl_2$ containing 0.05 g L^{-1} rifampicin and 0.025 g L^{-1} tetracycline for 2 hours in order to surface sterilize the roots. The antibiotic solution was thoroughly rinsed from the roots with several washes of sterile deionized distilled water, the roots were soaked for 5 minutes in sterile, aerated 0.5 mM $CaCl_2$ without antibiotics, and then transferred to 800 ml sterile, aerated 0.5 mM $CaCl_2$ for 22 hours. About half of the shoots were trimmed off after the antibiotic treatment in order to increase root exudation (Curl and Truelove 1986). The two root mats were wrung out by hand and the solutions combined for a total of 1,100 ml root exudate. The exudate was frozen at –20°C, thawed, and 750 ml were concentrated by rotary evaporation at 44°C to approximately 250 ml. The concentrate was filter sterilized (0.22 μm polyethersulfone, Millipore) and stored at 4°C. The total organic carbon (TOC) in the concentrated root exudate solution was 693 mg C L^{-1}.

MPN of PCP Degraders in the Presence of CWG Root Exudate

Inoculum from PTT soil was prepared as previously described by extracting 10 g PTT soil into 50 ml 2.5 mM phosphate buffer for 2 hours. For the control MPN, 130 ml PCP MPN medium was combined with 20 ml 1.5 mM $CaCl_2$ (as the root exudate was concentrated roughly by a factor of three). The root exudate MPN medium contained 130 PCP MPN medium plus 20 ml sterile, concentrated root exudate. Cultures were prepared as before, with 4.5 ml modified PCP MPN medium (containing 50 mg L^{-1} PCP and $CaCl_2$ or root exudate) inoculated with 0.5 ml of the appropriate inoculum dilution. Tubes were incubated at 30°C with shaking at 250 cpm. After 18 days, the cultures were filtered as before and PCP concentration determined spectrophotometrically at 317 nm.

There were five tubes of each dilution from 10^{-2} to 10^{-5} for each treatment, plus uninoculated and autoclaved inoculum controls.

Results and Discussion

MPN of PCP-Degraders in PTT Soil and Inoculum Used in Experiments

The initial MPN experiment estimated 2,450 PCP degraders per gram of moist PCP-contaminated PTT soil (Table 6). The MPN estimates of PCP-degraders used in PCP dose response and ^{14}C-PCP experiments were lower and ranged from 165 to 550 PCP degraders per gram of moist PTT soil. Later MPN estimates may have been 10-fold lower as a result of extended storage periods of the PTT soil. Sterilized soil inoculum and phosphate buffer control dilutions showed no PCP degradation in any of the MPN experiments.

Table 6. Most Probable Number (MPN) of PCP Degraders in PTT Inoculum Added to Inoculated CWG Treatments.

Experiment	MPN PCP-degraders per g soil	95% Confidence Interval
Initial MPN on PTT soil	2,450	740-8,100
PCP dose response #1	165	50-550
PCP dose response #2	245	75-800
^{14}C-PCP experiment #1	395	120-1,300
^{14}C-PCP experiment #2	550	165-1,800

MPN of PCP Degraders in the Presence of CWG Root Exudate

The MPN method estimated 8,500 PCP degraders (95% C.I.: 2,600-28,000) in the PTT soil inoculum in the presence of CWG root exudate and 85 PCP degraders (95% C.I.: 26-280) in the no exudate control. Thus, there were 100-fold more PCP-degraders

in the presence of CWG root exudates than in the control. Table 7 shows data from which these estimates were calculated, including the concentrations of PCP in each culture and how the dilutions were rated for PCP degradation.

Table 7. CWG Root Exudate Effect on the MPN of PCP Degraders in PTT Soil.

Control PCP MPN Medium			Root Exudate PCP MPN Medium		
Dilution	Final PCP Concentration (mg L^{-1})	Rating	Dilution	Final PCP Concentration (mg L^{-1})	Rating
10^{-2}	2	+	10^{-2}	<1	+
10^{-2}	2	+	10^{-2}	<1	+
10^{-2}	3	+	10^{-2}	1	+
10^{-2}	49	-	10^{-2}	<1	+
10^{-2}	49	-	10^{-2}	<1	+
10^{-3}	3	+	10^{-3}	<1	+
10^{-3}	4	+	10^{-3}	<1	+
10^{-3}	41	-	10^{-3}	2	+
10^{-3}	36	-	10^{-3}	1	+
10^{-3}	44	-	10^{-3}	<1	+
10^{-4}	4	+	10^{-4}	1	+
10^{-4}	38	-	10^{-4}	2	+
10^{-4}	45	-	10^{-4}	<1	+
10^{-4}	45	-	10^{-4}	<1	+
10^{-4}	37	-	10^{-4}	35	-
10^{-5}	46	-	10^{-5}	16	+
10^{-5}	46	-	10^{-5}	37	-
10^{-5}	45	-	10^{-5}	37	-
10^{-5}	40	-	10^{-5}	39	-
10^{-5}	41	-	10^{-5}	38	-
aic*	39	-	aic	39	-
aic	39	-	aic	39	-
0**	38	-	aic	39	-
0	37	-	0	37	-
0	37	-	0	38	-

*aic = autoclaved inoculum control, **0 = phosphate buffer uninoculated control

These results support the hypothesis that CWG root exudates encourage the proliferation of microorganisms capable of mineralizing PCP. However, this stimulation

could also be due to the greater concentration of total organic carbon (TOC) in the root exudate treatment than in the control treatment. The root exudates, which contained 693 mg L^{-1} TOC, contributed 92 mg L^{-1} TOC to the root exudate treatment. In comparison, if it is assumed that 50% of the 0.1 g L^{-1} yeast extract added to the MPN medium was readily available carbon, then both the control and root exudate MPN medium contained approximately 43 mg L^{-1} TOC from yeast extract. Thus, there was approximately 135 mg L^{-1} TOC in the root exudate treatment (92 mg L^{-1} TOC from root exudates plus 43 mg L^{-1} TOC from yeast extract) and 43 mg L^{-1} TOC in the control treatment.

Similar studies by Sato and Lee (1996) and Sato (1997) showed that the presence of easily utilizable carbon substrates in soil suspensions reduced the effective number of PCP-degrading microorganisms as compared to PCP as the sole carbon source. These authors observed greater PCP degradation in PCP only treatments as compared to glucose-, glycine-, glutamate- or cellulose-amended treatments. Although they did not attempt to equalize carbon concentration among treatments, they hypothesized that readily utilizable carbon compounds suppressed the proliferation of PCP-degrading organisms because: 1) PCP-degraders cannot compete with other microorganisms for nutrients, or 2) the microbes' ability to degrade PCP was lost in the presence of readily available nutrients. In contrast, I observed the opposite results in PTT soil inoculum in the presence of CWG root exudates, since the exudates increased numbers of PCP-degrading microorganisms. I believe that the PCP degraders in PTT soil either: 1) required additional carbon to degrade PCP efficiently, or 2) compound(s) in CWG root exudate triggered more effective PCP degradation. The latter may occur through a co-

metabolism mechanism. Gilbert and Crowley (1997) showed that the presence of *l*-carvone, an aromatic compound from spearmint, induced polychlorinated biphenyl degradation in *Arthrobacter* sp. strain B1B. Since plants are known to contain a significant amount of phenols (Pridham 1965), CWG root exudates most likely contain phenolic or similar aromatic compounds; thus enhanced co-metabolism of PCP by CWG rhizosphere inhabitants was possible. Additionally, the CWG rhizosphere may provide a secure niche for PCP-degrading microorganisms, since the rhizospheres of other plant species [Wimmera ryegrass, subterranean clover and maize (*Zea maize* L. cv. Pioneer 3183)] were found to be enriched in gram-negative bacteria (Sperber and Rovira 1959; Scher et al. 1984), and a separate study found all 40 PCP-degrading isolates to be gram-negative (Saber and Crawford 1985).

Recent rhizosphere research has focused attention on root border cells (BRD's) as a means by which plants influence the rhizosphere (Hawes 1990). BRD's are intact, respiring cells actively released from growing root tips that are believed to intercept possible plant pathogens and to otherwise influence rhizosphere microbial populations for the benefit of the plant (Hawes and Brigham 1992). Since CWG root exudates used in my experiment were passed through a 0.22 μm filter, they did not contain any BRDs. However, further studies should examine the potential of BRDs to contribute to PCP degradation because such intact cells have the potential to deliver a variety of compounds to microorganisms near the CWG root.

MPN experiments showed that PCP-contaminated PTT site soil contains microorganisms capable of PCP degradation. HPLC analysis of positive-rated dilution

cultures showed no PCP metabolite peaks, thus the PTT microbial consortia appeared to completely mineralize PCP. MPN estimates increased 100-fold in the presence of CWG root exudates as compared to controls. Microorganisms capable of PCP mineralization were either increased in number or are metabolically stimulated in the CWG rhizosphere due to the release of an unidentified component of CWG root exudates. Further studies must be done to confirm this proposed mechanism and to identify the CWG root exudate component responsible for stimulating microbial PCP degradation.

CHAPTER 5

SUMMARY

Research presented in the previous chapters showed that survival and growth of CWG plants in PCP-contaminated sand was strongly enhanced by the presence of microorganisms indigenous to a PCP-contaminated site. Of the two microbial consortia able to degrade PCP in liquid culture, one protected CWG plants from the toxic effects of PCP while the other did not. Conversely, of the two microbial consortia that did not degrade PCP well in liquid culture, one protected CWG plants from PCP toxicity and the other did not. Thus, protection of CWG from PCP toxicity appears to require more than the microorganisms' ability to degrade PCP, and microorganisms that do not degrade PCP in liquid culture may still provide protection to CWG plants. ^{14}C-PCP studies showed that inoculated treatments mineralized PCP to $^{14}CO_2$ while sterile CWG seedlings were not capable of PCP mineralization. Inoculated CWG seedlings absorbed and translocated less PCP than sterile or autoclaved-inoculum treated seedlings, suggesting that microorganisms intercepted and degraded a significant amount of PCP in the rhizosphere. In addition, most probable number (MPN) experiments showed PCP degradation by indigenous soil microorganisms was stimulated 100-fold in the presence of CWG root exudates.

Although these results further our understanding of how phytoremediation may operate through rhizostimulation mechanisms, they also demonstrate the complexity of rhizosphere interactions and the difficulty of using laboratory models to understand or

predict field situations. Nonetheless, PCP-degrading organisms most likely proliferate in the CWG rhizosphere due to a favorable niche or competitive advantage created by CWG root exudation. The enhanced proliferation of PCP-degrading microorganisms, in turn, promotes the health of CWG plants growing in PCP contaminated soil by limiting the plant's exposure to PCP.

Revegetation of contaminated soils limits erosion by stabilizing the soil and reduces contaminant leaching through plant transpiration. Since CWG is able to survive in soils containing high levels of PCP, it may represent a valuable treatment possibility for site managers. Additionally, indigenous PCP-degrading microbial consortia may be recruited by the CWG rhizosphere, enhancing PCP mineralization in soil as well as reducing PCP migration. Further research is required to understand the specific mechanisms of microbial recruitment and the ultimate fate of PCP metabolites in plants and the environment.

LITERATURE CITED

Adler, P.R., R. Arora, A. El Ghaouth, D.M. Glenn, and J.M. Solar. 1994. Bioremediation of phenolic compounds from water with plant root surface peroxidases. J. Environ. Qual. 23:1113-1117.

Alexander, M. 1982. Most probable number method for microbial populations. *In* A.L. Page, R.H. Miller, and D.R. Keeney (Ed.) Methods of soil analysis, Part 2. Chemical and microbiological properties—Agronomy Monograph no. 9 (2nd Ed.) Am. Soc. Agron. and Soil Sci. Soc. Am. Madison, WI.

Alexander, M. 1999. Biodegradation and Bioremediation. (2nd Ed.) Academic Press, Inc. San Diego, CA.

Alvey, S. and D.E. Crowley. 1996. Survival and activity of an atrazine-mineralizing bacterial consortium in rhizosphere soil. Environ. Sci. Technol. 30(5):1596-1603.

Anderson, T.A., E.A. Guthrie, and B.T. Walton. 1993. Bioremediation in the rhizosphere—Plant roots and associated microbes clean contaminated soil. Environ. Sci. Technol. 27(13):2630-2636.

Anderson, T.A. and B.T. Walton. 1995. Comparative fate of [14C]trichloroethylene in the root zone of plants from a former solvent disposal site. Environ. Toxicol. Chem. 14(12):2041-2047.

Apajalahti, J.H.A. and M.S. Salkinoja-Salonen. 1984. Adsorption of pentachlorophenol (PCP) by bark chips and its role in microbial PCP degradation. Microb. Ecol. 10:359-367.

Apajalahti, J.H.A. and M.S. Salkinoja-Salonen. 1987. Dechlorination and *para*-hydroxylation of polychlorinated phenols by *Rodococcus chlorophenolicus*. J. Bacteriol. 169(2):675-681.

Aprill, W. and R.C. Sims. 1990. Evaluation of the use of prarie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. Chemosphere 20:253-265.

Baker, A.J.M. and R.R. Brooks. 1989. Terrestrial higher plants which hyperaccumulate metallic elements – a review of their distribution, ecology and phytochemistry. Biorecovery. 1:81-126.

Barbeau, C., L. Deschênes, D. Karamanev, Y. Comeau, and R. Samson. 1997. Bioremediation of pentachlorophenol-contaminated soil by augmentation using activated soil. Appl. Microbiol. Biotechnol. 48:745-752.

Barber, D.A. and K.B. Gunn. 1974. The effect of mechanical forces on the exudation of organic substances by the roots of cereal plants grown under sterile conditions. New Phytol. 73:39-45.

Beard, A.P., A.C. McRae, and N.C. Rawlings. 1997. Reproductive efficiency in mink (*Mustela vison*) treated with the pesticides lindane, carbofuran, and pentachlorophenol. J. Reproduction and Fertility. 111:21-28.

Bellin, C.A., G.A. O'Connor, and Y. Jin. 1990. Sorption and degradation of pentachlorophenol in sludge-amended soils. J. Environ. Qual. 19:603-608.

Bengtsson, G., R. Lindqvist, and M.D. Piwoni. 1993. Sorption of trace organics to colloidal clays, polymers, and bacteria. Soil Sci. Soc. Am. J. 57:1261-1270.

Boyd, S.A., M.D. Mikesell, and J. Lee. 1989. Chlorophenols in soils, p. 209-228. *In* Reactions and movement of organic chemicals in soils, SSSA Special Publication no. 22. Soil Sci. Soc. Am Madison, WI

Brown, E.J., J.J. Pignatello, M.M. Martinson, and R.L. Crawford. 1986. Pentachlorophenol degradation: a pure bacterial culture and an epilithic microbial consortia. Appl. Environ. Microbiol. 52(1):92-97.

Burken, J.G. and J.L. Schnoor. 1996. Phytoremediation: Plant uptake of atrazine and role of root exudates. J. Environ. Engineer. 11:958-963.

Casterline, Jr., J.L., N.M. Barnett, and Y. Ku. 1985. Uptake, translocation, and transformation of pentachlorophenol in soybean and spinach plants. Environ. Res. 37:101-118.

Chanama, S. and R.L. Crawford. 1997. Mutational analysis of *pcpA* and its role in pentachlorophenol degradation by *Sphingomonas* (*Flavobacterium*) *chlorophenolica* ATCC 39723. Appl. Environ. Microbiol. 63(12):4833-4838.

Chaudhry, G.R., and S. Chapalamadugu. 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. 55(1):59-79.

Chin-A-Woeng, T.F.C., W. de Priester, A.J. van der Bij, and B.J.J. Lugtenberg. 1997. Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. Molec. Plant-Microbe Interact. 10(1):79-86

Christodoulatos, C., G.P. Korfiatis, N.M. Talimcioglu, and M. Mohiuddin. 1994. Adsorption of pentachlorophenol by natural soils. J. Environ. Sci. Health. A29(5):883-898.

Christodoulatos, C. and M. Mohiuddin. 1996. Generalized models for prediction of pentachlorophenol adsorption by natural soils. Wat. Environ. Res. 68(3):370-378.

Cochran, W.G. 1950. Estimation of bacterial densities by means of the "most probable number". Biometrics 6(2):105-110.

Cooney, C.M. 1996. Sunflowers remove radionuclides from water in ongoing phytoremediation field tests. Environ. Sci. Technol. 30(5):194A.

Crosby, D.G. 1981. Environmental chemistry of pentachlorophenol. Pure & Appl. Chem. 53:1051-1080.

Cunningham, S.D., T.A. Anderson, A.P. Schwab, and F.C. Hsu. 1996. Phytoremediation of soils contaminated with organic pollutants. Adv. Agron. 56:55-114.

Cunningham, S.D. and W.R. Berti. 1993. Remediation of contaminated soils with green plants: an overview. In Vitro Cell. Dev. Biol. 29P:207-212.

Cunningham, S.D., W.R. Berti, and J.W. Huang. 1995. Phytoremediation of contaminated soils. Trends Biotechnol. 13:393-397.

Curl, E.A. and B. Truelove. 1986. The Rhizosphere. Advanced Series in Ag. Sci. 15, Springer-Verlag, Berlin, Germany.

Deichmann, W.B. and S. Witherup. 1944. J. Pharmacol. Exp. Ther. 80:233.

Doughten, R.A. 1997. Biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) and pentachlorophenol (PCP) in soils. M.S. Thesis, Montana State University, Bozeman, MT.

Dushenkov, V., P.B.A. Nanda Kumar, H. Motto, and I. Raskin. 1995. Rhizofiltration: The use of plants to remove heavy metals from aqueous streams. Environ. Sci. Technol. 29(5):1239-1245.

Edgehill, R.U. 1996. Influence of chromium (VI) and acidic conditions on removal of pentachlorophenol from soil by *Arthrobacter* strain ATCC 33790. Bull. Environ. Contam. Toxicol. 57:737-742.

Elliot, L.F., C.M. Gilmour, J.M. Lynch, and D. Tittemore. 1984. Bacterial colonization of plant roots. *In* R.L Todd and J.E. Giddens (Eds.) Microbial-Plant Interactions, pp. 1-16. Soil Sci. Soc. Am., Madison, WI.

Ferro, A.M., R.C. Sims, and B. Bugbee. 1994. Hycrest crested wheatgrass accelerates the degradation of pentachlorophenol in soil. J. Environ. Qual. 23:272-279.

Gaines, T.B. 1969. Acute toxicity of pesticides. Toxicol. Appl. Pharmacol. 14(3):515-534.

Gilbert, E.S. and D.E. Crowley. 1997. Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter* sp. strain B1B. Appl. Environ. Microbiol. 63(5):1933-1938.

Graham, J.H., R.T. Leonard, and J.A. Menge. 1981. Membrane-mediated decrease in root exudation responsible for phosphorous inhibition of vesicular arbuscular mycorrhiza formation. Plant Physiol. 68:548-552.

Haby, P.A. and D.E. Crowley. 1996. Biodegradation of 3-chlorobenzoate as affected by rhizodeposition and selected carbon substrates. J. Environ. Qual. 25:304-310.

Häggblom, M.M., L.J. Nohynek, and M.S. Salkinoja-Salonen. 1988. Degradation and O-Methylation of chlorinated phenolic compounds by *Rhodococcus* and *Mycobacterium* strains. Appl. Environ. Microbiol. 54(12):3043-3052.

Haque, A., I. Scheunert, and F. Korte. 1978. Isolation and identification of a metabolite of pentachlorophenol-^{14}C in rice plants. Chemosphere 1:65-69.

Hatzios, K.K. and D. Penner. 1982. Metabolism of herbicides in higher plants, p. 15-81. Burgess Publishing Co., Minneapolis, MN.

Hawes, M.C. 1990. Living plant cells released from the root cap: A regulator of microbial populations in the rhizosphere? Plant and Soil 129:19-27.

Hawes, M.C. and L.A. Brigham. 1992. Impact of root border cells on microbial populations in the rhizosphere. Adv. Plant Pathol. 8:119-148.

Kadlec, R.H. 1999. Constructed wetlands for treating landfill leachate. *In* G. Mulamoottil, E.A. McBean and F. Rovers (Ed.) Constructed Wetlands for the Treatment of Landfill Leachates. CRC Press, LLC, Boca Raton, FL.

Karamanev, D.G. and R. Samson. 1998. High-rate biodegradation of pentachlorophenol by biofilm developed in the immobilized soil bioreactor. Environ. Sci. Technol. 32:994-999.

Keith, L.H. and W.A. Telliard. 1979. Priority Pollutants I-a perspective view. Environ. Sci. Technol. 13(4):416-423.

Klibanov, A.M., T. Tu, and K.P. Scott. 1983. Peroxidase-catalyzed removal of phenols from coal-conversion waste waters. Science. 221:259-261.

Kroschwitz, J.I. and M. Howe-Grant (Eds.). 1993. Chlorophenols. pp. 156-168 *In* Encyclopedia of chemical technology, 4th Ed. John Wiley & Sons, New York, NY.

Kumar, P.B.A.N., V. Dushenkov, H. Motto, and I. Raskin. 1995. Phytoextraction: The use of plants to remove heavy metals from soils. Environ. Sci. Technol. 29:1232-1238.

Kuwatsuka, S. and M. Igarashi. 1975. Degradation of PCP in soils. II. The relationship between the degradation of PCP and the properties of soils, and the identification of the degradation products of PCP. Soil Sci. Plant Nutr. 21(4):405-414.

Lafrance, P., L. Marineau, L. Perreault, and J. Villeneuve. 1994. Effect of natural dissolved organic matter found in groundwater on soil adsorption and transport of pentachlorophenol. Environ. Sci. Technol. 28(13):2314-2320.

Lamar, R.T. and D.M. Dietrich. 1990. In situ depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. Appl. Environ. Microbiol. 56(10):3093-3100.

Lamar, R.T. and J.W. Evans. 1993. Solid-phase treatment of a pentachlorophenol-contaminated soil using lignin-degrading fungi. Environ. Sci. Technol. 27:2566-2571.

Langebartels, C. and H. Harms. 1985. Analysis for nonextractable (bound) residues of pentachlorophenol in plant cells using a cell wall fractionation procedure. Ecotoxicol. and Environ. Safety 10:268-279.

Larsen, S., H.V. Hendriksen, and B.K. Ahring. 1991. Potential for thermophilic (50°C) anaerobic dechlorination of pentachlorophenol in different ecosystems. Appl. Environ. Microbiol. 57(7):2085-2090.

Lee, L.S., P.S.C. Rao, P. Nkedi-Kizza, and J.J. Delfino. 1990. Influence of solvent and sorbent characteristics on distribution of pentachlorophenol in octanol-water and soil-water systems. Environ. Sci. Technol. 24(5):654-660.

Lee, J.-Y. and L. Xun. 1997. Purification and characterization of 2,6-dichloro-*p*-hydroquinone chlorohydrolase from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. 179:1521-1524.

Lynch, J.M. 1982. Interactions between bacteria and plants in the root environment. *In* M.E. Rhodes-Roberts and F.A. Skinner (Ed.), Bacteria and Plants, p. 1-23. Academic Press, Inc. London.

McBean, E.A. and F. Rovers. 1999. Landfill leachate characteristics as inputs for the design of wetlands used as treatment systems. *In* G. Mulamoottil, E.A. McBean and F. Rovers (Ed.) Constructed Wetlands for the Treatment of Landfill Leachates. CRC Press, LLC, Boca Raton, FL.

McDermott, T.R. and M.L. Kahn. 1992. Cloning and mutagenesis of the *Rhizobium meliloti* isocitrate degydrogenase gene. J. Bacteriol. 174(14):4790-4797.

McGinnis, G.D., H. Borazjani, D.F. Pope, D.A. Strobel, and L.K. McFarland. 1991. On-site treatment of creosote and pentachlorophenol sludges and contaminated soil. Ada, OK: Robert S. Kerr Environmental Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency publication EPA-600/2-91-019, Project CR-811498.

Metzger, W.C., D.A. Klein, and E.F. Redente. 1986. Bacterial physiological diversity in the rhizosphere of range plants in response to retorted shale stress. Appl. Environ. Microbiol. 52(4):765-770.

Miller, H.J., G.Henken, and J.A. VanVeen. 1989. Variation and composition of bacterial populations in the rhizospheres of maize, wheat, and grass cultivars. Can. J. Microbiol. 35:656-660.

Mueller, J.G., P.J. Chapman, and P.H. Pritchard. 1989. Creosote-contaminated sites. Environ. Sci. Technol. 23(10):1197-1201.

Mueller, J.G., D.P. Middaugh, S.E. Lantz and P.J. Chapman. 1991. Biodegradation of creosote and pentachlorophenol in contaminated groundwater: Chemical and biological assessment. Appl. Environ. Microbiol. 57(5):1277-1285.

Newman, L.A., S.E. Strand, N. Choe, J. Duffy, G. Ekuan, M. Raszaj, B.B. Shurtleff, J. Wilmoth, P. Heilman, and M.P. Gordon. 1997. Uptake and biotransformation of trichloroethylene by hybrid poplars. Environ. Sci. Technol. 31(4):1062-1067.

Norton, J.M. and M.K. Firestone. 1991. Metabolic status of bacteria and fungi in the rhizosphere of ponderosa pine seedlings. Appl. Environ. Microbiol. 57(4):1161-1167.

Okeke, B.C., A Paterson, J.E. Smith, and I.A. Watson-Craik. 1997. Comparative biotransformation of pentachlorophenol in soils by solid substrate cultures of *Lentinula edodes*. Appl. Microbiol. Biotechnol. 48:563-569.

Orser, C.S., J. Dutton, C. Lange, P. Jablonski, L. Xun, and M. Hargis. 1993*a*. Characterization of a *Flavobacterium* glutathione S-transferase gene involved in reductive dechlorination. J. Bacteriol. 175(9):2640-2644.

Orser, C.S., C.C. Lange, L. Xun, T.C. Zahrt, and B.J. Schneider. 1993*b*. Cloning, sequence analysis, and expression of the *Flavobacterium* pentachlorophenol-4-monooxygenase gene in *Escherichia coli*. J. Bacteriol. 175(2):411-416.

Pfender, W.F. 1996. Bioremediation bacteria to protect plants in pentachlorophenol-contaminated soil. J. Environ. Qual. 25:1256-1260.

Pignatello, J.J., M.M. Martinson, J.G. Steiert, R.E. Carlson, and R.L. Crawford. 1983. Biodegradation and photolysis of pentachlorophenol in artificial freshwater streams. Appl. Environ. Microbiol. 46(5):1024-1031.

Pridham, J.B. 1965. Low molecular weight phenols in higher plants. Ann. Rev. Plant Physiol. 16:13-36.

Prikryl, Z. and V. Vancura. 1980. Root exudates of plants VI. Wheat root exudation as dependent on growth, concentration gradient of exudates and the presence of bacteria. Plant and Soil 57:69-83.

RAM Environmental, LLC. 1998. Sampling Report, March 1996-September 1998, Pine Tree Timber Facility. Submitted to the Montana Dept. of Environmental Quality, Environmental Remediation Division, Site Response Section, Helena, MT, June 1999. RAM Environmental, LLC., Bozeman, MT. RAM Project Code: 93-165.

Ravanel, P and M. Tissut. 1986. Toxicity of pentachlorophenol on isolated plant mitochondria. Phytochem. 25(3):577-583.

Rawlings, N.C., S.J. Cook, and D. Waldbillig. 1998. Effects of the pesticides carbofuran, chlorpyrifos, dimethoate, lindane, triallate, trfluralin, 2,4-D, and pentachlorophenol on the metabolic endocrine and reproductive endocrine system in ewes. J. Toxicol. Environ. Health. Part A, 54:21-36.

Reddy, B.R. and N. Sethunathan. 1983. Mineralization of parathion in the rice rhizosphere. Appl. Environ. Microbiol. 45(3):826-829.

Reynolds, A. 1997. Personal communication, Superfund Program, Montana Department of Environmental Quality, Helena, MT.

Rise, D. 1996. Montana Department of Agriculture Pesticide Sampling Program (Draft). Montana Department of Agriculture Ground Water Program, Helena, MT, unpublished.

Rise, D. 1999. Personal communication, Ground Water Program, Montana Department of Agriculture, Helena, MT.

Rovira, A.D. 1979. Biology of the soil-root interface. *In* J.L. Harley and R.S. Russell (Eds.), The Soil-Root Interface, p.145-160. Academic Press, Inc., London.

Rugh, C.L., H.D. Wilde, N.M. Stack, D.M. Thompson, A.O. Summers, and R.B. Meagher. 1996. Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial merA gene. Proc. Natl. Acad. Sci. U.S.A. 93(8):3182-3187.

Saber, D.L. and R.L. Crawford. 1985. Isolation and characterization of Flavobacterium strains that degrade pentachlorophenol. Appl. Environ. Microbiol. 50(6):1512-1518.

Saboo, V.M. and M.A. Gealt. 1998. Gene sequences of the *pcpB* gene of pentachlorophenol-degrading *Sphingomonas chlorophenolica* found in nondegrading bacteria. Can. J. Microbiol. 44:667-675.

Sandmann, E.R.I.C. and M.A. Loos. 1984. Enumeration of 2,4-D-degrading microorganisms in soils and crop plant rhizospheres using indicator media; high populations associated with sugarcane (*Saccharum officinarum*). Chemosphere. 13:1073-1084.

Sato, K. 1997. Effect of cellulose and glucose on the proliferation of PCP (pentachlorophenol)-degrading microorganisms in soil suspension. Soil Sci. Plant Nutr. 43(4):937-941.

Sato, K. and S. Lee. 1996. Effect of glycine, glutamate and glucose on proliferation of PCP (pentachlorophenol)-degrading microorganisms in soil. Soil Sci. Plant Nutr. 42(4):905-910.

Schäfer, W. and H. Sandermann, Jr. 1988. Metabolism of pentachlorophenol in cell suspension cultures of wheat (*Triticum aestivum* L.). Tetrachlorocatechol as a primary metabolite. J. Agric. Food Chem. 36:370-377.

Scheel, D., W. Schäfer, and H. Sandermann, Jr. 1984. Metabolism of pentachlorophenol in cell suspension cultures of soybean (*Glycine max* L.) and wheat (*Triticum aestivum* L.). General results and isolation of lignin metabolites. J. Agric. Food Chem. 32:1237-1241.

Schellenberg, K., C. Leuenberger, and R. Schwarzenbach. 1984. Sorption of chlorinated phenols by natural sediments and aquifer materials. Environ. Sci. Technol. 18:652-657.

Schenk, T., R. Müller, F Mörsberger, M.K. Otto, and F. Lingens. 1989. Enzymatic dehalogenation of pentachlorophenol by extracts from *Arthrobacter* sp. strain ATCC 33790. J. Bacteriol. 171(10):5487-5491.

Scher, F.M., J.S. Ziegle, and J.W. Kloepper. 1984. A method for assessing the root-colonizing capacity of bacteria on maize. Can. J. Microbiol. 30:151-157.

Scheunert, I., Z. Qiao, and F. Korte. 1986. Comparative studies of the fate of atrazine-14C and pentachlorophenol-14C in various laboratory and outdoor soil-plant systems. J. Environ. Sci. Health. B12(6):457-485.

Schmitt, R., J. Kaul, T.V.D. Trenck, E. Schaller, and H. Sandermann, Jr. 1985. β-D-Glucosyl and O-malonyl-β-D-glucosyl conjugates of pentachlorophenol in soybean and wheat: Identification and enzymatic synthesis. Pest. Biochem. Physiol. 24:77-85.

Schnoor, J.L., L.A. Licht, S.C. McCutcheon, N.L. Wolfe, and L.H. Carreira. 1995. Phytoremediation of organic and nutrient contaminants. Environ. Sci. Technol. 29(7):318A-323A.

Schwetz, B.A., J.M. Norris, G.L. Sparschu, V.K. Rowe, P.J. Gehring, J.L. Emerson, and C.G. Gerbig. 1973. Toxicology of chlorinated dibenzo-p-dioxins, p. 55-69. *In* E.H. Blair (Ed.) Chlorodioxins-Origin and Fate, Advances in Chemistry Series 120. American Chemical Society, Washington, D.C.

Sperber, J.I. and A.D. Rovira. 1959. A study of the bacteria associated with the roots of subterranean clover and Wimmera rye-grass. J. Appl. Bacteriol. 22(1):85-95.

Stanlake, G.J. and R.K. Finn. 1982. Isolation and characterization of a pentachlorophenol-degrading bacterium. Appl. Environ. Microbiol. 44(6):1421-1427.

Steiert, J.G. and R.L. Crawford. 1985. Microbial degradation of chlorinated phenols. Trends in Biotechnol. 3(12):300-305.

Steiert, J.G. and R.L. Crawford. 1986. Catabolism of pentachlorophenol by a *Flavobacterium* sp. Biochem. Biophys. Res. Commun. 141:825-830.

Stuart, S.L. and S.L. Woods. 1998. Kinetic evidence for pentachlorophenol-dependent growth of a dehalogenating population in a pentachlorophenol- and acetate-fed methanogenic culture. Biotechnol. Bioeng. 57(4):420-429.

Suzuki, T. 1977. Metabolism of pentachlorophenol by a soil microbe. J. Environ. Sci. Health. B12(2):113-127.

Tuomela, M., M. Lyytikäinen, P. Oivanen, and A. Hatakka. 1999. Mineralization and conversion of pentachlorophenol (PCP) in soil inoculated with the white-rot fungus *Trametes versicolor*. Soil Biol. and Biochem. 31:65-74.

United States Department of Energy. 1994. Summary report of a workshop on phytoremediation research needs. Plume Focus Area and Division of Energy Biosciences, DOE/EM-0224, July 24-26, Santa Rosa, CA.

United States Environmental Protection Agency. 1999. National primary drinking water regulations; Technical factsheet on: Pentachlorophenol [Online] Available: http://www.epa.gov/OGWDW/dwh/t-soc/pentachl.html. [1999, May 28].

United States Environmental Protection Agency. 1998. Unified Air Toxics Website: EPA Regulations [Online] Available: http://www.epa.gov/ttnuatwl/eparegs.html. [1998, November 24]

Walton, B.T. and T.A. Anderson. 1990. Microbial degradation of trichloroethylene in the rhizosphere: Potential application to biological remediation of waste sites. Appl. Environ. Microbiol. 56(4):1012-1016.

Watanabe, I. 1973. Isolation of pentachlorophenol decomposing bacteria from soil. Soil Sci. Plant Nutr. 19(2):109-116.

Watanabe, I. 1977. Pentachlorophenol-decomposing and PCP-tolerant bacteria in field soil treated with PCP. Soil Biol. Biochem. 9:99-103.

Weiss, U.M., P. Moza, I. Scheunert, A. Haque, and F. Korte. 1982. Fate of pentachlorophenol-14C in rice plants under controlled conditions. J. Agric. Food Chem. 30:1186-1190.

Xun, L. and C.S. Orser. 1991*a*. Purification of a *Flavobacterium* pentachlorophenol-induced periplasmic protein (PcpA) and nucleotide sequence of the corresponding gene (*pcpA*). J. Bacteriol. 173(9):2920-2926.

Xun, L. and C.S. Orser. 1991*b*. Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. 173(14):4447-4453.

Xun, L. E. Topp, and C.S. Orser. 1992. Confirmation of oxidative dehalogenation of pentachlorophenol by a *Flavobacterium* pentachlorophenol hydroxylase. J. Bacteriol. 174(17):5745-5747.

Youngman, R.J. and A.D. Dodge. 1979. Mechanisms of paraquat action: Inhibition of the herbicidal effect by a copper chelate with superoxide dismutating activity. Z. Naturforsch. 34c:1032-1035.

Yu, J. and O.P. Ward. 1996. Investigation of the biodegradation of pentachlorophenol by the predominant bacterial strains in a mixed culture. Internat. Biodeter. Biodegrad. 181-187.