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PHYTOREMEDIATION STRATEGIES FOR RECALCITRANT CHLORINATED ORGANICS

A

DISSERTATION

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

William Edwin Schnabel, M.S.

Fairbanks, Alaska

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PHYTOREMEDIATION STRATEGIES FOR RECALCITRANT CHLORINATED ORGANICS

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Abstract

The purpose of the research was to investigate novel strategies for the phytoremediation of recalcitrant chlorinated organic soil contaminants. The recalcitrance of many chlorinated organics is related to chemical stability and bioavailability. Mycorrhizal fungi have the potential to enhance the degradation of such compounds through the action of lignolytic enzyme systems, and to increase the bioavailability of such compounds through increased root surface area and reach. Furthermore, the addition of surfactants has the potential to increase compound bioavailability via increased solubility. The organochlorine pesticide aldrin, and the polychlorinated biphenyl 3,3'4,4'-tetrachlorobiphenyl (TCB) were chosen as representative recalcitrant contaminants. Feltleaf willow (*Salix alaxensis*) and balsam poplar (*Populus balsamifera*) were chosen as vegetative species likely to be useful for phytoremediation in sub-arctic ecosystems. Mixed-culture mycorrhizal fungi were first shown to be capable of taking up the hydrophobic contaminants *in vitro*. In the same experiments, surfactant addition increased the level of contaminant uptake. In subsequent vegetative uptake studies, mycorrhizal infection was highly correlated with the uptake of aldrin and TCB in the willow systems. In the poplar systems, this correlation was not as strong. Once taken up into the vegetative matrix of either species, most of the carbon originating from the chlorinated compounds existed as bound transformation products. Additionally, water-soluble transformation products of aldrin were formed in all of the soils tested, and such transformations were enhanced in the presence of vegetation. TCB transformation

products were not detected in any of the soils tested. Surfactant addition did not impact the fate of either contaminant in the vegetative uptake studies. The surfactants, in the concentrations added, did not sufficiently solubilize the contaminants into the soil solution. The results of these studies indicated that the phytoremediation of recalcitrant chlorinated organics such as aldrin and TCB could be enhanced through the action of mycorrhizal fungi, and that surfactant addition has the *potential* to increase mycorrhizal uptake. Field studies were recommended, involving the use of specific degradative fungal species and effective surfactants.

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Chapter 1: Introduction

Humankind has fostered an intimate relationship with the plant kingdom since the dawn of man. At first, plants were used for making tools, as a shelter, as a source of heat, and as a source of collectable food and medicine. After a time, humans learned to cultivate vegetation, which resulted in humanity's profound transition from a scattering of hunter/gatherers, to an assemblage of settled farmers—the rudimentary framework around which modern society is built. As technology advanced, humans employed science to extract useful compounds from vegetative materials, thus bolstering the plant kingdom's position in the human psyche. Now, at the dawn of the second millennium, plants are once again being used for a novel purpose. Today, plants are being employed to break down the chemical wastes that modern science has engendered

Phytoremediation, or the use of vegetation for the remediation of unwanted compounds, is a developing technology in the field of environmental engineering. Currently, plants are being used for a variety of remediative purposes, many of which are based on vegetative uptake and transformation of noxious organics, microbial breakdown of organics through increased bioactivity in the rhizosphere, or extraction and hyper-accumulation of heavy metals. Phytoremediation has not, however, been proven effective for the remediation of strongly hydrophobic, recalcitrant organic compounds.

The purpose of this research was to investigate strategies for broadening the reach of phytoremediation. Two classes of compounds, polychlorinated biphenyls (PCB's) and organochlorine pesticides, were chosen as common soil contaminants generally

considered to be recalcitrant to phytoremediation. It was hypothesized that through the manipulation of mycorrhizal fungi activity and/or through chemical surfactant addition, a vegetative system could be induced to increase the rate of contaminant breakdown. This hypothesis was tested during the course of three distinct experimental phases.

The first phase, or the *In Vitro* Study, was designed to determine whether ectomycorrhizal fungi indigenous to Alaskan soils had the ability to take up PCB's and organochlorine pesticides (see Chapter 3). It was hypothesized that since these fungi play a key role in the uptake of vegetative nutrients, they may have the ability to increase the vegetative uptake and/or transformation of soil contaminants. Additionally, chemical surfactants were added to various treatment groups to determine the effects of such amendments upon fungal uptake. Furthermore, the surfactants used were chosen specifically because they contained nitrogen and phosphorus, two essential fungal and vegetative nutrients. It was hypothesized that since mycorrhizal fungi are believed to break down organic litter in order to obtain nitrogen and phosphorus, then the same fungi might break down surfactants containing nitrogen and phosphorus, and fortuitously degrade the associated soil contaminants.

The second phase, or the Mycorrhizal Development Study, was conducted in an effort to understand mycorrhizal growth characteristics (see Chapter 6). In particular, it was hypothesized that plants having a more robust population of mycorrhizal fungi would be better equipped to take up and/or transform soil contaminants. Consequently, the Mycorrhizal Development Study was conducted to determine how to enhance mycorrhizal development under specific conditions. Although there is considerable

disagreement in the literature concerning the conditions under which mycorrhizal development is best induced, several researchers have reported that mycorrhizae develop best when the concentration of mineral nutrients in the soil solution is low (see Chapter 2). Consequently, one group of plants was fertilized with a solution of mineral nutrients considered to be "nominal" for plant growth. Mycorrhizal development in plants fed sequentially lower concentrations of mineral nutrients was compared to mycorrhizal development in the "nominal" group. Additionally, leaf litter and chemical surfactants were added to selected treatment groups to determine if such amendments would affect vegetative or fungal growth.

The final phase, or the Uptake Study, was developed to determine the effects of mycorrhizal development and/or surfactant addition upon the fate of PCB's and organochlorine pesticides in vegetated systems (see Chapters 4 and 5). Two Alaskan vegetative species, feltleaf willow (*Salix alaxensis*) and balsam poplar (*Populus balsamifera*), were grown in semi-enclosed phytoreactors spiked with chlorinated contaminants. Contaminant fate was determined via ^{14}C labels associated with the added compounds. This experiment was designed to emulate field conditions as closely as possible, while maintaining controlled experimental conditions. It was determined that if mycorrhizal development and/or surfactant addition was shown to increase contaminant breakdown, then the Uptake Study could serve as a guide to broaden the reach of phytoremediation. The theoretical transformation mechanisms associated with the Uptake Study are depicted in Figure 1.

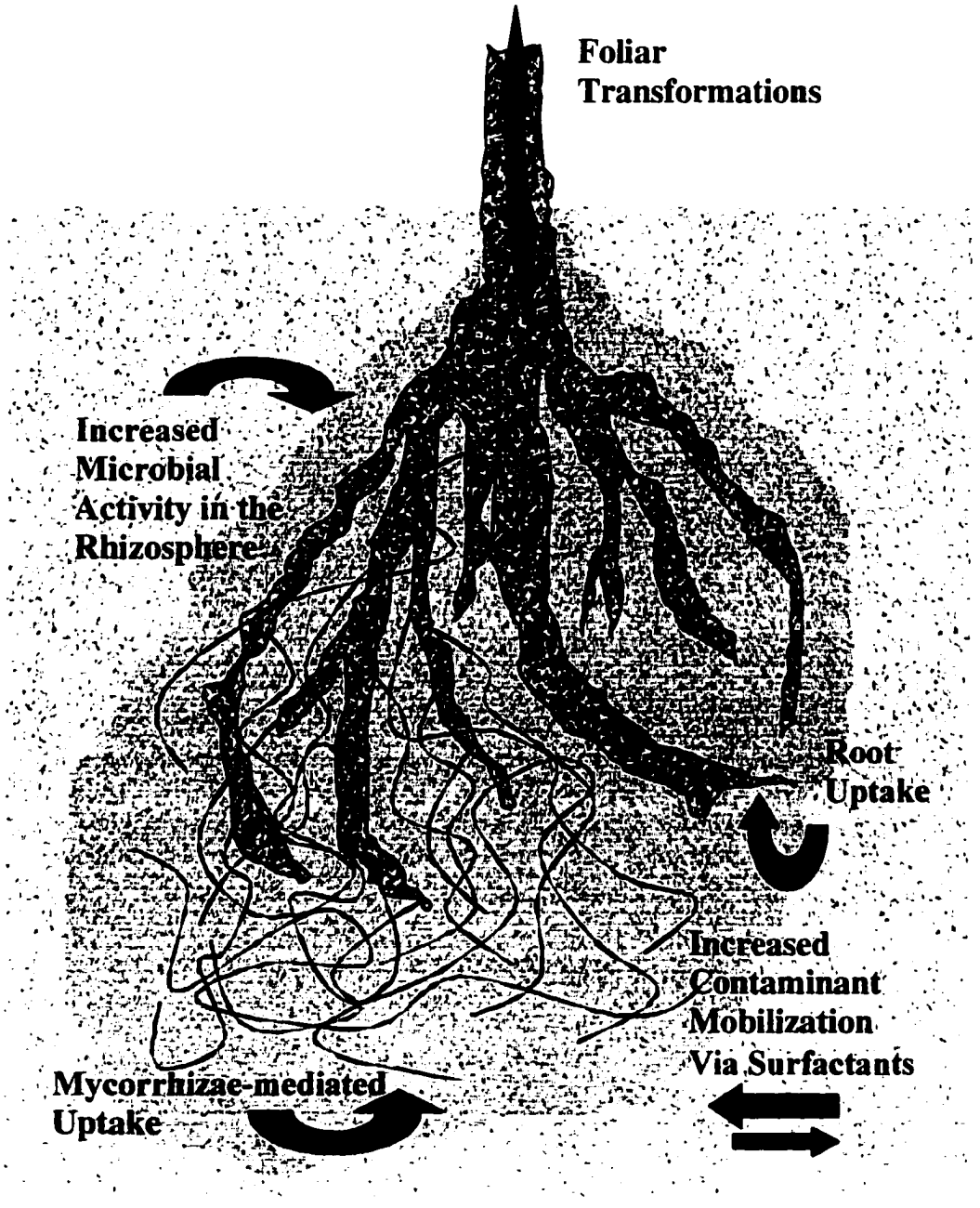

Figure 1: Potential Transformation Mechanisms

Hydrophobic contaminants mobilized via surfactants could potentially be transformed through increased microbial activity in the rhizosphere, mycorrhizae-mediated uptake and/or transformation, root uptake and/or transformation, and foliar transformation of xylem-mobile products. It is hypothesized that though the enhancement of one or all of these mechanisms, PCB's and organochlorine pesticides could be remediated.

Chapter 2: Literature Review

Although many pertinent studies are cited throughout the remaining chapters of this document, it is useful to examine the relevant studies to date in one concise section. The following literature review was compiled at the beginning of the study, and continued to be updated throughout the course of the research program. This chapter reviews the physical properties of 3,3'4,4'-tetrachlorobiphenyl, aldrin, and dieldrin; the relationship of those physical properties to recalcitrance; the general principals and state of the art of phytoremediation; and the burgeoning field of mycorrhizae-mediated phytoremediation.

Section 2.1: Physical Properties

The behavior of contaminants in the environment is intrinsically related to the physical properties associated with each contaminant. It is necessary, therefore, to examine the chemistry of the compounds used. Although dieldrin was never added as a parent compound in this research, dieldrin is commonly formed from the epoxidation of aldrin in biological systems (Gannon and Decker, 1958; Lichtenstein and Schulz, 1960), and should therefore be considered.

2.1.1: TCB

The compound 3,3'4,4'-tetrachlorobiphenyl (TCB) is one of 209 different polychlorinated biphenyl congeners. TCB is a biphenyl molecule with four chlorine substituents, resulting in a molecular weight of 292 g/mol (Figure 2). The chemical formula is $C_{12}H_6Cl_4$. In pure form, TCB is a white crystalline solid with a melting point of 177-179 °C, and a boiling point of 250 °C (ChemService, 1996). The aqueous solubility of TCB has been reported to be 0.175 mg/L (Hutzinger, Safe and Zitko, 1974). The octanol-water partition coefficient (Log_{10} K_{ow}) has been reported as 6.4 (Hawker and Connell, 1988).

2.1.2: Aldrin

Aldrin is the common name for 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexadyhydro-endo-exo-1,4:5,8-dimethanonaphthalene (Stecher, Windholz and Leahy, 1968). The chemical formula for aldrin is $C_{12}H_8Cl_6$, resulting in a molecular weight of 365 g/mol (Figure 3). In pure form, aldrin is a white crystalline solid with a melting point of 104 °C (Stecher *et al.*, 1968). Measurements of octanol/water partition coefficient (Log_{10} K_{ow}) for aldrin have varied, ranging from 5.5 (Connell and Markwell, 1990) to 7.4 (Briggs, 1981). The aqueous solubility of aldrin has been reported as 0.1-0.2 mg/L (Willis and McDowell, 1982). The vapor pressure of aldrin was measured as 6.5 x

10^{-5} mmHg at 20 °C (WHO, 1991). Aldrin is considered to be relatively non-volatile, non water-soluble compound.

2.1.3: Dieldrin

Dieldrin is the common name for 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,8,8a-octadyhydro-endo-exo-1,4:5,8-dimethanonaphthalene (Stecher *et al.*, 1968). Dieldrin closely resembles aldrin, and is formed from the epoxidation of aldrin's non-chlorinated ring. Dieldrin has the chemical formula $C_{12}H_8Cl_6O$, and a molecular weight of 381 g/mol (Figure 4). In pure form, dieldrin has a melting point of 177 °C (Stecher *et al.*, 1968). The Log_{10} K_{ow} has been reported between 5.5 (Worthing, 1983), and 6.2 (Briggs, 1981). Aqueous solubility has been reported as 0.1-0.25 mg/L (Worthing, 1983). Dieldrin vapor pressure was reported to be 3.2 mmHg at 20 °C (WHO, 1991). Dieldrin, similar to aldrin, is considered to be a relatively non-volatile, non water-soluble compound.

Section 2.2: Physical Properties vs. Recalcitrance

2.2.1: Bioavailability

It has long been recognized that the recalcitrance of organochlorine pesticides (Lichtenstein and Schulz, 1959) and PCB's (Weber and Mrozek, 1979) in natural environments is strongly related to the organic content of the soil. Soils with a higher organic carbon content tend to retain contaminants for a much longer period of time than

similar soils with low organic carbon content. In a recent study, researchers found that biological reductive dechlorination of PCB's in sediments was dependent upon the aqueous phase PCB concentration (Zwiernik, Quensen and Boyd, 1999), hence the bioavailability of the contaminants. In the study, the aqueous solubility was decreased with the addition of hydrocarbon residues to the sediments, which had the effect of increasing the effective organic content of the sediments.

Although the biological mechanisms studied in the present research are assumed to take place under aerobic rather than anaerobic conditions, the effect of aqueous concentration on bioavailability would be the same regardless of the conditions. Furthermore, although parameters intrinsic to the system under study such as chemical toxicity and biological composition should be considered important, the overriding factor governing the biological fate of strongly hydrophobic contaminants appears to be the sorption/desorption processes between the contaminant and the system components (Kannan *et al.*, 1998).

2.2.2: Equilibrium Concentrations

As bioavailability and/or recalcitrance have been shown to be related to the amount of contaminant in the aqueous phase, a large amount of research has been devoted to understanding the parameters involved in sorption/desorption of contaminants to soils and/or sediments. In one widely cited study, researchers derived an equation for the partition coefficient, K_{oc}, of hydrophobic solutes between sediment organic carbon and the aqueous phase (Karickhoff, Brown and Scott, 1979). In the study, the partition

coefficient was related in two separate equations between the octanol-water coefficient (K_{ow}) and aqueous solubility (S),

$$\text{Log } K_{oc} = 1.00 \text{ Log } K_{ow} - 0.21$$

and

$$\text{Log } K_{oc} = -0.54 \text{ Log } S + 0.44,$$

where S represents the aqueous solubility expressed as mole fraction. K_{oc} was then related by definition to the partition coefficient (K_p) between the total sediment and the aqueous phase,

$$K_p = K_{oc} \text{ oc},$$

where oc represents the mass fraction of organic carbon in the soil. By employing such equations, researchers could then estimate the equilibrium concentrations of a broad range of solutes based upon the K_{ow} and/or solubility. Furthermore, the study found that the linear partition coefficients were relatively independent of sediment solute concentrations and ionic strength of the aqueous suspensions (Karickhoff *et al.*, 1979).

2.2.3: Sorption/Desorption Kinetics

Researchers have argued that models based solely upon equilibrium (as described above) do not adequately describe the sorption/desorption processes of fluctuating systems such as frequently flooded topsoils. One model describes the kinetics of sorption/desorption based upon not only K_{ow} and organic carbon content, but also solution diffusivity, soil density, and soil porosity (Wu and Gschwend, 1986). These researchers found that the rate of hydrophobic compound desorption decreases with

increasing K_{ow}, organic carbon, and aggregate size, and increases with water flow. Other researchers found that the sorption/desorption kinetics of aged organic compounds were temperature dependant (Cornelissen *et al.*, 1997). Colder systems, it was found, tended to retain sorbed contaminants longer than warmer systems. Hence, although the temperature dependence of hydrophobic contaminant biodegradation is often attributed to the temperature dependence of biological activity itself (Ghadiri, Rose and Connell, 1995), hydrophobic contaminants are also less likely to be bioavailable under cooler conditions.

PCB's in particular have engendered a spate of recent sorption kinetics research. It has been reported, for example, that PCB's tend to desorb in a two-phase model, whereby PCB's desorb from sediments first relatively quickly, then slowly over an extended period (Ghosh *et al.*, 1999). The desorption rate constants for the labile pool were found to be two orders of magnitude higher than the rate constants for the slowly desorbing pool. Both pools, however, were shown to desorb more slowly with increasing overall chlorination, decreasing ortho chlorination, and decreasing temperature. This study was in agreement with an earlier study, wherein PCB contaminated soils were submerged into water and the relative PCB desorption rates were measured (Girvin *et al.*, 1997). In the earlier study, the labile fraction was found to consist of 80-90% of the total PCB concentration, and most of this fraction desorbed within 48 hours of contact with water. Although this study demonstrated that PCB's were able to reach equilibrium in a matter of hours or days, it should be noted that the organic content of the soils studied was relatively low (<0.2%) and likely had a large impact on the desorption kinetics.

2.2.4: Surfactant Use

The effective soil solution concentration of a hydrophobic contaminant can be increased through the use of surfactants. In a recent study, researchers modeled the bioavailability of phenanthrene in the presence and absence of nonionic surfactants (Guha and Jaffe, 1996). The results of this study indicated that surfactants above their critical micelle concentration increased bioavailability by aiding in the desorption and mass transfer of solute through the aqueous matrix. Furthermore, the study showed that the surfactants aided in the actual biodegradation through an increase in membrane permeability. This technology has led to the development of bioremediation strategies whereby PCB's were first solubilized from soil particles via surfactant addition, then transformed by PCB-degrading microorganisms (Layton *et al.*, 1998). Such a strategy could potentially be appropriate for phytoremediation purposes, but literature reports of successful surfactant-mediated phytoremediation strategies are scarce.

In summary, the environmental fate of strongly hydrophobic contaminants such as aldrin and PCB's is related to bioavailability, which is in turn related to the organic content of the soil and the lipophilicity of the contaminant. Although the equilibrium coefficients between sorptive phases are fairly well defined, the kinetics governing desorption are highly variable. Desorption of hydrophobic compounds from soil to solution can be enhanced through the use of surfactants. Desorption of toxic hydrophobic contaminants is not always desired. In the environment, for example, extremely

hydrophobic PCB congeners have been shown to be more recalcitrant to bioaccumulation than moderately hydrophobic congeners, due to soil sorption and limited membrane permeability (Kannan *et al.*, 1998). In this instance, less bioavailability was equated with lower toxicity. For phytoremediation purposes, however, a contaminant must be made bioavailable before remediation can occur.

Section 2.3: Phytoremediation

2.3.1: General Principals

The term "phytoremediation" refers to the use of vegetative systems to enhance the degradation of soil or water contaminants. Although the impacts of vegetation on soils have been observed for millennia, it wasn't until the late Twentieth Century that researchers began to study vegetative ecosystems exclusively for the purpose of remediating contaminants. Although numerous review articles detail the various applications of phytoremediation, most agree that vegetation enhances contaminant degradation through 1) direct vegetative uptake and transformation; 2) exudation of contaminant-degrading enzymes; and 3) enhancement of bacterial and fungal populations in the rhizosphere (Schnoor *et al.*, 1995). In most systems, it is likely that all three mechanisms contribute to various extents.

Poplars (*Populus spp.*) have received considerable attention as a genus useful for the phytoremediation of organic contaminants. Poplars are known to grow rapidly and produce a large root mass, two parameters considered important in phytoremediation. Organic compounds associated with poplar phytoremediation studies include chlorinated solvents (Newman *et al.*, 1998), pesticides (Burken and Schnoor, 1996), and strongly hydrophobic compounds such as pentachlorophenol (Burken and Schnoor, 1998).

Willows (*Salix spp.*), on the other hand, have not received as much attention from phytoremediation researchers as have poplars. Like poplars, however, they are fast growing and produce considerable root mass. Recent literature describing willow use includes studies on the phytoremediation of chlorinated solvents (Newman *et al.*, 1998) and heavy metals (Greger and Landberg, 1999).

2.3.2: PCB Phytoremediation

Plants were first shown to contribute to the metabolism of PCB's in the mid 1970's. In one of the first of such studies, researchers demonstrated that aquatic plant *Ranunculus fluitans* was able to take up ^{14}C-labeled 2,2'-dichlorobiphenyl directly from the water column and bioconcentrate it to a factor of 814 over the course of four weeks (Moza *et al.*, 1974). Although most (97%) of the radioactivity in the plant tissues was found to be parent product, 3% was shown to be metabolized. Furthermore, only 34% of the radiolabel remaining in the water column was found to be a parent product. Although the metabolites were not specifically identified, TLC and GC analysis indicated that they were likely a mixture of dihydroxy derivatives, monohydroxy derivatives, and fully

dechlorinated product. It was not determined whether the metabolism took place inside or outside of the vegetative matrix, but a higher amount of metabolized product was detected in vegetated samples than non vegetated controls. Subsequent experiments with higher chlorinated congeners (2,4',5-trichlorobiphenyl and 2,2'4,4',6-pentachlorobiphenyl) revealed similar hydroxylated products, but showed decreased metabolism with increased chlorine content (Moza *et al.*, 1976). In a further experiment using the same congeners applied to soils in which carrots were grown, the carrots were found to take up 3% of the applied radioactivity in the 2,4',5-trichlorobiphenyl samples (Moza *et al.*, 1979). Approximately 18% of the radioactivity in the carrots was identified as non-extractable residue or methylated product. As before, the pentachlorobiphenyl was found to be more recalcitrant to metabolism.

More recent studies have argued that terrestrial plants, in this case tomatoes and barley, lack the ability to translocate or metabolize PCB's (Quiping *et al.*, 1991; Quiping *et al.*, 1992). These researchers hypothesized that all PCB's or metabolites found in the upper portion of plants in earlier studies were the result of foliar sorption of volatilized compounds. These studies, however, reported only on the PCB activity in the plant tops. No mention was made concerning the possibility of contaminant metabolism in the root zone. The finding that stem-injected PCB's were not xylem mobile is not surprising, given the correlation between xylem mobility and K_{ow} (Trapp, McFarlane and Matthies, 1994).

In order to determine whether PCB metabolism occurs in the vegetative matrix, researchers have grown vegetative isolates in the laboratory to test for such metabolism. In one study, axenic cultures of Paul's Scarlet rose sorbed approximately half of the 2-chlorobiphenyl available in solution, and released most of the compound back into solution as a glycosylated derivative (Fletcher, Groeger and McFarlane, 1987). Killed cells of the same species were found to sorb the parent compound from solution as well, but no metabolites were released. In a recent study, axenic cultures of *Solanum nigrum* roots were shown to transform a variety of PCB's congeners belonging to the commercial mixture Delor 103 (Mackova *et al.*, 1997). Furthermore, PCB transformation was increased in this study through the addition of plant growth regulators kinetin, 2,4-dichlorophenoxy-acetic acid, benzylaminopurin, and naphthaleneacetic acid.

Vegetation has been shown to contribute to the degradation of PCB's not only inside of the vegetative matrix, but also by contributing to the growth of PCB-degrading microorganisms. In one experiment, it was demonstrated that 3 species of PCB-degrading bacteria (*Alcaligenes eutrophus*, *Pseudomonas putida*, and *Corynebacteria sp.*) were able to grow on a variety of phenolic compounds commonly found in root exudates (Donnelly, Hegde and Fletcher, 1994). Furthermore, it was found that the bacterial cultures retained their ability to cometabolize a wide variety of PCB congeners even when such phenolics served as the sole carbon source. A subsequent assay of vegetative species revealed that all of the seventeen species tested exuded phenolic compounds to the rhizosphere, thus indicating that the presence of vegetation likely contributes to the microbial breakdown of PCB's in the environment (Fletcher and Hegde, 1995). A final

study concluded that in one promising species (*Morus rubra* L.), degradation-enhancing root phenolics were released continuously throughout the growing season, followed by a dramatic increase shortly before leaf senescence (Hegde and Fletcher, 1996).

Although there are a variety of studies indicating that vegetation has the potential to contribute to the degradation of PCB's, there is little information available describing successful field projects. This lack of information indicates that PCB phytoremediation strategies have yet to be proven effective.

2.3.3: Pesticide Phytoremediation

One of the first proposed applications for the phytoremediation of pesticide contaminated soils was for the remediation of soils at agrochemical dealerships. As such, studies have abounded concerning the potential for remediating agrochemicals with vegetation (Nair *et al.*, 1993) (Anderson, Coats and Kroger, 1994) (Burken and Schnoor, 1996; Burken and Schnoor, 1998). Such studies have targeted pesticides less recalcitrant than aldrin, however, and may not describe methods useful for the phytoremediation of the strongly hydrophobic organochlorine pesticides.

As described earlier, the phytoremediation potential for hydrophobic pesticides is related to bioavailability. Although aldrin and dieldrin are strongly hydrophobic and tend to sorb to soil particles, crops have been demonstrated to take up DDT and dieldrin directly through the roots (Beall and Nash, 1969). Furthermore, plants have been shown to metabolize pesticides such as aldrin through cytochrome P-450-linked enzymatic activities (Borlakoglu and John, 1989). As root uptake of hydrophobic contaminants has

been well-modeled (Trapp *et al.*, 1994), it stands to reason that such information could be applied to the phytoremediation of aldrin. To date, only the sorptive properties of vegetation have been studied with regards to aldrin phytoremediation (Bras, Santos and Alives, 1999). The contributions of vegetative uptake and rhizosphere enhancement remain largely unexamined.

Section 2.4: Mycorrhizae

2.4.1: General Principals

"Mycorrhizae" describes the mutualistic vegetative-fungal relationship that develops in the roots of many vegetative species. Although the existence of mycorrhizal associations has been recognized for over 100 years, it wasn't until recently that the prevalence and importance of such relationships has been recognized (Allen, 1991). In short, the fungal symbiont of a mycorrhizal association contributes water, nutrients, or root surface area and reach to the vegetative partner, and in exchange, the plant delivers energy in the form of photosynthates to the fungi. It has been estimated that virtually all terrestrial woody species benefit from mycorrhizal associations to some extent (Laursen, 1985). Herbaceous and ericaceous species are also widely reported to develop mycorrhizal associations (Allen, 1991).

In boreal woody species such as willow and poplar, the dominant form of mycorrhizal association is termed ectomycorrhizae (Helm, Allen and Trappe, 1996). Ectomycorrhizal associations are indicated by a relatively thick fungal sheath around the

root tips, fungal penetration between (but not within) the root cells, and radiating hyphae. Most woody plants form ectomycorrhizal associations, and the number of fungal species capable of forming such associations with a given vegetative species is tremendous (Allen, 1991).

2.4.2: Quantification of Ectomycorrhizal Fungal Biomass

The amount of fungal biomass in an ectomycorrhizal system has traditionally been difficult to quantify. The simplest method involves the microscopic enumeration of infected root tips, reported as a percentage of the whole. Aside from the tedium, a major drawback of this method is that mycorrhizal systems tend to have a higher total number of root tips than non-mycorrhizal systems, thus throwing into question the comparability of two different systems (Nylund and Wallander, 1992). Another microscopic method involves the isolation of hyphal fragments from soil, and the subsequent estimation of fungal biomass based upon hyphal length, fragmentation, and density per mass soil (Hanssen, Thingstad and Goksoyr, 1974). Again aside from the tedium, a major drawback of this method is that the procedure does not distinguish between mycorrhizal fungi and other fungal species free living in the bulk soil. A widely employed method makes use of the finding that fungal cell walls contain chitin, whereas the cell walls of most higher plants do not (Bartnicki-Garcia, 1968). The relative amount of mycorrhizal infection, then, can be measured through hydrolysis of the root material and subsequent colorimetric assay of the hydrolysates (Vignon *et al.*, 1986). Since the mass of cell wall per unit fungal mass is variable, however, the chitin method is a more direct measure of

fungal surface area than true fungal biomass. A final method involves the quantification of ergosterol, a sterol prominent in fungal cell membranes but relatively absent in most higher plants (Martin, Delaruelle and Hilbert, 1990). Since membranes are present in both the outer structures and the cytoplasm of fungal cells, the ergosterol assay is more representative of true fungal biomass than is the chitin assay (Nylund and Wallander, 1992). The ergosterol assay, however, invloves the use of HPLC analysis, which can be more time-consuming than the relatively quick colorimetric analysis associated with the chitin assay.

In our experiments, we chose to employ the chitin assay for ectomycorrhizal enumeration. As stated, this method is more of a measure of fungal surface area than true fungal biomass. It was decided, however, that fungal surface area might be more directly related to uptake than fungal biomass. Additionally, although it was possible that different plants could be infected with various fungal species having differing amounts of chitin per unit cell wall, it was assumed that the chitin assay would be an adequate indicator of the relative abundance of general, mixed-culture infection.

2.4.3: Degradation of Plant Cell Components

As described above, one of the primary functions of the mycorrhizal fungal symbiont is the acquisition of nutrients for the host plant. Although fungal mycelium increase root reach and are therefore able to access nutrients outside of the depletion zones, mycorrhizal fungi also have the enzymatic capability to access organically-bound nutrients. In an early study, five species of ectomycorrhizal fungi were shown to

mineralize holocellulose, lignocellulose, and lignin (Trojanowski, Haider and Huttermann, 1984). Since these compounds are key components of plant cell walls, the degradation of such material would be essential for the acquisition of the nutrients contained inside. Numerous hydrolytic enzymes have been detected in ectomycorrhizal fungi extracts, including protease, esterase, α-D-galactopyranosidase, β-D-galactopyranosidase, α-D-mannopyranosidase, β-D-xylopyranosidase, α-D-glucopyranosidase, β-D-glucopyranosidase, and alkaline phosphatase (Bae and Barton, 1989). Such enzymes were implicated in the use of complex carbohydrates. In a recent study, ectomycorrhizal fungi were shown to actively degrade birch litter. This study reported elevated levels of protease, polyphenol oxidase, and phosphomonerase in the colonized zone (Bending and Read, 1995). Although the reach of degradative capabilities among the numerous ectomycorrhizal fungal species has yet to be elucidated, it seems clear that a wide variety of ectomycorrhizal fungal species have the capacity to degrade recalcitrant soil constituents.

2.4.4: Degradation of Xenobiotics

In the mid 1980's, researchers proposed the use of the white rot fungus *Phanerochaete chrysosporium* for the degradation of persistent organic pollutants (Bumpus *et al.*, 1985). The study reported the mineralization of DDT, 3,3'4,4'-tetrachlorobiphenyl, 2,4,5,2',4',5'-hexachlorobiphenyl, 2,3,7,8-tetrachlorodibenzo-p-dioxin, lindane, and benzo[a]pyrene to various extents in pure culture. The degradation was attributed to a non-specific, lignin-degrading secondary enzyme system exuded

under low nutrient conditions. A subsequent study employing the same fungi reported 15% and 23% mineralization of chlordane and lindane respectively over a 30-day period (Kennedy, Aust and Bumpus, 1990). Although the aldrin, dieldrin, heptachlor, and mirex also studied were poorly mineralized, there was a substantial amount (1-10%) of metabolite formation observed. More recently, four species of white rot fungi were assayed for their ability to degrade PCB's in the commercial mixture Delor 106 (Novotny *et al.*, 1997). Under nitrogen limiting conditions, PCB disappearance ranged from 0-50% after three weeks for the four species tested. The degradation of PCB's was linked to the activities of Mn-dependant peroxidase, Mn-independent peroxidase, lignin peroxidase, and laccase enzymes.

Mycorrhizal systems have been proposed as systems capable of remediating sites contaminated with recalcitrant xenobiotics. First, mycorrhizal fungi have been shown to exhibit the same lignin-degrading activity as the well-studied white rot fungi (Trojanowski *et al.*, 1984). Second, mycorrhizal fungi are reported to make up a significant proportion of the soil biota. Indeed, researchers have estimated that mycorrhizal fungi comprise the largest component of soil microbial biomass in many forests (Read, 1984). Finally, while white rot remediation strategies require the constant addition of degradable carbon to the systems, mycorrhizal fungi require only the cultivation of the appropriate vegetative species (Donnelly and Fletcher, 1995).

Studies supporting the use of mycorrhizal fungi for remediation have only begun to surface within the past decade. In one such study, ericoid and ectomycorrhizal fungal species cultured in vitro were shown to degrade atrazine at a higher rate than white rot

fungi (Donnelly, Entry and Crawford, 1993). In the same study, white rot was found to degrade 2,4-dichlorophenoxyacetic acid (2,4-D) faster than the mycorrhizal species. In both instances, degradation occurred best under nitrogen limiting conditions. In a subsequent study, two different ectomycorrhizal species were found to mineralize up to 17% of the added 2,4-D in liquid culture (Meharg, Cairney and Maguire, 1997a). Furthermore, mineralization was found to be greater when the mycorrhizal fungi were grown in symbiosis with pine than when the fungi were cultured alone. A further study described the degradation of 2,4,6-trinitrotoluene (TNT) by ectomycorrhizal fungi (Meharg, Dennis and Cairney, 1997b). In this study, although the fungi were found to degrade TNT, it was determined that nitrogen or carbon limiting conditions did not contribute to the degradation. The lignolytic activity induced under such conditions, it was observed, was not required for the degradation of TNT. Finally, numerous ectomycorrhizal species were found to degrade various PCB congeners *in vitro* (Donnelly and Fletcher, 1995). No individual species was found to degrade all of the congeners degraded, thus implying that different mycorrhizal fungi species have qualitatively different degradative capabilities.

In summary, mycorrhizal fungi are posed to contribute greatly to the field of phytoremediation. Mycorrhizal fungi have been shown to exhibit many of the same degradative capabilities as the white rot fungi, but do not require maintenance such as the addition of carbon amendments. Furthermore, intrinsic to the application of a mycorrhizal remediation system are the additional degradative benefits of the associated vegetation. Although mycorrhizal phytoremediation systems apparently have a great

potential to degrade contaminants in the field, relatively few field studies are reported on in the literature.

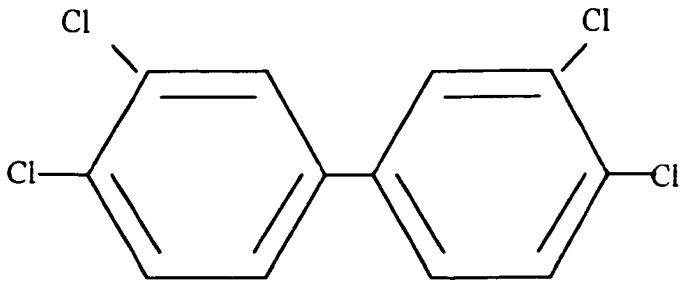

Figure 2: Molecular Structure of 3,3'4,4'-Tetrachlorobiphenyl

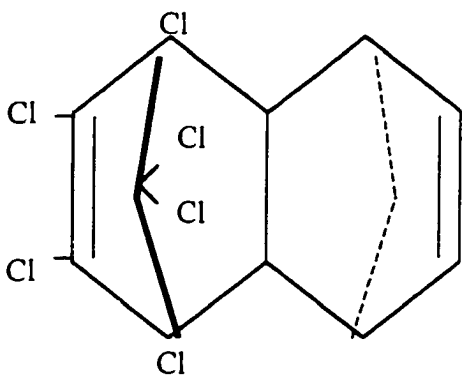

Figure 3: Molecular Structure of Aldrin

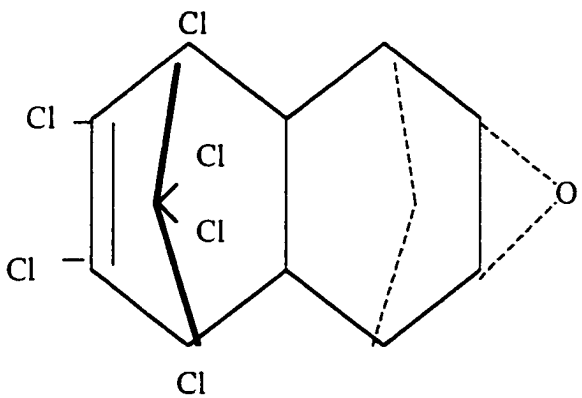

Figure 4: Molecular Structure of Dieldrin

Chapter 3: *In Vitro* Study

This chapter is a format-modified version of a manuscript submitted to the *International Journal of Phytoremediation*[1]. The manuscript is currently in the review process. Raw data supporting this experiment is contained in Appendix A.

Section 3.1: Abstract

Mixed liquid cultures of ectomycorrhizal fungi were incubated in the presence of common soil contaminants (aldrin and PCB's) and three commercial surfactants (Surfonic® L24-9, Surfonic® T-20, and Rexophos JV-05-015). Two surfactants (Surfonic® L24-9 and Surfonic® T-20) significantly increased hyphal growth under carbon-limiting conditions, thus implying surfactant biodegradability. All three surfactants increased the hyphal uptake of contaminant-derived radiolabel during a 10-day incubation. In addition, surfactants enhanced hyphal radiolabel uptake during an extended 30-day incubation, while hyphal growth slowed considerably after ten days. Results of this experiment provided evidence that surfactant addition may be a useful augmentation to mycorrhizae-mediated phytoremediation.

[1] Submitted as: Schnabel, W. and White, D. 2000a. Surfactant addition enhances the hyphal uptake of PCB's and aldrin by mycorrhizal fungi in liquid culture. *International Journal of Phytoremediation*, January, 2000.

Section 3.2: Introduction

Mycorrhizal fungi enhance a plant's ability to access and take up nutrients from the soil. The term, mycorrhizae, refers to the mutualistic relationship between a mycorrhizal fungi and a host plant. Fungi, in physical contact with the plant root, receive simple sugars from the host plant in return for mineral nutrients. Since the fungi infuse the soil with hyphae and degradative enzymes, they are able to access nutrient pools not otherwise available to plant roots (Cairney and Burke, 1994).

Recent studies demonstrated that mycorrhizal fungi have the capacity to degrade numerous organic compounds such as PCB's (Donnelly and Fletcher, 1995), 2,4-dichlorophenol (Meharg *et al.*, 1997a), and TNT (Meharg *et al.*, 1997b). Since mycorrhizae are a fungal-vegetative relationship, mycorrhizal remediation of contaminants in soils is considered, in a broad sense, phytoremediation. It has been postulated that mycorrhizal fungi can degrade recalcitrant compounds using enzyme systems similar to the white rot fungi (Bae and Barton, 1989; Cairney and Burke, 1994). Mycorrhizae-mediated phytoremediation (myco-phytoremediation), however, has benefits over remediation strategies involving white rot fungi. For example, the mycorrhizal relationship can be encouraged through the development of aboveground plants. Stimulating plant growth may be an easier way to enhance mycorrhizal degradation of contaminants than attempting to manipulate soil conditions to favor exaggerated growth of the saprotrophic white rot fungi. Also, since a myco-phytoremediation strategy would necessarily involve the use of vegetative systems,

additional benefits could be imparted through an increase in free-living rhizosphere organisms (e.g., bacteria) and plant uptake (Anderson, Guthrie and Walton, 1993; Burken and Schnoor, 1998).

Although phytoremediation has been successful for many contaminants (Schnoor *et al.*, 1995), remediation of strongly hydrophobic and halogenated organics has been somewhat problematic. Extremely hydrophobic compounds such as polychlorinated biphenyls (PCB's) are often sequestered in the soil and made unavailable for biological uptake (Pal, Weber and Overcash, 1980). Surfactant addition could augment biodegradation by mobilizing hydrophobic contaminants in the soil (Guha and Jaffe, 1996), thereby increasing the efficacy of a myco-phytoremediation strategy.

Most of the relevant studies to date have sought to determine the degradation potentials of individual mycorrhizal fungal species under specific conditions. In the field, however, vegetation is often in symbiosis with a consortium of fungal species that vary both spatially and temporally throughout the rhizosphere (Helm *et al.*, 1996). Given the considerable ecological variation between contaminated sites, it may be beneficial to take a more generalized approach to myco-phytoremediation. Developing methods of enhancing the degradative capacity of mixed culture systems could provide a widely applicable remediation strategy.

The purpose of this study was to test the hypothesis that surfactant addition could enhance hyphal uptake of PCB's and aldrin by a mixed culture of ectomycorrhizal fungi. This is the first step in a series of experiments designed to develop a phytoremediation

strategy employing vegetative systems, mixed-culture mycorrhizal fungi, and chemical surfactants.

Section 3.3: Materials and Methods

3.3.1: Mycorrhizal Fungi Inoculum

Root samples were collected from feltleaf willow (*Salix alaxensis*) and balsam poplar (*Populus balsamifera*) at eight sites across Alaska. After washing the root samples with water, ectomycorrhizal root tips were removed under a dissecting microscope and sorted according to morphotype (Helm *et al.*, 1996). Representative root tips of the morphotypes found at each site were placed onto culture plates containing fungal growth media (described below) and incubated at 20 °C in darkness for approximately ten days. Successive cultures were prepared from each of the resulting colonies and maintained on similar media. After one month of culturing, plugs from 48 culture plates were combined to form a mixed-culture inoculum of ectomycorrhizal fungi.

3.3.2: Incubation Media

Liquid media was prepared using a modified Meharg media (Meharg *et al.*, 1997a). This formulation consisted of 140 mg/L $MgSO_4 \bullet 7H_2O$, 50 mg/L $(NH_4)_2HPO_4$, 40 mg/L $K_2HPO_4 \bullet 3H_2O$, 33 mg/L $CaCl_2 \bullet 2H_2O$, 25 mg/L NaCl, 30 mg/L $ZnSO_4 \bullet 7H_2O$, 12.5 µg/L FeEDTA, 12.5 µg/L citric acid, and 10 µg/L thiamine. In addition, media for

the radiolabelled samples contained 100 mg/L glucose. Media for the growth experiment (carbon-limited) contained no added glucose. Media for culture plates contained 13 g/L agar in addition to the above constituents. After mixing, the media solution was autoclaved for 30 minutes. After the solution was allowed to cool at room temperature for 30 minutes, erythromycin (4 mg/L) and tetracycline (4 mg/L) were added to the media to inhibit bacterial activity. Amendments included aldrin, biphenyl, 2,2'-dichlorobiphenyl, 3,3'4,4'-tetrachlorobiphenyl, and 2',3,3',4,5-pentachlorobiphenyl (Ultra Scientific, N. Kingstown, RI). Radiochemicals included aldrin-(1,2,3,4,10-^{14}C), 2',3,3',4,5-pentachlorobiphenyl-(3,4,5-phenyl ring-UL-^{14}C), and 3,3',4,4'-tetrachlorobiphenyl-(UL-^{14}C). All radiochemicals were obtained from Sigma-Aldrich, St. Louis, MO. Surfactants included Surfonic® L24-9, Surfonic® T-20, and Rexophos JV-05-015 (Huntsman Corporation, Guelph, Ontario, Canada).

3.3.3: Procedure

Media (20 ml), inoculum (1 ml), and amendment solution (1 ml) were added to 40 ml amber glass reaction vessels. The cultures were then allowed to incubate in darkness for either 10 or 30 days at 20°C on a shaker table (60 rpm). After incubation of the non-radiolabelled samples (Section 3.4.1), hyphae were filtered on 0.7 μm glass microfiber filter paper, dried overnight at 100°C, then weighed. For the radiolabelled samples (Section 3.4.2), the fungal hyphae were filtered over a 0.7 μm glass microfiber filter, then washed with 10 ml ethanol. To ensure complete desorption of hydrophobic compounds, the hyphal mass was subsequently rinsed with 10 ml of methylene chloride, followed by

an additional rinse of 10 ml of ethanol. The rinsed hyphal mass was then dried overnight at 100°C, weighed, and analyzed in an R.J. Harvey OX500 biological oxidizer.

Section 3.4: Results and Discussion

3.4.1: Growth Experiment

Various amendments were added to triplicate reaction vessels (8 mg/reactor) under carbon-limiting conditions to test their impacts upon hyphal growth. To demonstrate carbon limitation, a set of control cultures were incubated in carbon-rich media (100 mg/L glucose) under replicate conditions. After ten days, the fungi incubated in the carbon-rich media had produced approximately ten times the hyphal mass as the fungi incubated in the carbon-limiting media. The control vessels incubated in carbon-limiting media produced approximately 0.3 mg dry weight of fungal hyphae (Figure 5). Since no glucose was added to the media, this growth was attributed to carbon in the inoculum solution. The aldrin and biphenyl compounds were neither inhibitory to hyphal growth, nor did they act as significant growth substrates under the incubation conditions. Conversely, two of the three surfactants tested (Surfonic® T-20 and Surfonic® L24-9) produced hyphal masses significantly greater than the control samples. This result indicates that the mixed culture was able to use both surfactants as a growth substrate.

In the field, it might prove beneficial to employ detergents that not only serve as surfactants, but also provide nutrients such as carbon, nitrogen, or phosphorus to the

fungal symbiont. Biodegradation of the surfactants could potentially lead to fortuitous degradation of hydrophobic contaminants incorporated into the surfactant micelles.

3.4.2: Uptake Experiment

Radiolabelled compounds (0.5 mg/reactor) were employed to assess the impact of surfactant addition (1.0 mg/reactor) on fungal uptake of chlorinated organics. Aldrin was chosen as a representative recalcitrant organochlorine pesticide. The compounds 3,3',4,4'-tetrachlorobiphenyl (TCB) and 2',3,3',4',5'-pentachlorobiphenyl (PtCB) were chosen as PCB congeners likely to persist in soils. All values reported in this section represent the highest single value resulting from three replicate samples.

Effect of Surfactant Addition: The radiolabel retained by the hyphal mass for each treatment set is shown in Figure 6. As these values represent the amount of radiolabel remaining after rinsing the hyphal mass with ethanol and methylene chloride, the measured radiolabel is assumed to result from hyphal uptake rather than surface sorption. In all instances, the TCB was taken up to a higher degree than PtCB. This result is consistent with findings of other researchers suggesting that more highly substituted PCB congeners are often less bioavailable (Chiou *et al.*, 1977; Donnelly and Fletcher, 1995; Pal *et al.*, 1980). Additionally, both PCB congeners were taken up to a higher degree than aldrin in all cases except in the incubation with Surfonic® L24-9.

For comparative purposes, the uptake values for each surfactant were normalized to the highest observed uptake value for each contaminant (see Figure 7). Each of the surfactants increased the relative uptake of at least two of the three contaminants over the

controls. Surfonic® T-20 showed the most promise for additional research, as this surfactant increased hyphal uptake for all contaminants by at least a factor of two during the ten-day incubation period.

Many factors exist under field conditions that could alter the magnitude of the trends observed *in vitro*. Surfactant addition might prove to be especially useful in organic soils, for example, due to problematic sorption of hydrophobic compounds to soil organic matter. The presence of vegetation in symbiotic association with mycorrhizal fungi would undoubtedly affect the growth and uptake patterns of the fungi. Additionally, activities of other rhizosphere organisms, such as bacteria, could potentially affect the efficacy of surfactant addition.

Effect of Incubation Time: A replicate set PtCB and aldrin samples were incubated for thirty days to assess the effects of incubation time. The radiolabel uptake per unit hyphal mass increased in every instance between ten and thirty days for the PtCB samples, regardless of surfactant presence or type (Figure 8). A similar pattern was observed for the aldrin samples (data not shown). Control experiments revealed that by the tenth day of incubation, approximately 80% of the glucose added to these reactors was either mineralized or used for cell growth. In the ensuing twenty days, the mass of the fungal hyphae remained relatively constant as readily available carbon became limiting. The continued uptake of radiolabel, observed during a stationary phase of hyphal growth, suggests that uptake was not strictly dependent upon cell growth. This result implies that in the field, mycorrhizal systems likely have the capacity to take up contaminants under conditions of both high and low hyphal growth.

Section 3.5: Conclusion

This study provided evidence that surfactant addition could enhance mycorrhizae-mediated phytoremediation. Results indicated that a mixed culture of ectomycorrhizal fungi grown *in vitro* used two of the three added surfactants as a growth substrate under carbon-limiting conditions. In the field, the physical association between a biodegradable surfactant micelle and a hydrophobic target compound could potentially enhance the biodegradability of the target compound. Additionally, each of the three surfactants increased the radiolabel uptake of at least two of the three chlorinated compounds under high growth conditions. Although radiolabel uptake does not necessarily equal contaminant degradation, increased radiolabel uptake does imply increased bioavailability, which is an important precursor to biodegradation. Finally, an extended incubation suggested that mycorrhizal fungi have the capacity to take up hydrophobic contaminants during periods of low hyphal growth. Surfactant addition has the potential to enhance myco-phytoremediation efforts under a variety of conditions.

Section 3.6: Acknowledgments

This work was supported by the ENSR Corporation and the U.S. Army (through the U.S. Army Corps of Engineers). Dot Helm and the staff at the University of Alaska Fairbanks Agricultural and Forestry Experiment Station Palmer Research Center provided additional support.

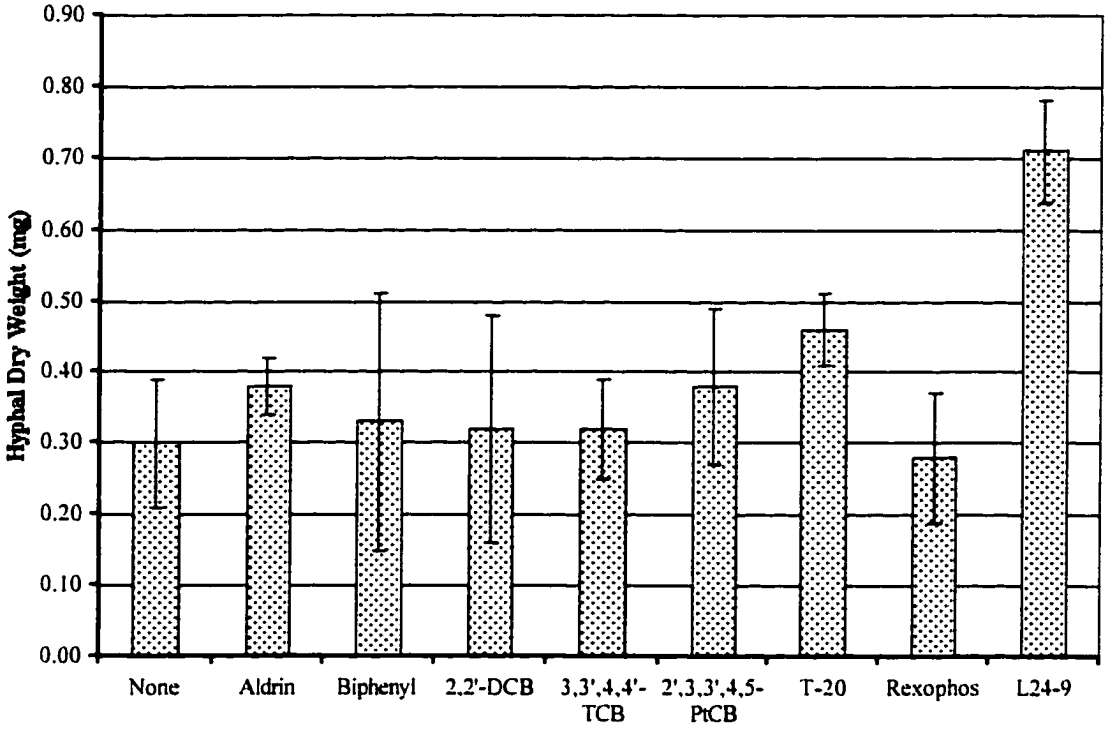

Figure 5: Hyphal Growth Under Carbon-Limiting Conditions

Hyphal weights of mixed culture ectomycorrhizal fungi grown in carbon-limiting media are shown. Media amendments are listed along the bottom axis. Error bars represent 95% confidence interval about the mean of 3 replicates.

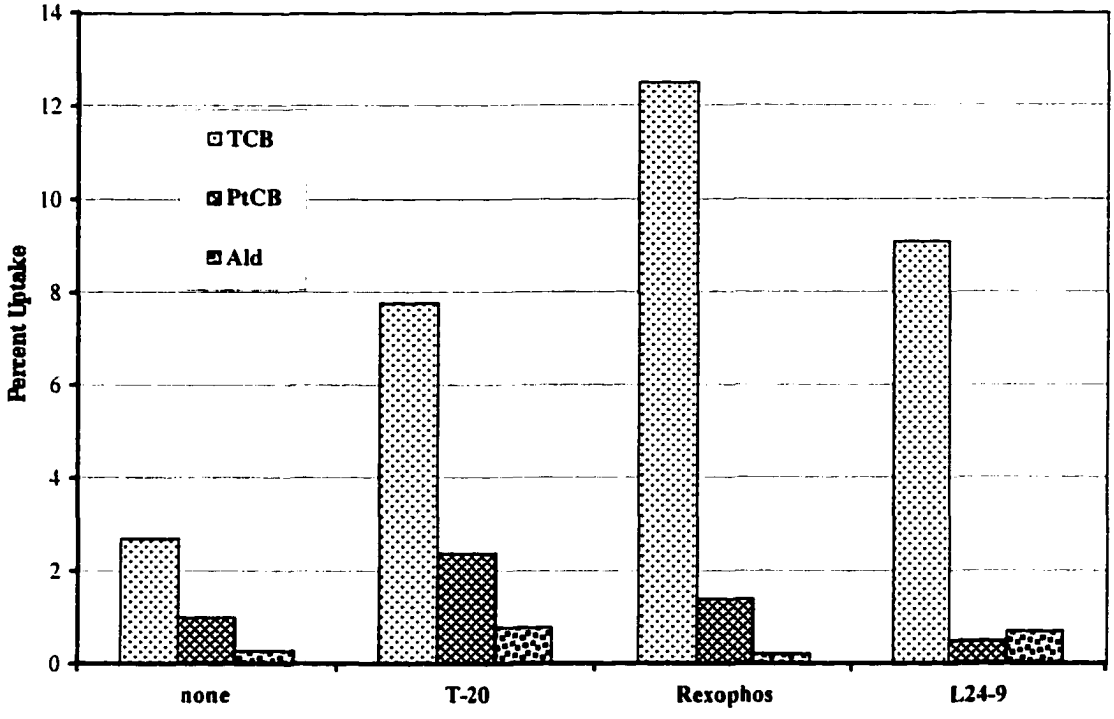

Figure 6: Uptake of TCB, PtCB, and Aldrin with Surfactants

Uptake represented as a percent of added radiolabel is shown for fungi incubated in the presence of the listed surfactants.

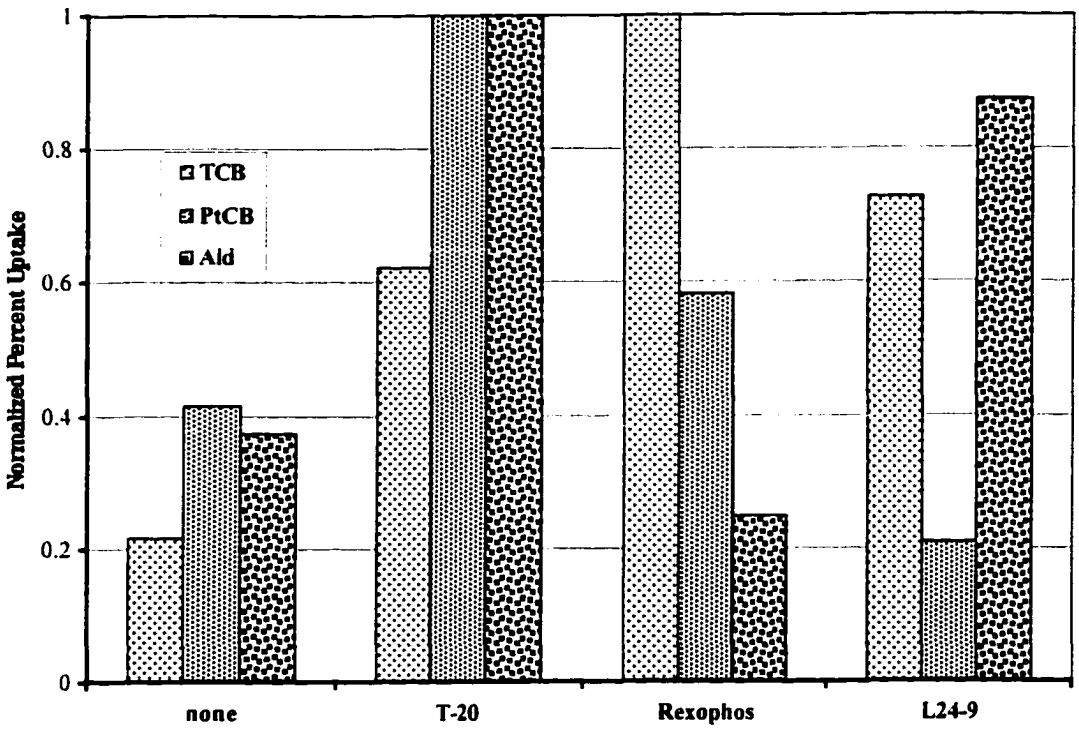

Figure 7: Normalized Uptake of TCB, PtCB, and Aldrin with Surfactants

For comparative purposes, uptake is normalized relative to the highest amount of uptake for TCB (≈ 12%), PtCB (≈ 2%), and Aldrin (≈ 1%) observed throughout the treatment groups.

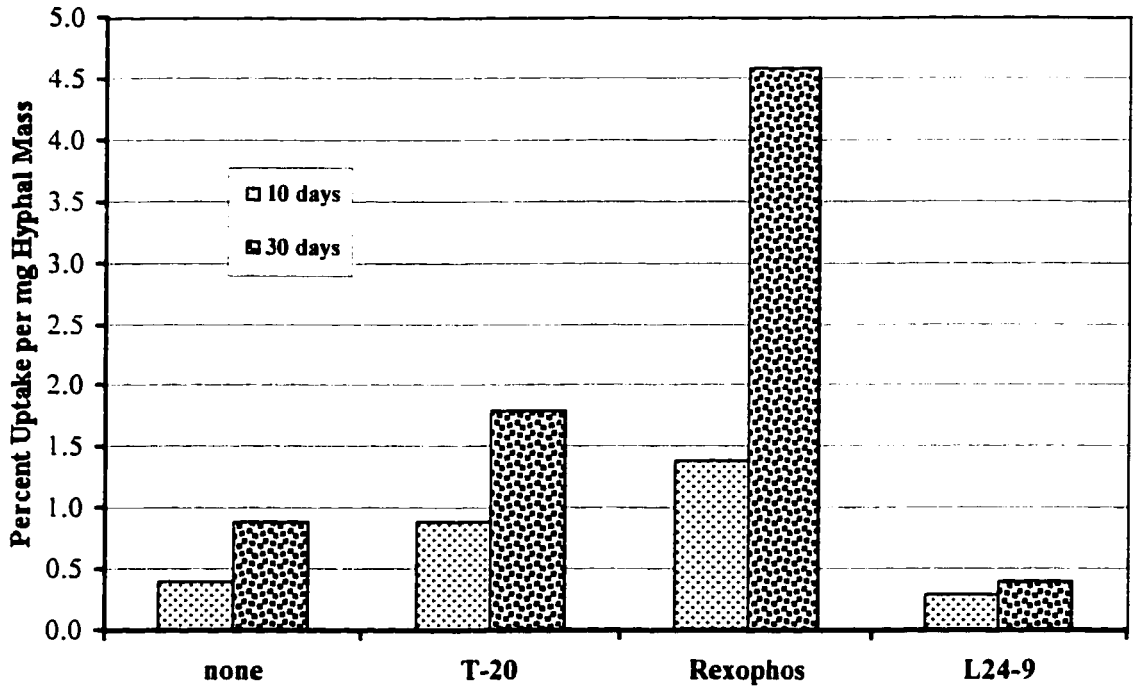

Figure 8: PtCB Uptake After 10 and 30 Days Incubation in Surfactants

Percent uptake per unit hyphal mass is shown for incubations of 10 and 30 days. Hyphal mass did not significantly increase after 10 days of incubation.

Chapter 4: TCB Uptake Study

This chapter is a format-modified version of a manuscript submitted to the *International Journal of Phytoremediation*[2]. The manuscript is currently in the review process. Raw data supporting this experiment is contained in Appendix B.

Section 4.1: Abstract

The objective of this study was to investigate the effects of mycorrhizal infection on vegetative uptake of polychlorinated biphenyls. Feltleaf willow (*Salix alaxensis)* and balsam poplar (*Populus balsamifera*) were grown in soil spiked with 6 mg/kg 3,3',4,4'-tetrachlorobiphenyl-(UL-^{14}C). The fungicide Daconil$_{2787}$® was employed to suppress indigenous mycorrhizal infection. After 100 days of greenhouse incubation in semi-enclosed phytoreactors, mycorrhizal infection was found to be approximately 3 fold higher in the untreated willows versus the fungicide-amended willows. Radiolabel uptake was found to correlate most highly with mycorrhizal infection in the willow roots (R=0.83). Over the same time period, mycorrhizal infection in the poplars was not significantly affected by fungicide addition. In the poplar phytoreactors, radiolabel uptake was most highly correlated with water use (R=0.70). The overall vegetative radiolabel uptake was low (≈ 1%), but the limited uptake was attributed to soil sorption processes rather than vegetative limitations.

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[2] Submitted as: Schnabel, W. and White, D. 2000. The effect of mycorrhizal fungi on the fate of PCB's in two vegetated systems. *International Journal of Phytoremediation*. March, 2000.

Section 4.2: Introduction

The high cost and regulatory obstacles associated with polychlorinated biphenyl (PCB) remediation are often prohibitive to soil clean up. Consequently, many researchers are currently investigating novel PCB remediation strategies, which are both effective and cost-efficient. For example, white rot fungi have been shown to be capable of degrading PCB's, and their use has been proposed as a viable remediation strategy (Bumpus *et al.*, 1985). Additionally, various vegetative species have demonstrated the ability to release root phenolics into the rhizosphere, and could thus contribute to the growth of PCB-degrading bacteria *in situ* (Fletcher and Hegde, 1995). Finally, physical-biological hybrid methods have been proposed involving the use of surfactant-mediated soil washing and PCB-degrading bacteria (Layton *et al.*, 1998). The key components of such systems include 1) PCB-degrading soil microorganisms, 2) vegetative contributions to the rhizosphere, and 3) PCB mobilization through surfactants. Since it is likely that these components could act concurrently to promote the breakdown of PCB's in the soil, it may prove beneficial to work towards a remediation strategy which encompasses all of them.

Mycorrhizal (symbiotic vegetative-fungal) systems amended with surfactants could potentially be employed to remediate a wide variety of recalcitrant soil contaminants, including PCB's. Such myco-phytoremediation systems could promote contaminant breakdown via increased bacterial activity, increased fungal activity, vegetative uptake, and contaminant mobilization. Identification of mycorrhizal uptake

characteristics *in situ* is a first step towards the development of such a remediation strategy. Mycorrhizal fungi, similar to white rot, have been shown to degrade a variety of organic compounds. Such compounds include lignin (Trojanowski *et al.*, 1984), TNT (Meharg *et al.*, 1997b), and PCB's (Donnelly and Fletcher, 1995). Additionally, vegetation itself has been widely reported to hasten contaminant breakdown in the surrounding soil (Schnoor *et al.*, 1995). The relationship between *in situ* vegetative PCB uptake and mycorrhizal infection is poorly understood, however, and requires further study. Once the effects of mycorrhizal infection on uptake are more clearly understood, researchers can begin to investigate methods of increasing mycorrhizal-mediated uptake (e.g. inoculation strategies, surfactant addition).

It may prove beneficial to investigate the degradative characteristics of indigenous mixed-culture systems rather than to investigate the degradative capabilities of specific organisms under strict conditions. In the field, vegetation exists in symbiosis with a consortium of mycorrhizal fungal species that vary both spatially and temporally throughout the rhizosphere (Helm *et al.*, 1996). Additionally, it has been shown that a wide-variety of mycorrhizal fungi demonstrate PCB-degrading capabilities (Donnelly and Fletcher, 1995). Finally, both the vegetative and fungal components of a mycorrhizal system interact closely and exchange nutrients with free-living rhizosphere microorganisms (Allen, 1991). By examining the degradative characteristics of mixed-culture systems, researchers can develop strategies for inducing entire systems to promote the remediation of soil contaminants.

The purpose of this study was to investigate the effect of mycorrhizal infection on the fate of PCB's in vegetated soil. Congener #77, 3,3'4,4'-tetrachlorobiphenyl (TCB), was chosen as a representative PCB molecule based upon its intermediate level of chlorine substitution, mobility (Pal *et al.*, 1980), and toxicity (Van den Berg *et al.*, 1998), as well as its relatively high resistance to biodegradation in vegetated systems (Donnelly *et al.*, 1994). Feltleaf willow and balsam poplar were chosen as vegetative test species because they represent a successional range of woodland development. While both species are relatively fast growing and easy to cultivate, willow often thrives in nutrient-poor soils, whereas poplar generally thrives in soils higher in mineral and organic nutrients (Viereck and Little, 1972). The present study represents a plank in the development of an integrated phytoremediation strategy whereby mycorrhizal vegetation, free-living soil microorganisms, and surfactant amendments are employed to accomplish the *in situ* degradation of PCB's and other extremely recalcitrant soil contaminants.

Section 4.3: Materials & Methods

4.3.1: Plant Sampling

Cuttings (approximately 15 cm) were collected from adult feltleaf willow (*Salix alaxensis)* and balsam poplar (*Populus balsamifera*) branches near Fairbanks, AK. In order to avoid genotypical variation, all cuttings were taken from the same specimens. Poplar cuttings were collected in early April, prior to bud break. Willow cuttings were

collected in early July, shortly before planting. Cuttings were stored at 4 °C in darkness until planting.

Prior to planting, poplar buds were trimmed to one bud per cutting, and all leaves were removed from willow cuttings. All cuttings were then scrubbed with a solution of 0.5% NaOCL to remove soil and organic material. After rinsing, the cuttings were planted into 300ml foam cups containing approximately 200 ml of clean silica sand. The plants were then placed onto a benchtop mister for rooting. During rooting, the plants were fertilized once per week with a 0.07% solution of 15-30-15 Miracle-Gro® commercial fertilizer (Scotts Miracle-Gro Products, Inc.).

4.3.2: Soils

The soil used for plant growth was a homogenized mixture of field soil and commercial quartz sand. Soil samples were collected from an uncontaminated site on Ft. Wainwright, Alaska, located near a site contaminated with chlorinated organic compounds. Both feltleaf willow and balsam poplar were observed to be growing near the collection site. To ensure adequate drainage through the phytoreactors, this soil was mixed with quartz sand (#8 and #16 mesh) at a ratio of 1:1:1 by mass. The final mixture was classified by sieve analysis as a sand, containing 90.8% sand, 8.9% silt, and 0.3% clay. Nutrient analysis performed at the UAF Agricultural and Forestry Experiment Station Plant and Soil Test Laboratory, Palmer Alaska, revealed that the soil mixture contained 1 ppm NH_4^+-N, 2 ppm NO_3^--N, 2 ppm P, and 18 ppm K. Additionally, the final soil contained 1.2% organic matter, based upon loss on ignition.

4.3.3: Phytoreactors

Semi-enclosed phytoreactors were constructed to contain individual plants (Figure 9). Where possible, glass or Teflon® construction materials were employed to reduce sorption of the analyte onto phytoreactor components. One-liter amber glass jars were used to house the soil and plant roots. Feedwater influent ports and ventilation ports were drilled into the caps, and feedwater effluent ports were drilled into the bottom of the vessels. The feedwater effluent and ventilation streams contained activated carbon to trap organic compounds escaping from the phytoreactors. The feedwater inlet ports were sealed off except during injection of feedwater. All ports were sealed with silicone caulk. The glass portions of the phytoreactors were covered with aluminum foil to discourage algal growth and reflect radiant heat. The phytoreactors were filled with 800 ml (1320 g dry weight) soil. This volume of soil was found to hold 185 ml of water at saturation. After the soils were spiked and the carrier solvents allowed to evaporate (see below), rooted cuttings were inserted through holes in the caps, and sealed with lanolin.

The semi-enclosed phytoreactors were designed specifically for fate studies on hydrophobic and recalcitrant soil contaminants. The phytoreactors were designed to approximate soil moisture, soil gas, and ambient air conditions found in a natural setting. Furthermore, the placement of caps on the phytoreactors allowed the plant tops to be exposed to the ambient air while preventing foliar sorption of volatilized radiolabel.

4.3.4: Dosing

Each phytoreactor was spiked with 8 mg 3,3',4,4'-tetrachlorobiphenyl (TCB). Radiolabelled TCB-(UL-^{14}C), obtained from Sigma-Aldrich, was mixed with unlabelled TCB (Ultra Scientific) to obtain a specific activity of 0.025 μCi/mg TCB. Two milliliters of a methylene chloride carrier solution (4 mg TCB/ml) were mixed into the soil matrix. The soil was mixed daily and left uncapped under greenhouse lighting for a period of 4 days to allow the carrier solution to evaporate. The final soil TCB concentration was 6.1 mg TCB/kg dry soil.

4.3.5: Inoculation

To ensure adequate microbial populations, the phytoreactors were inoculated with mixed-culture native Alaskan soil microorganisms after evaporation of the methylene chloride. Root and rhizosphere soil samples were obtained from three feltleaf willow and three balsam poplar stands near Fairbanks, AK. The soils and homogenized root material (100 g/sample) were then mixed into a carboy containing 2 L deionized water. In addition, 200 ml of a previously cultured mixed ectomycorrhizal fungi suspension were added to the carboy (Schnabel and White, 2000a). The carboy was shaken vigorously to produce a suspension of soil, organic material, and regionally indigenous soil organisms. This suspension was then added to the phytoreactors in 20 ml aliquots.

4.3.6: Plant Treatment

Plants were grown in a well-ventilated greenhouse at the University of Alaska Fairbanks Experiment Farm. Rooted cuttings were planted into dosed phytoreactors in late August, and allowed to grow until early December (100 days). A matrix of high-intensity sodium vapor lamps was located approximately one meter above the tops of the original cuttings. To simulate regional peak growing season, the lights were left on continuously. During peak daylight hours, photosynthetic-active radiation (PAR) was measured to be 249±19 $\mu mol/m^2$-sec at the tops of the original cuttings. The PAR was measured to be 112±21 $\mu mol/m^2$-sec at night. Greenhouse temperature was maintained at 20±5 °C.

Plants were watered on an as-needed basis with tap water. To encourage rapid growth during the early stage of development (40 days), all plants were fertilized with a 0.07% solution of 15-30-15 Miracle-Gro® commercial fertilizer (≈ 7.5mM nitrogen, 0.6mM phosphorus, and 0.9mM potassium) with each watering event. In addition to N, P, and K, the Miracle-Gro® contained small quantities of boron, copper, iron, manganese, molybdenum, and zinc. During the latter stages of development (60 days), all plants were fertilized with a solution containing only nitrogen and phosphorus (5 mM-N, 1 mM-P) on every second watering event. This latter fertilization regimen was found in our lab to be optimal for mycorrhizal development in feltleaf willow and balsam poplar under similar greenhouse conditions (unpublished data). During the latter stages of growth, the plants required water approximately once every 4 days. In selected phytoreactors, the

fungicide Daconil$_{2787}$® (Montsanto) was added with every watering event to inhibit mycorrhizal development. Feedwater for the fungicide-treated plants included 750 ppm chlorothalonil, the active ingredient in Daconil$_{2787}$®.

At the end of the growing period, the plants were harvested and reactor components were separated and prepared for analysis. Roots, leaves and stems were removed from the phytoreactors and stored at –20 °C for further analysis. Activated carbon traps were placed into sealed glass containers to await extraction and analysis. Soils were returned to the phytoreactors and stored at 4 °C for further analysis.

4.3.7: Microbial Enumeration

Bulk soil bacteria and fungi were enumerated following a previously reported plating method (Wollum, 1982). Bacterial media was prepared by adding 3 g of tryptic soy broth and 15 g of agar to 1 L deionized water. The media mixture was autoclaved for one hour, then stirred continuously while cooling at room temperature. After approximately 45 minutes, the cooled media was poured onto sterile Petri plates. The plates were then allowed to cure for 3 days prior to inoculation. Fungal media was prepared by adding 10 g potato flour, 10 g glucose, and 15 g agar to 1L deionized water. The mixture was autoclaved for one hour and allowed to cool on a stir plate for 45 minutes. After cooling, 40 mg erythromycin and 40 mg tetracycline hydrochloride (Fisher Scientific) were added to inhibit bacterial growth. The media was then poured into sterile Petri plates and allowed to cure for 3 days.

Bulk soils from vegetated pots both treated and untreated with fungicide were sampled and prepared for microbial enumeration. Soil suspension dilutions ranging to 10^{-8} were prepared in autoclaved tap water, and the plates were inoculated as described (Wollum, 1982). Bacterial plates were counted after an incubation period of 6 days at 20 °C. Fungal plates were counted after an incubation of 9 days at 20 °C.

4.3.8: Biological Oxidation

Radiolabel concentration in reactor components was quantified via biological oxidation. Root material was first removed from the cuttings and shaken to remove large clumps of soil. Next, the roots were immersed in acetone and scrubbed thoroughly to remove any fine soil particles or surface-sorbed organic compounds. The acetone was then squeezed out of the root mass, and a small quantity of water (≈ 10 ml) was used to rinse away any remaining acetone. The water was then squeezed out of the root mass, and the roots were allowed to drip dry for five minutes. The scrubbed soil, water, and acetone rinsates were subsequently added to the bulk soils for subsequent extraction. After drying and weighing, a subsample (≈ 2 g) of the root mass was analyzed in an R.J. Harvey OX500 biological oxidizer. The remainder of the root mass was then oven dried at 80 °C for two days. After drying, the root material was crushed to a fine powder using a mortar and pestle and mixed thoroughly. A subsample (≈ 0.4 g) of this dried root material was then oxidized as before (see "Drying Effects" section). Scintillation cocktail from the biological oxidizer was counted in a Beckman LSC6000IC liquid

scintillation counter. New growth stems and leaves were treated similar to the root material, with the exception that water and acetone rinses were not performed. Woody material from the original cuttings was not analyzed.

4.3.9: Drying Effects

In order to account for heterogeneous distribution of radiolabel in the root material, all roots were dried, crushed, and mixed thoroughly before the final oxidation. Although this step was considered to be essential, there was a possibility that such treatment would cause the analyte to volatilize during the drying process. Consequently, a subsample of the fresh roots was oxidized immediately after harvest, and these measurements were compared with the measurements from the dried and mixed roots. Averaged over all samples, the radiolabel concentration in the fresh subsamples was approximately the same as the radiolabel concentration in the well-mixed dried samples (dried/fresh = 1.2 ± 0.1). Consequently, it was determined that drying the samples did not significantly volatilize the radiolabel.

4.3.10: Radiolabel Extractions

To test for sorbed volatile organics, the effluent air traps were extracted into hexane. Carbon from the effluent air traps was emptied into individual 20 ml scintillation vials. Five ml of hexane were then added to each vial. After vigorous shaking, 10 ml of ScintiSafe Plus 50% scintillation fluid (Fisher Scientific) were added, and the radioactivity was quantified on the scintillation counter.

To differentiate compounds based on polarity, the effluent water traps were extracted into acetone and hexane. Carbon from the effluent water traps was emptied into individual 40 ml glass vials. Twenty ml of acetone were then added to each vial. The vials were then shaken vigorously and the suspensions allowed to settle for 5 minutes. Five ml of the acetone extract were then removed from each vial and added to 10 ml ScintiSafe Plus 50% scintillation fluid in a counting vial. The remaining acetone was poured off, and the extraction procedure was repeated using hexane. The extracts were then read on the scintillation counter. To ensure that no material remained sorbed to the carbon, a subsample of the extracted carbon was analyzed via biological oxidizer and was found to contain no radiolabel.

For selected samples, radiolabel in the root material was extracted into hexane. Approximately 0.5 g of dried, pulverized root material was weighed out and added to 40 ml glass vials containing 20 ml hexane. The vials were then capped and shaken vigorously on a shaker table for one hour. The contents of the vials were then allowed to settle overnight. After all fine particles had settled, 5 ml of hexane were sampled from each vial and added to 10 ml ScintiSafe Plus 50% scintillation fluid for counting. An additional 10 ml of hexane were sampled and stored for analysis via GC-ECD (HP 5890 Series II gas chromatograph). The GC results were then compared with the scintillation results to determine the form of the extracted radiolabel.

Radiolabel in the soils was extracted into acetone and hexane. Soils from the phytoreactors were added to fluorinated, 2 L TCLP extraction bottles. The empty phytoreactors were then rinsed with 200 ml acetone, and the acetone was added to the

extraction bottles. In addition, the soil, water, and acetone rinsates from the root material (see "Biological Oxidation" section) were added to the TCLP bottles. The soils were then tumbled in a TCLP tumbler at 30 rpm for 10 minutes. The acetone was drained from the bottles and stored in VOC-compatible glass containers at 4 °C until further analysis. After the acetone extraction, the empty phytoreactors were rinsed with 200 ml hexane, and the hexane was added to the soils and tumbled as before. The hexane was then drained into new VOC-compatible glass bottles, and an additional 100 ml of hexane were added to the soils. The soils were rinsed with this second aliquot of hexane, and the rinsates were drained and combined with the original hexane extracts. The final hexane extracts were capped and stored at 4 °C until further analysis. All hexane and acetone extracts were then analyzed for radiolabel via liquid scintillation. Additionally, selected subsamples of the extracts were analyzed for compound-specific quantification via GC-ECD.

4.3.11: Chitin Assay

Mycorrhizal development in the root material was assayed via root chitin content. As prescribed in an earlier study (Vignon *et al.*, 1986), the root material was acid-hydrolyzed, and the hydrolysates were assayed colorimetrically on a Beckman DU 520 Spectrophotometer. Chitin content was found in a previous experiment to correlate highly with visual counts of ectomycorrhizal-infected feltleaf willow and balsam poplar root tips (unpublished data). Additionally, the chitin method allowed for a large number of samples to be quantified much more efficiently than quantification through direct

counts. In our laboratory, a mixed-culture of indigenous ectomycorrhizal fungi grown *in vitro* was found to contain 85 mg chitin/g fungal mycelium (dry weight).

Section 4.4: Results & Discussion

4.4.1: Effect of Fungicide

The addition of Daconil$_{2787}$® had different effects on the willow and poplar systems. Fungicide addition did not significantly affect plant growth in either system (Table 1). Mycorrhizal formation, however, was found to be substantially inhibited by the presence of fungicide in the willow phytoreactors. In the poplar systems, this effect was not observed. The pattern of radiolabel uptake was similar to the pattern of mycorrhizae formation. For the willows, radiolabel uptake was lower in the phytoreactors amended with fungicide. Uptake in the poplar phytoreactors was approximately the same regardless of fungicide treatment.

It remains unclear why the fungicide suppressed mycorrhizal formation in the willow systems but not the poplar systems. As described previously, the soils and inoculum used for both sets of phytoreactors were identical. It has been demonstrated that mycorrhizal infection varies qualitatively between poplars and willows growing closely together, however, so the discovery of qualitative differences between the mycorrhizal responses in our phytoreactors is not surprising (Helm *et al.*, 1996).

The addition of fungicide affected the composition of the bulk soil in all of the phytoreactors. In an experiment performed under conditions similar to those used for the uptake study, soils amended with fungicide were compared with non-fungicide control soils. Fungal and bacterial colony-forming units (CFU) were enumerated from both sets of soil. After incubation, it was found that the soils amended with fungicide produced an average of $2x10^2$ fungal CFU/g, whereas the control soils produced an average of $1x10^3$ fungal CFU/g. Thus, fungicide addition suppressed the fungal response by a factor of five in the bulk soils. Conversely, fungicide addition significantly increased the number of bacterial colony-forming units in the same soils. Soils amended with fungicide contained an average of $9x10^6$ bacterial CFU/g, compared with $2x10^5$ bacterial CFU/g in the control soil. Presumably, the increased bacterial presence in fungicide-amended soils was the result of decreased competitive pressures exerted by the fungi. The fungicide may also have served as a bacterial substrate.

The disparity between the bulk soil composition of the fungicide and control soils indicates that under the experimental conditions the fungicide suppressed fungal activity. Although enumeration via pour plates often under-approximates the actual number of soil organisms, one can expect the trends observed using pour plates to reflect the trends in the soils being tested. Consequently, it was assumed that the bulk soils amended with fungicide in the uptake experiment contained comparatively more free-living bacteria and fewer free-living fungi than the soils not amended with fungicide.

4.4.2: Mass Balance on Radiolabel

Average radiolabel recoveries in the vegetated phytoreactors ranged from 80% to 89% (Table 2). A small percentage of the total radiolabel (between 0.6% and 1.2%) was found in the root material compared to the bulk soil. This is due in part to the large soil mass (≈1.3 kg dry weight) compared to the relatively small root mass (≈ 2 g dry weight) harvested.

A significant amount of radiolabel in each phytoreactor remained unaccounted for. It is possible that some of this radiolabel was mineralized in the soil and escaped through the effluent carbon traps. Such a result is unlikely, however, since mineralization rates are usually low for congeners with more than two or three chlorine atoms (Pal *et al.*, 1980). In a similar study, for example, researchers measured only 0.1% mineralization of hexachlorobiphenyl after 102 days (Epuri and Sorensen, 1997). Furthermore, no radiolabel was detected in any of the effluent air traps. Volatilization of parent compound, therefore, did not likely present a significant loss of radiolabel. Additionally, the finding that no radiolabel was detected in the water traps indicates that neither TCB nor any organic metabolites were present in significant amounts in the effluent water stream. Finally, periodic assays of the water leaving the traps indicated that no soluble radiolabel escaped the carbon traps in the effluent water stream.

Most of the radiolabel not accounted for in the mass balance likely remained sorbed to the soil after extraction. Although the extracted soils were not tested individually for residual radiolabel, 5 subsamples were combusted from the homogenized post-extraction waste soil pile. Residual radiolabel from the combined soils was found to

represent 23 ± 7% of the original soil concentration. A recent study investigating field-contaminated sediments indicated that PCB's in soil generally display two-phase desorption kinetics (Ghosh *et al.*, 1999). For tetrachlorobiphenyls, the study showed that most of the tested material desorbed relatively quickly, and the remaining fraction was slow to desorb. In our experiment, most of the radiolabel unaccounted for was likely strongly-sorbed, and relatively non-extractable.

4.4.3: Root Extractions

One subsample of dried root material from each treatment group was extracted using the outlined procedure. As indicated in Table 3, only a fraction of the radiolabel taken up into the root material was extracted into hexane. This trend was observed in both plant systems regardless of fungicide application. The extractable radiolabel was subsequently identified via GC-ECD as parent product in all four samples. The presence of extractable parent product in the root material indicated that the vegetative systems had at least one mechanism for the mass transfer of TCB across the root cell membrane. By definition, the non-extractable fraction of the radiolabel in the root material took the form of bound residue. Although the existence of bound residue suggests that the parent TCB compound was metabolized or otherwise transformed at some point, these experiments did not indicate whether the transformation occurred inside or outside of the vegetative matrix.

The total radiolabel concentration in the dry root material was as much as 9 times higher than the radiolabel concentration in the surrounding soil (Table 3). By contrast, the extractable radiolabel concentration in the root material was between 0.2 and 3.0 times as high as the surrounding soil. Thus, since bound residue can be detected only by using labeled compounds, conventional uptake studies for unlabeled compounds in the field may underestimate the amount of uptake actually occurring.

No radiolabel was detected in the aboveground portion of any of the plants. Since the roots were found to contain measurable amounts of TCB, this finding suggests that the TCB was not xylem mobile. Such a result was expected, based upon the results of previous transport studies (Quiping *et al.*, 1992), and the predictions of transport models based upon K_{ow} (Burken and Schnoor, 1998). Although it is possible that xylem mobile TCB transformation products were formed, translocated, and volatilized out of the leaves, it was expected that such an occurrence would result in detectable amounts of radiolabel stored in the leaves.

4.4.4: Uptake Correlations

Several vegetative parameters were measured and plotted against the radiolabel concentration in the root material. These parameters were chosen based upon the supposition that root uptake would likely be related to either plant growth (root mass, total plant mass, root to shoot ratio), transpiration rates (water use per unit mass per time), or mycorrhizal fungi infection (chitin content per unit root mass, total chitin content). Of all the parameters correlated, willow root uptake was most highly correlated

with mycorrhizal infection (Figure 10). Poplar root uptake, on the other hand, was most highly correlated with vegetative parameters such as water usage (Figure 11).

It is likely that there were several mechanisms contributing to vegetative radiolabel uptake. The data presented above indicate that mycorrhizal symbiosis may have been a mechanism for uptake in the willow systems. Increasing the level of mycorrhizal development could potentially increase the amount of uptake in similar systems. In the poplar phytoreactors, mycorrhizal development was not shown to be related to radiolabel uptake. A possible explanation, however, is that since the fungicide added was ineffective at inhibiting mycorrhizal development, the effects of suppressed mycorrhizal activity were not measurable. Regardless, the poplar phytoreactors demonstrated that uptake could possibly be enhanced in similar systems via physical-chemical manipulations such as increased contaminant solubility (surfactant addition) or increased vegetative transpiration rates.

4.4.5: Soil Extractions

As discussed above, selected acetone and hexane soil extracts were analyzed via GC-ECD. These results were then compared to the results of the LSC analysis to indicate whether the extracted radiolabel represented parent product. Over all the samples tested (n = 10) TCB accounted for 101 ± 9% of the extract radiolabel concentration. Likewise, TCB accounted for 116 ± 22% of the acetone extract radiolabel concentrations. Although we did not attempt to determine the existence of radiolabelled metabolic products in the

soil extracts, it was concluded that the quantity of such products compared to the quantity of parent product would be minimal.

4.4.6: Phytoremediation Potential

The rate-limiting step in the uptake of TCB was the desorption of TCB from the soil particles to the soil solution. The partition coefficient for hydrophobic compounds between soil organic carbon and an aqueous solution has been described as

$$\log K_{oc} = -0.54 \log S + 0.44,$$

where S is aqueous solubility expressed as mole fraction (Karickhoff *et al.*, 1979). The aqueous solubility of TCB has been reported as 0.175 ppm (Hutzinger *et al.*, 1974). Based upon the above equation and the soil organic content (1.2%), the soil solution equilibrium concentration would have been approximately 10 ppb. In rough terms, both the poplars and willows took up 50 ml of water per day for 100 days. Using these numbers, approximately 40 μg TCB would have been available for plant uptake over the course of the experiment. In general terms, however, the plants took up twice this amount of TCB. Although the above argument is based upon estimates and averages as opposed to individually measured parameters, it seems clear that the roots took up most or all of the radiolabel available in the soil solution. This conclusion is supported by the absence of radiolabel in the effluent water or effluent carbon traps. Furthermore, as the roots took up more TCB than was theoretically available to them based on equilibrium

coefficients, it appears likely that the presence of vegetation enhanced the desorption of TCB from the soil.

Although the plants likely took up all available radiolabel, the mass balances of this experiment indicate that the phytoremediation potential for similar systems is low. An uptake and/or transformation rate of approximately 1% per year would be unacceptable for most phytoremediation applications. Since the rate-limiting step was determined to be soil surface desorption, however, there is a possibility that the addition of surfactants could increase vegetative uptake and/or transformation in future systems. Surfactant addition in other systems has been shown to dramatically increase bioavailability (Guha and Jaffe, 1996).

Section 4.5: Conclusions

Although soils contaminated with PCB's such as 3,3'4,4'-tetrachlorobiphenyl are not generally considered to be amenable to phytoremediation, the results of this experiment indicate that there is a potential to increase vegetation-mediated transformation through the development of healthy mycorrhizal systems. Additionally, as vegetative uptake was found to be limited by contaminant bioavailability, surfactant addition has the capacity to further enhance phytoremediation potential. Additional research into both of these arenas could yield a viable integrated system for the phytoremediation of PCB's.

Section 4.6: Acknowledgements

This work was supported by the ENSR Corporation and the U.S. Army (through the U.S. Army Corps of Engineers). Dr. Pat Holloway and the staff at the University of Alaska Fairbanks Agricultural and Forestry Experiment Station, Fairbanks, Alaska, provided additional support.

Table 1: Fungicide Effects in TCB Uptake Study

	Plant Dry Weight (g)	Mycorrhizae Formation (mg Chitin/g Dry Root)	Radiolabel Uptake (μCi/10 kg Dry Root)
Willow	12.3 ± 1.3	4.4 ± 1.4	11.1 ± 4.2
Willow + Fungicide	12.0 ± 2.4	1.4 ± 0.6	5.1 ± 1.4
Poplar	9.4 ± 1.2	1.7 ± 1.3	6.9 ± 3.6
Poplar + Fungicide	8.6 ± 2.2	1.8 ± 1.1	8.5 ± 7.1

The effects of amending vegetative feedwater with 750 ppm Daconil$_{2787}$® are shown. Sample size (n = 5-6) for plant dry weight and radiolabel uptake; (n = 4) for mycorrhizae formation. Error is reported as 95% confidence interval.

Table 2: Mass Balances in TCB Uptake Study

	% In Soil	% In Roots	Total
Non-Veg	72.8 ± 2.4	n/a	72.8 ± 2.4
Non-Veg + Fungicide	68.4 ± 6.3	n/a	68.4 ± 6.3
Willow	79.7 ± 4.9	1.2 ± 0.5	80.9 ± 5.4
Willow + Fungicide	80.1 ± 3.5	0.7 ± 0.2	80.7 ± 3.7
Poplar	81.4 ± 6.3	0.6 ± 0.4	82.0 ± 6.7
Poplar + Fungicide	88.9 ± 3.8	0.6 ± 0.3	89.5 ± 4.0

Radiolabel recoveries from all phytoreactor components are listed. No radiolabel was detected in the plant tops, effluent air, or effluent water traps. Sample size (n = 3) for non-vegetated phytoreactors; (n = 6) for vegetated systems. Error is reported as 95% confidence interval.

Table 3: Root Radiolabel Concentrations in TCB Uptake Study

Plant	Fungicide	Total Radiolabel Concentration (μCi/10kg)	BF	Extractable Radiolabel Concentration (μCi/10kg)	BF
Willow	No	11.3	7.4	2.8	1.8
Willow	Yes	3.0	2.0	0.4	0.2
Poplar	No	7.7	5.1	1.5	1.0
Poplar	Yes	13.9	9.1	4.5	3.0

Total radiolabel concentrations identified via combustion are compared to radiolabel concentrations determined via hexane extraction (n = 1). All radiolabel was identified via GC-ECD as parent product. "BF" represents bioconcentration factor (concentration in roots/concentration in soil) calculated from the two quantification methods.

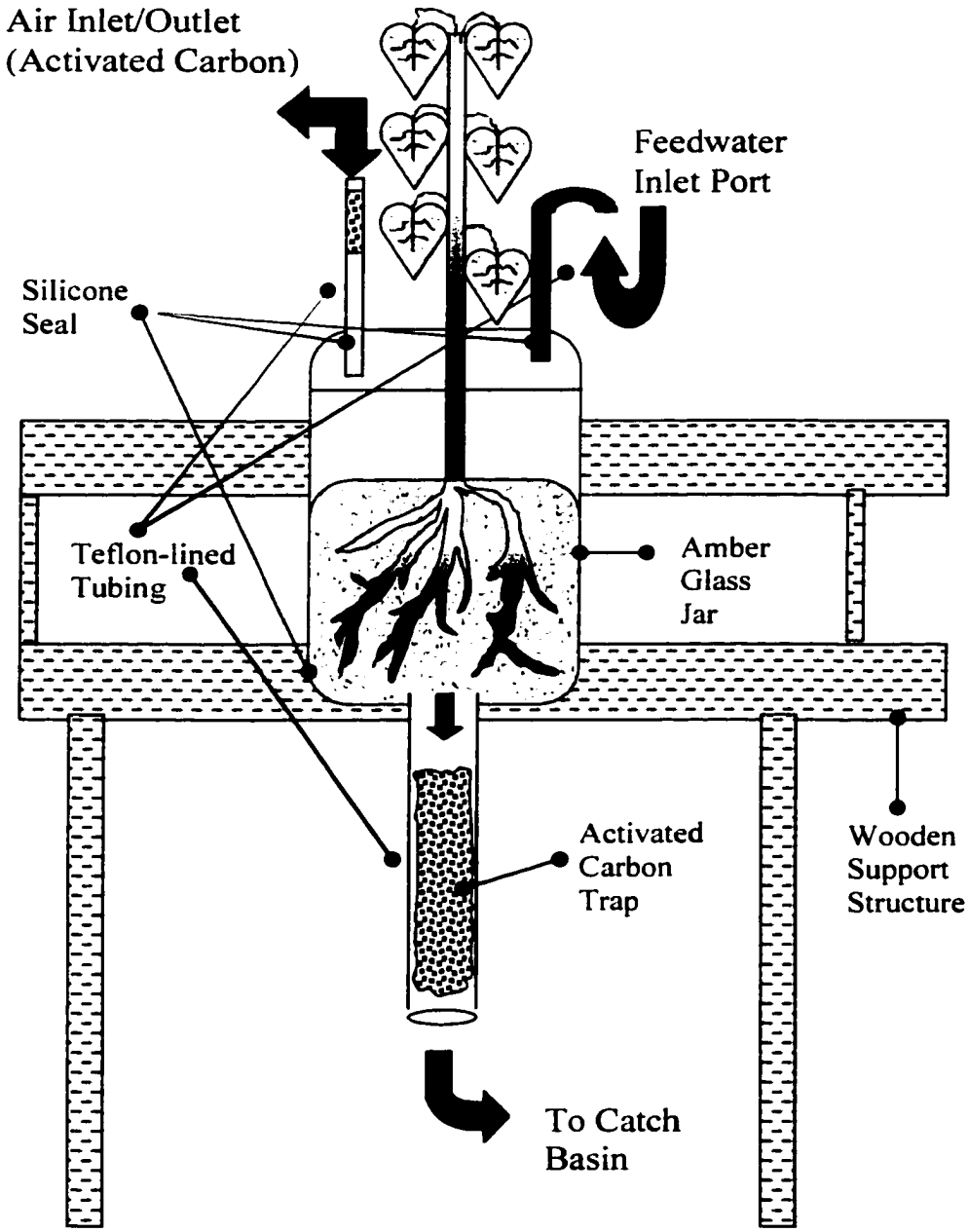

Figure 9: Phytoreactor Design

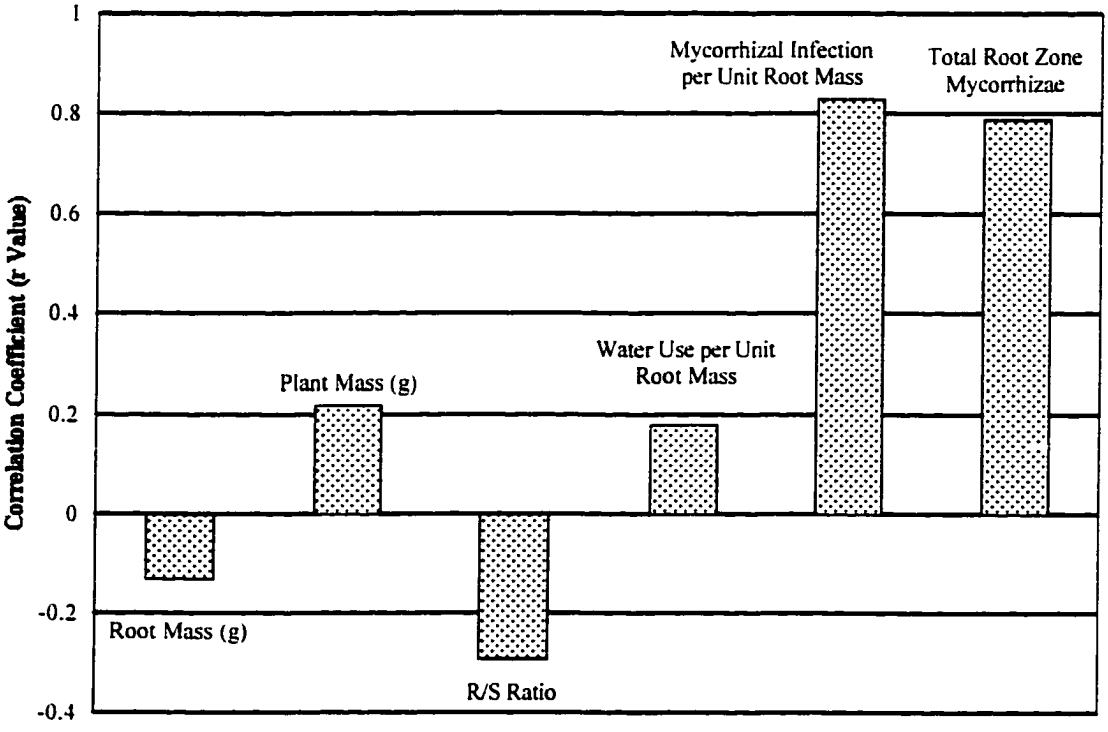

Figure 10: Willow Root Radiolabel Correlations in TCB Uptake Study

Final root radiolabel concentrations in *Salix alaxensis* are shown correlated to the listed vegetative and mycorrhizal parameters (n = 8). "R/S Ratio" refers to the dry mass of the roots divided by the dry mass of the shoots.

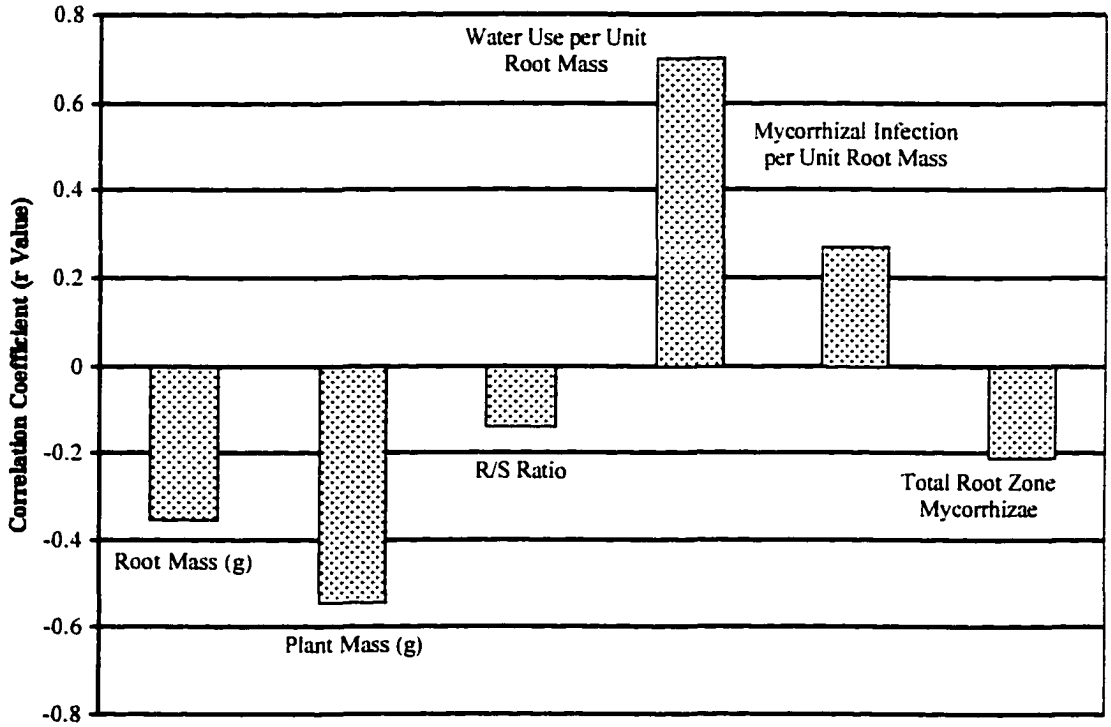

Figure 11: Poplar Root Radiolabel Correlations in TCB Uptake Study

Final root radiolabel concentrations in *Populus balsamifera* are shown correlated to the listed vegetative and mycorrhizal parameters (n = 8). "R/S Ratio" refers to the dry mass of the roots divided by the dry mass of the shoots.

Chapter 5: Aldrin Uptake Study

This chapter is a format-modified version of a manuscript submitted to the *International Journal of Phytoremediation*[3]. The manuscript is currently in the review process. Raw data supporting this experiment is contained in Appendix B.

Section 5.1: Abstract

The objective of this study was to investigate the phytoremediation potential of mycorrhizal systems for the remediation of aldrin-contaminated soils. Feltleaf willow (*Salix alaxensis)* and balsam poplar (*Populus balsamifera*) were grown in soil spiked with 0.8 mg/kg aldrin- (1,2,3,4,10-^{14}C). Daconil$_{2787}$® was employed to suppress indigenous mycorrhizal infection. After 100 days of greenhouse incubation, mycorrhizal infection in the fungicide-amended willows was found to be 2.5 fold lower than in controls. Mycorrhizal infection in the poplar systems was unaffected by fungicide addition. Mycorrhizae were correlated with radiolabel uptake in the willow systems (r = 0.79), and not as strongly in the poplar systems (r = 0.58). Most of the radiolabel in the root material was bound product regardless of mycorrhizal infection, but 12-21% was found to be extractable dieldrin. Aldrin was not detected in any vegetative matrix. Dieldrin constituted less than 1% of the radiolabel in the willow leaf material, accumulating to approximately 5 μg/kg. Dieldrin was not detected in the poplar leaves

[3] Submitted as: Schnabel, W., and White, D. 2000. The effect of mycorrhizal fungi on the fate of aldrin: Phytoremediation potential. *International Journal of Phytoremediation*. March, 2000.

(MDL ≈ 1 μg/kg), although the poplars took up approximately the same amount of radiolabel as the willows. Water-soluble transformation products were formed in the vegetated soils (6-12%) and non-vegetated controls (1-2%).

Section 5.2: Introduction

The organochlorine pesticide aldrin was first manufactured in the early 1950's, and was employed worldwide as an agricultural insecticide until the early 1970's. Although its use has been banned or severely restricted in many countries since the mid-1970's, aldrin residues persist in soils long after application has ceased (Miglioranza *et al.*, 1999). Aldrin and its epoxide, dieldrin, are considered to be toxic at moderate concentrations to a wide variety of terrestrial organisms, including humans (WHO, 1991). Due to its lipophilicity and its persistence in the environment, aldrin tends to bioconcentrate through the food web and is found in humans today (Alawi, Tamimi and Jaghabir, 1999).

Numerous studies have been performed concerning the environmental fate of field-applied aldrin. Early studies concluded that aldrin persistence increased with soil organic content, and decreased with increased soil moisture and temperature (Lichtenstein and Schulz, 1959). Aldrin has been shown to be readily epoxidized to dieldrin in soils (Lichtenstein and Schulz, 1960), and dieldrin has been shown to be taken up into crops via root sorption (Beall and Nash, 1971). Additionally, plants themselves

have been shown to transform aldrin into its epoxide (Gannon and Decker, 1958). Results of these studies and others were complied into a comprehensive review published by the World Health Organization (WHO, 1991).

Although the fate of aldrin is well studied, scant information is available concerning the applicability of phytoremediation for aldrin-contaminated sites. Recent studies have been centered on the kinetics of microbial-mediated aldrin and dieldrin degradation (Bandala *et al.*, 1998; Ghadiri *et al.*, 1995), but the utility of adding large plants to such systems remains unstudied. In other systems, plants have been shown to enhance the degradation of recalcitrant organic compounds via root exudates (Fletcher and Hegde, 1995), root uptake (Schnabel *et al.*, 1997), and through the degradative contributions of root-associated mycorrhizal fungi (Meharg *et al.*, 1997b). Through the contribution of one or all of these mechanisms, phytoremediation could be a plausible remediation strategy for aldrin-contaminated sites.

The purpose of this study was to examine the phytoremediation potential of vegetative systems in aldrin-contaminated soil. Particular emphasis was placed upon the effects of mycorrhizal fungi, given that mycorrhizal fungi have been shown to be effective degraders of recalcitrant organics (Donnelly and Fletcher, 1995; Trojanowski *et al.*, 1984). The study was completed in a greenhouse, yet was designed to emulate the growth conditions frequently encountered in a sub-arctic summer season.

Section 5.3: Materials & Methods

The present study was run in parallel with a study described elsewhere (Schnabel and White, 2000b). Experimental procedures and analytical methods are detailed more fully in the previous report.

5.3.1: Plants and Soils

Feltleaf willow (*Salix alaxensis*) and balsam poplar (*Populus balsamifera*) cuttings were collected from individual specimens in the area surrounding Fairbanks, AK. The cuttings were greenhouse rooted in clean quartz sand prior to placement into phytoreactors. Soils were collected from an uncontaminated site on Ft. Wainwright, AK. To ensure adequate drainage through the phytoreactors, site soils were homogenized with quartz sand (#8 and #16 mesh) at a ratio of 1:1:1 by mass. The final mixture was classified by sieve analysis as a sand, containing 90.8% sand, 8.9% silt, and 0.3% clay. Initial soil nutrients included 1 ppm NH_4^+-N, 2 ppm NO_3^--N, 2 ppm P, and 18 ppm K (UAF Agricultural and Forestry Experiment Station Plant and Soil Test Laboratory, Palmer Alaska). The experimental soil initially contained 1.2% organic matter, based upon loss on ignition.

5.3.2: Phytoreactors

Semi-enclosed phytoreactors were constructed as depicted in Figure 12. Activated carbon traps were placed in the ventilation and effluent water ports to prevent

the escape of organic compounds. Teflon® or glass was used where possible to prevent sorption onto phytoreactor components. The phytoreactors were filled with 800 ml (1320 g) dry soil. The soils were spiked with 1 mg aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,8,8a-hexahydro-endo-exo-1,4:5,8-dimethanonapthalene, Ultra Scientific), using methylene chloride and toluene (3:2) as a carrier solvent. The aldrin contained 0.2 μCi/mg aldrin-1,2,3,4,10-^{14}C (Sigma-Aldrich). The final aldrin concentration was 0.76 mg/kg soil (0.15 μCi/kg dry soil). Phytoreactors were left uncapped for four days under greenhouse lighting prior to inoculation and plant insertion to allow the carrier solvents to evaporate. After the equilibration period, an inoculum slurry of soil, root homogenate, and a mixed culture of regionally derived soil microorganisms was mixed into the soils. The inoculum solution was designed to represent the soil biota of a generic early to mid-successional boreal site. The rooted cuttings were then placed into the phytoreactors, and the phytoreactors were capped and sealed to prevent foliar sorption of organic vapors.

5.3.3: Greenhouse Operation

Plants were grown in a well-ventilated greenhouse on the University of Alaska Fairbanks Experiment Farm. The growth period was 100 days, beginning in late August. The plants were subjected to continuous lighting to simulate regional conditions during peak growing season (photosynthetic active radiation = 249±19 μmol/m^2-sec day, 112±21 μmol/m^2-sec night). Greenhouse temperature was maintained at 20±5 °C.

Plants were watered on an as-needed basis with tap water. During the early stage of development (40 days), all plants were fertilized with a 0.07% solution of 15-30-15

Miracle-Gro® commercial fertilizer. During the latter stages of development (60 days), all plants were fertilized with a pH neutral solution containing only nitrogen and phosphorus (5 mM nitrogen, 1 mM phosphorus) on every second feeding event. This latter fertilization regimen was found in our lab to be optimal for mycorrhizal development in feltleaf willow and balsam poplar under similar greenhouse conditions (unpublished data). In selected phytoreactors, the fungicide $Daconil_{2787}$® (Montsanto) was added with every watering event to inhibit mycorrhizal development. Feedwater for the fungicide-treated plants included 750 mg/L chlorothalonil, the active ingredient in $Daconil_{2787}$®.

5.3.4: Analytical Methods

Radiolabel quantification was accomplished via combustion in an R.J. Harvey OX500 biological oxidizer (BO). Scintillation counting (LSC) was performed on a Beckman LSC6000IC scintillation counter. Root material was rinsed in acetone and water, and a subsample was combusted after rinsing. Roots were then oven dried at 80 °C for 2 days, pulverized and homogenized. A second root subsample was then combusted. Radiolabel loss during the drying procedure was found to be minimal (94 ± 11% recovered). The dried roots were used for subsequent analyses to ensure homogeneity. New growth stems and leaves were combusted fresh, with no water or acetone rinses. Woody material from the original cuttings was not analyzed.

Activated carbon from the effluent air traps was extracted into hexane. The effluent water traps were extracted into acetone and hexane to differentiate compounds

based on polarity. Extracts were counted via LSC. The activated carbon was then combusted and analyzed via LSC to ensure that no radiolabel remained sorbed to the carbon.

On selected samples, dried root material was extracted into hexane. The activity of the extracts was counted via LSC. Extracts were then analyzed qualitatively via gas chromatograph, electron capture (GC-ECD), using an HP 5890 Series II Gas Chromatograph. Soils were extracted sequentially into acetone and hexane. Extracts were counted via LSC and analyzed qualitatively via GC-ECD.

On selected samples, leaf material was oven dried at 80 °C for two days and homogenized. A subsample of the leaf material was combusted and counted via LSC. The remaining leaf material was extracted into methylene chloride via Soxhlet extraction and analyzed qualitatively via GC-ECD (EPA Method 8081).

Mycorrhizal fungi inhabiting the root material was quantified via chitin concentration, as described previously (Vignon *et al.*, 1986). Colorimetric analyses were performed on a Beckman DU 520 spectrophotometer. In our laboratory, a mixed-culture of indigenous ectomycorrhizal fungi grown *in vitro* was found to contain 85 mg chitin/g fungal mycelium (dry weight).

Section 5.4: Results

5.4.1: Fungicide/Mycorrhizal Effects

The effects of fungicide addition on dry weight, mycorrhizae formation and radiolabel uptake are described in Table 4. As shown in the table, the fungicide had no significant effect on plant growth for the willows or poplars. Mycorrhizal development was significantly decreased by the addition of fungicide to the willow phytoreactors, and was relatively unaffected in the poplar systems. Although the radiolabel uptake was measured to be higher in the untreated systems than in the fungicide amended systems, the differences were not significant at the 95% confidence level.

In order to elucidate the relationship between mycorrhizal infection and root uptake, root chitin content was plotted against root radiolabel uptake in Figure 13 and Figure 14 for the willows and poplars respectively. As shown in Figure 13, radiolabel uptake in the willow roots appeared to correlate strongly with the amount of mycorrhizal fungi present ($r^2 = 0.63$). In the poplars, this correlation was not as strong ($r^2 = 0.34$). As stated previously, however, mycorrhizal development in the poplars was not strongly affected by the addition of fungicide. It is likely, then, that the limited variability of mycorrhizal development in the poplars contributed to the ambiguous findings.

To put into perspective the relative contribution of mycorrhizal infection to uptake, root radiolabel concentrations were correlated to mycorrhizal infection as well as a series of growth-related parameters (Figure 15 and Figure 16). In the willow systems, root uptake was found to be most highly correlated with mycorrhizal infection ($r = 0.79$). In the poplar systems, root uptake was correlated most strongly (negative correlation)

with water use (r = -0.70), however was somewhat correlated with mycorrhizal infection (r = 0.58).

5.4.2: Mass Balance

A much larger proportion of the radiolabel was recovered from the soil than was recovered from the vegetation (Table 5). This result was expected, given that the root mass (≈ 2 g dry weight) was much lower than the total mass of the soil (≈ 1.3 kg dry weight). On the whole, the root radiolabel concentration was one order of magnitude higher than the soil radiolabel concentration. The radiolabel recovered from the leaves was approximately one order of magnitude lower than the radiolabel recovered from the roots. Accordingly, the leaf radiolabel concentration was of the same order of magnitude as the soil concentration. No radiolabel was detected in the plant stems, effluent air traps, or effluent water traps. Any radiolabel sorbed to the reactor components was rinsed into the soils during the extraction procedure.

5.4.3: Effluent Experiments

Water exiting selected phytoreactors was assayed for radiolabel via LSC on Days 44, 46, 57 and 88. The amount of radiolabel detected in the effluents varied considerably, and followed no discernable chronological patterns. To estimate the total amount of radiolabel lost in the effluent water streams, the average daily effluent radiolabel content for each treatment type was multiplied by the total number of days (Figure 17). The results indicate that a significant amount of radiolabel was lost in the

effluent water streams of all the tested vegetated phytoreactors over the course of the experiment. Furthermore, the effluent radiolabel loss appeared to be lower in the non-vegetated phytoreactors than in the vegetated systems. This disparity is reflected in the phytoreactor mass balances (Table 5), wherein an apparently higher amount of radiolabel was recovered from the non-vegetated soils than the vegetated soils.

On three occasions, radiolabel-laden effluent waters were subjected to liquid/liquid extraction with methylene chloride to determine the approximate K_{ow} of the radiolabelled compounds. As a result, no radiolabel was detected in the organic layer of any of the 26 extractions performed. Considering the detection limits of the scintillation counter, it was estimated that the Log_{10} K_{ow} of the effluent radiolabelled compound(s) were somewhat lower than −1. Given that the Log_{10} K_{ow} of aldrin and dieldrin are reported to be approximately 7 and 6 respectively (Briggs, 1981), the effluent radiolabel almost certainly represented a degradation product(s) other than dieldrin.

On one occasion, radiolabel-laden effluent waters were collected upstream of the effluent activated-carbon traps. Half of the volume in each of these samples was then routed through the traps, and the remaining volume was left unfiltered. Comparisons of radiolabel concentrations between the two sets indicated that the traps did not capture any of the radiolabel exiting the phytoreactors. Additionally, extraction and biological oxidation of the activated carbon remaining in the traps at the end of the study revealed no sorbed radiolabel. As a result, the traps were found to be entirely ineffective at capturing the water-soluble radiolabel. As part of this filtration experiment, effluent waters (both filtered and unfiltered) were subsampled and acidified to determine whether

the radiolabel in the effluent waters represented dissolved CO_2. As acidification (pH ≈2) did not result in the loss of radiolabel from any of the samples tested, it was deduced that the radiolabel did not represent mineralized parent product.

In field studies, dihydrochlordene dicarboxylic acid has been shown to be a major hydrophilic metabolite of aldrin (Stewart and Gaul, 1977). Although no further qualitative analyses were performed on the effluent waters, this compound may have been present in the leachates.

5.4.4: Root Extractions

One subsample of dried root material from each treatment group was analyzed qualitatively. The roots were extracted into hexane as described previously (Schnabel and White, 2000b). As shown in Table 6, the extractable radiolabel concentration for each of the samples tested was significantly lower than the total radiolabel residing in the root material. As the root material was dried and ground to a fine powder prior to extraction, it was determined that the radiolabel remaining in the root material after extraction represented non-extractable bound residue. Additionally, GC-ECD analysis revealed that most or all of the radiolabel in the extracts took the form of dieldrin rather than aldrin. Thus, although the abundance of bound residue implies the existence of other degradation pathways, a significant amount of aldrin was epoxidized to dieldrin in the roots themselves, or in the surrounding soil. This result was not surprising, given that microbial (Lichtenstein and Schulz, 1960) and vegetative (Gannon and Decker, 1958) systems have long been known to epoxidize aldrin.

Bioconcentration factors were calculated based upon the radiolabel concentration in the roots and extracts versus the radiolabel concentration in the original soil. As shown in Table 6, bioconcentration factors based upon total root radiolabel concentration (via combustion) were approximately one order of magnitude higher than those based upon solvent-extractable radiolabel. Consequently, field studies employing unlabeled analytes may significantly underestimate the actual amount of contaminant uptake and transformation.

5.4.5: Leaf Extractions

As discussed, selected leaf samples were subjected to Soxhlet extraction and analyzed via GC-ECD (EPA Method 8081). Prior to extraction, a subsample of this leaf material was combusted via BO to quantify the radiolabel. The results of these analyses are presented in Table 7. As shown in the table, the amount of extractable dieldrin was significantly lower than the expected values based upon combustion. Less than 1% of the radiolabel present in any leaf sample was extractable as dieldrin. Furthermore, dieldrin was detected only in the willow leaves, and not the poplar leaves (MDL $\approx$ 1 μg/kg). Aldrin was not detected in any of the leaf samples (MDL $\approx$ 1 μg/kg).

5.4.6: Soil Extractions

As stated previously, the soils were extracted with acetone and hexane at the end of the experiment. In addition to quantification via LSC, one sample from each treatment group was analyzed via GC-ECD to determine the relative amounts of aldrin and dieldrin

contained in the extracts. These results are reported in Table 8. As shown in the table, the addition of fungicide decreased the conversion of aldrin to dieldrin in every treatment group. Furthermore, the presence of vegetation not amended with fungicide appeared to enhance the conversion of aldrin to dieldrin over the non-vegetated controls. Although the epoxidation of aldrin into dieldrin would not necessarily be considered a remediative process, increased conversion rates do indicate increased biological activity and/or bioavailability, which could lead to other transformations.

Section 5.5: Discussion

5.5.1: Mass Balance

A significant proportion of the added radiolabel was lost during the course the experiment. Three viable explanations exist to account for this observation. First, some amount of radiolabel remained sorbed to the soil after multiple extractions into acetone and hexane. Although phytoreactor soils were not tested individually after extraction, five subsamples of the combined post-extraction experimental soils were combusted via BO to test for residual radiolabel. This test revealed that $10 \pm 2\%$ of the original radiolabel remained soil bound after extraction. Thus, soil binding likely accounted for only a fraction of the radiolabel lost during the experiment. Another plausible explanation for the lost radiolabel is that a consequential amount of aldrin volatilized from the soil during the four-day equilibration period at the beginning of the experiment. Although it was necessary to evaporate the carrier solvent from the dosed soils before

transplanting the vegetation, this step allowed for the possibility of radiolabel loss. Since volatilization is generally considered to be the major source of loss from soils recently treated with aldrin (WHO, 1991), it is reasonable to assume that volatilization played a role in the experimental losses. Finally, a notable amount of water-soluble radiolabel was measured in the effluent water streams (see "Effluent Experiments"). The variable nature of these observations resulted in very rough estimates of total loss however, and these losses were not considered in the mass balance.

5.5.2: Impact of Mycorrhizae

The results of the fungicide amendments indicated that mycorrhizal infection was associated with higher levels of root radiolabel uptake, especially in the willow systems. Such a finding does not conclusively demonstrate a causative relationship. There is a possibility that fungicide addition decreased the activity of pesticide-degrading bacteria such as *Pseudomonas fluorescens*. In a control experiment, however, soil bacterial populations were found to be approximately one order of magnitude higher in similar soils treated with Daconil$_{2787}$® than in soils left untreated (Schnabel and White, 2000b). This increased bacterial population was attributed to decreased competitive pressure from soil fungi. As the fungicide-treated soils in the uptake study likely had higher bacterial populations as well, but did not show increased uptake, the soil bacterial activity was probably not strongly related to root uptake. There is also a possibility that fungicide addition decreased the activity of free-living degradative fungi. In the same control experiment as described above, soil fungal populations were found to decrease by a factor

of five with the addition of fungicide. Given that mycorrhizal fungi and white rot fungi (e.g. *Phanerochaete chrysosporium*) both possess enzyme systems capable of contaminant degradation (Donnelly and Fletcher, 1995), and that mycorrhizal fungi often constitute a much larger proportion of the soil biomass in vegetated systems than do white rot fungi (Allen, 1991), it stands to reason that decreased mycorrhizal activity was most likely responsible for the decreased vegetative uptake in the presence of fungicide.

5.5.3: Transformations in the Soil

The effluent experiments indicated that the radiolabel exiting the phytoreactors was a water-soluble degradation product. It was estimated that roughly 6-12% of the total radiolabel experienced this fate, and that the vegetated phytoreactors likely exhibited more degradation than the non-vegetated controls. Given the limited replication of these samples, however it was difficult to associate the formation of water-soluble product with any vegetative parameters beyond the mere existence of vegetation.

The relative amount of transformation in the soil agrees with the findings of previous researchers. Using the rate equations presented by (Ghadiri *et al.*, 1995),

Aldrin, 30 °C	$C_t = 1.959\, e^{-0.0164t}$
Aldrin, field	$C_t = 3.627\, e^{-0.0022t}$
Dieldrin, 30 °C	$C_t = 2.412\, e^{-0.0249t}$
Dieldrin, field	$C_t = 3.359\, e^{-0.0027t}$

where t is expressed in weeks and C_t is expressed in mg/kg; one would expect approximately 20% and 30% of aldrin and dieldrin respectively to be degraded in 100

days at 30 °C in moist soils. Under "cooler" outdoor conditions, one would expect those values to be 3% and 4% of aldrin and dieldrin respectively. The values obtained from the effluent data fall within this range for an experiment conducted at 20 °C. In the previous study, aldrin and dieldrin degradation was attributed to free-living soil microorganisms (Ghadiri *et al.*, 1995). In the current study, transformation to water-soluble metabolites occurred in the absence of vegetation, but this process was enhanced when plants were added to the system. It remains unclear whether the addition of vegetation contributed different mechanisms of transformation (e.g. mycorrhizal degradation), or whether the activity of free-living microorganisms was merely enhanced through rhizosphere effects. Regardless, the addition of vegetation appeared to increase transformation rates, and hence could be especially important in boreal sites where microbial activity is inhibited due to low temperatures.

5.5.4: Fate of Radiolabel in the Vegetative Matrix

Extraction of the root material indicated that most (≈ 80-90%) of the radiolabel taken up existed as bound transformation product. The remainder was found to be extractable dieldrin. Extraction of the leaf material indicated that most (>99%) of the radiolabel translocated to the leaves took the form of a transformation product. Again, the remainder was found to be dieldrin. As the phytoreactors were sealed to prevent volatilization, these results indicate that in the willows, there was at least some amount of translocation of dieldrin from the roots to the leaves. This translocation was likely limited, however, based upon the xylem immobility of strongly hydrophobic compounds

(Goodman *et al.*, 1992). Consequently, it is reasonable to assume that the transformation of most of the leaf-associated radiolabel took place before the radiolabel was translocated to the leaves.

The question remains concerning whether the transformation products detected in the roots and leaves were transformed inside or outside of the vegetative matrix. Although a significant amount of transformation from aldrin to water-soluble metabolite was detected in vegetated and non-vegetated soils, these metabolites would not necessarily be concentrated in the roots due to their lipophobicity (Burken and Schnoor, 1998). Alternatively, although the hydrophobic parent product would be expected to accumulate in the root tissue, the concentration of aldrin and/or dieldrin in the soil solution was likely much lower than the concentration of the water-soluble metabolite.

5.5.5: Field Applicability

The purpose of this study was to examine aldrin uptake and/or transformation characteristics with regards to phytoremediation potential. In order for phytoremediation to be successful, the aldrin must be transformed at a higher rate than in non-vegetated soils. Furthermore, the mere epoxidation from aldrin to dieldrin would be an ineffective remediation strategy, as dieldrin is considered to be more recalcitrant than aldrin (Bandala *et al.*, 1998). Rough estimates indicated that approximately 6-12% of the available aldrin was transformed to water-soluble metabolites in the vegetated soils, and 1-2% was transformed in the non-vegetated soils over the course of the 100-day growth period. The identity of the metabolite(s) was not confirmed, yet would be an important

consideration for phytoremediation development. Other considerations would include the organic content of the soils, the hydrogeologic state of the system, and the ambient temperature of the soils. Regardless, the results of this study agree with previous findings that aldrin and/or dieldrin do not accumulate indefinitely in biologically active soils (Ghadiri *et al.*, 1995; Lichtenstein *et al.*, 1970).

Vegetative uptake and transformation to non-extractable product would likely be considered a remediative process. In this study, mycorrhizal development was correlated to uptake in the willow plants, and to a lesser extent in the poplars. Most of the radiolabel taken up in either system was transformed at some point into non-extractable product. Regardless of mycorrhizae, however, vegetative uptake was minimal compared to other degradative processes. Vegetative uptake could likely be enhanced through the addition of surfactants to the soil solution (Guha and Jaffe, 1996). If uptake were enhanced, either through surfactant addition or increased mycorrhizal activity, consideration would need to be given to the form of the product in the vegetative matrix. Although dieldrin was not translocated to a great extent in the willows compared to the total amount of residues, it did accumulate in the leaves to approximately 5 μg/kg. This might be considered unacceptable for phytoremediation purposes. On the other hand, the poplars were not shown to translocate dieldrin, even though the dieldrin concentrations in the poplar roots were as high or higher than the dieldrin concentrations in the willow roots. Consequently, poplars might make better candidates for further studies.

Section 5.6: Conclusions

The results of this study indicate that phytoremediation is a viable option for soils contaminated with aldrin. Vegetated systems could contribute to the transformation of aldrin to water-soluble products in the soil, and bound products in the plant matrix. The presence of vegetation was shown to enhance transformations in the soil. Mycorrhizal fungi were shown to be associated with the uptake of aldrin or its resides in willow systems, and postulated to be associated with uptake in poplar systems. Furthermore, as a large proportion of extractable parent product was found in the soil at the end of the study, mobilization through surfactant addition could potentially increase transformations in similar systems. As poplars were not shown to translocate aldrin or its epoxide (dieldrin) through the xylem and thus retained all the contaminants of concern below ground, further studies involving *Populus balsamifera*, mycorrhizal enhancement, and surfactant addition are encouraged.

Section 5.7: Acknowledgements

The ENSR Corporation and the U.S. Army (through the U.S. Army Corps of Engineers) supported this work. Dr. Pat Holloway and the staff at the University of Alaska Fairbanks Experiment Farm, Fairbanks, Alaska, provided additional support.

Table 4: Fungicide Effects in Aldrin Uptake Study

	Plant Dry Weight (g)	Mycorrhizae Formation (mg Chitin/ g Dry Root)	Radiolabel Uptake (μCi/10 kg Dry Root)
Willow	13.2 ± 2.4	3.3 ± 1.2	30.2 ± 20.4
Willow + Fungicide	11.2 ± 2.3	1.3 ± 0.5	22.0 ± 9.9
Poplar	9.4 ± 1.2	1.3 ± 0.9	33.0 ± 17.1
Poplar + Fungicide	9.8 ± 3.2	1.4 ± 0.7	17.7 ± 7.8

The effects of amending vegetative feedwater with 750 ppm Daconil $_{2787}$® are shown. Sample size (n = 5-6) for plant dry weight and radiolabel uptake; (n = 4) for mycorrhizae formation. Error is reported as 95% confidence interval.

Table 5: Mass Balances in Aldrin Uptake Study

	% In Soil	% In Roots	% In Leaves	Total
Non-Vegetated	52.95 ± 4.1	n/a	n/a	52.95 ± 4.1
Non-Veg. + Fungicide	47.6 ± 10.4	n/a	n/a	47.6 ± 10.4
Willow	34.7 ± 5.0	3.0 ± 1.1	0.3 ± 0.2	38.0 ± 6.3
Willow + Fungicide	38.1 ± 4.9	2.6 ± 1.0	0.1 ± 0.0	40.9 ± 6.0
Poplar	35.9 ± 8.1	1.9 ± 1.3	0.3 ± 0.1	38.1 ± 9.5
Poplar + Fungicide	40.9 ± 3.9	2.0 ± 1.3	0.1 ± 0.1	43.0 ± 5.3

Radiolabel recoveries from all phytoreactor components are listed. No radiolabel was detected sorbed to the effluent air or effluent water traps. Sample size (n = 3) for non-vegetated phytoreactors; (n = 6) for vegetated systems. Error is reported as 95% confidence interval

Table 6: Root Radiolabel Extractions in Aldrin Uptake Study

	Total Radiolabel Conc. (μCi/10kg)	Total BF	Extractable Radiolabel Conc. (μCi/10kg)	Ext. BF	Percent Extractable Radiolabel	Extract Dieldrin Content (%)
Willow	19.0	12.6	2.3	1.5	12%	166%
Willow + Fungicide	10.2	6.7	1.1	0.7	11%	93%
Poplar	23.8	15.7	4.2	2.8	18%	141%
Poplar + Fungicide	10.2	6.8	2.1	1.4	21%	59%

Total root radiolabel concentrations identified via combustion are compared to radiolabel concentrations determined via hexane extraction (n = 1). "BF" represents bioconcentration factor (concentration in roots/concentration in soil) calculated from the two quantification methods.

Table 7: Leaf Extractions in Aldrin Uptake Study

	Leaf Radiolabel Conc. via BO (μg dieldrin /kg leaf)	Leaf BF Based on Radiolabel	Leaf Dieldrin Conc. via GC-ECD (μg dieldrin /kg leaf)	Leaf BF Based on Extractable Dieldrin	Dieldrin % of Total Leaf Radiolabel
Willow	802	1.0	5.3	0.007	0.66%
Willow + Fungicide	807	1.0	6.2	0.008	0.76%
Poplar	1083	1.4	<1.0	<0.001	<0.09%
Poplar + Fungicide	1792	2.3	<1.0	<0.001	<0.06%

Total leaf radiolabel concentrations identified via combustion are compared to leaf dieldrin concentrations analyzed via Soxhlet extraction and GC-ECD (EPA Method 8081). Values represent average of two samples per treatment. "BF" represents bioconcentration factor (concentration in roots/concentration in soil) calculated from the two quantification methods.

Table 8: Aldrin to Dieldrin Conversion in Soils

Extractant	Sample	Ext. Aldrin Conc. (mg/L)	Ext. Dieldrin Conc. (mg/L)	% Conversion to Dieldrin
Acetone	Non-Vegetated	1.30	0.30	19
	Non-Veg. + Fungicide	1.65	0.15	8
	Willow	1.20	1.10	48
	Willow + Fungicide	3.45	< 0.10	< 3
	Poplar	1.30	0.60	32
	Poplar + Fungicide	2.45	< 0.10	< 4
Hexane	Non-Vegetated	1.40	0.20	13
	Non-Veg. + Fungicide	1.55	0.10	6
	Willow	0.65	0.55	46
	Willow + Fungicide	1.55	0.15	9
	Poplar	1.30	0.65	33
	Poplar + Fungicide	1.60	< 0.10	< 6

Extract concentrations for six samples are presented. Samples were extracted first with acetone, then with hexane. No dieldrin was added to the soils; dieldrin concentrations represent biologically-mediated epoxidation products.

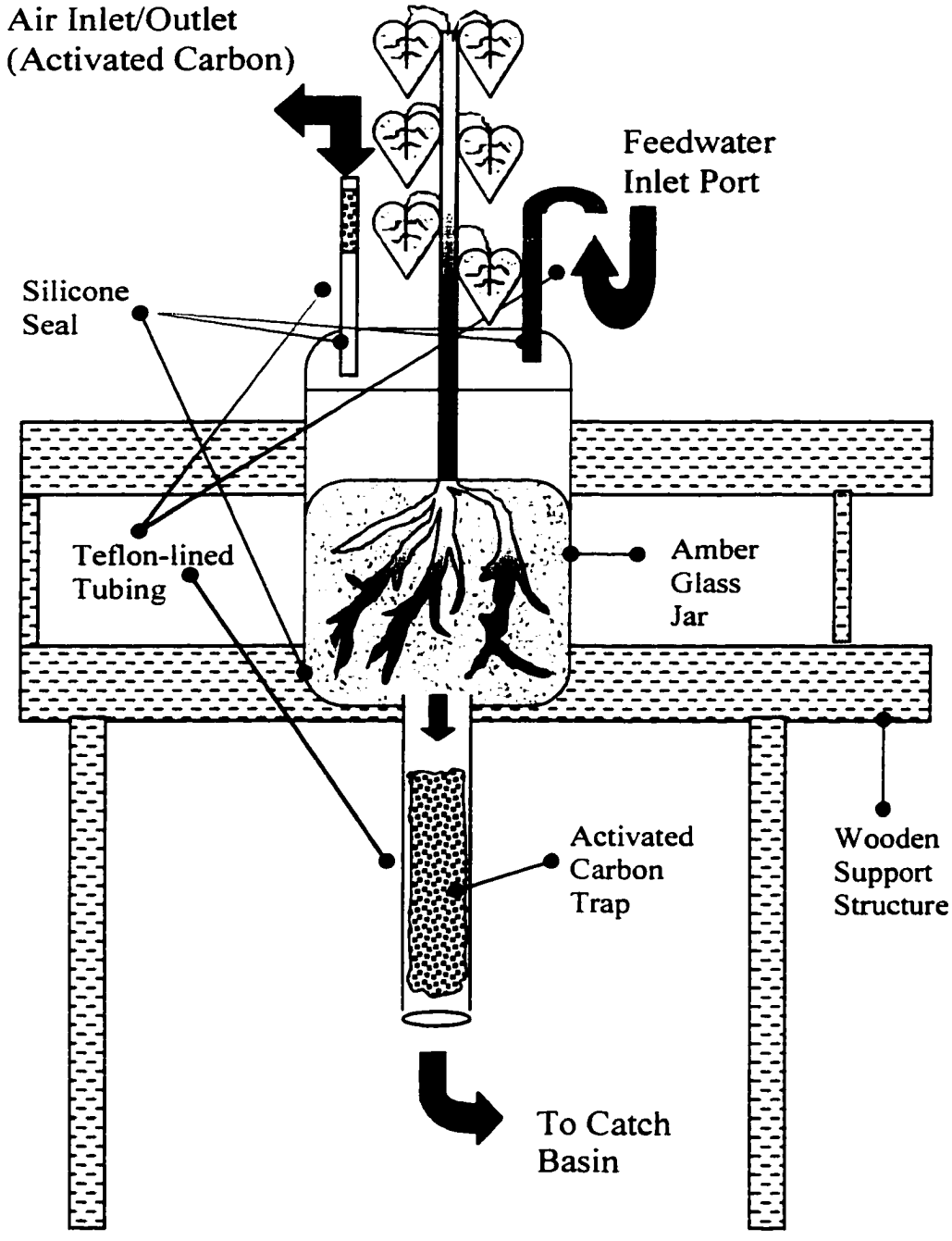

Figure 12: Phytoreactor Design

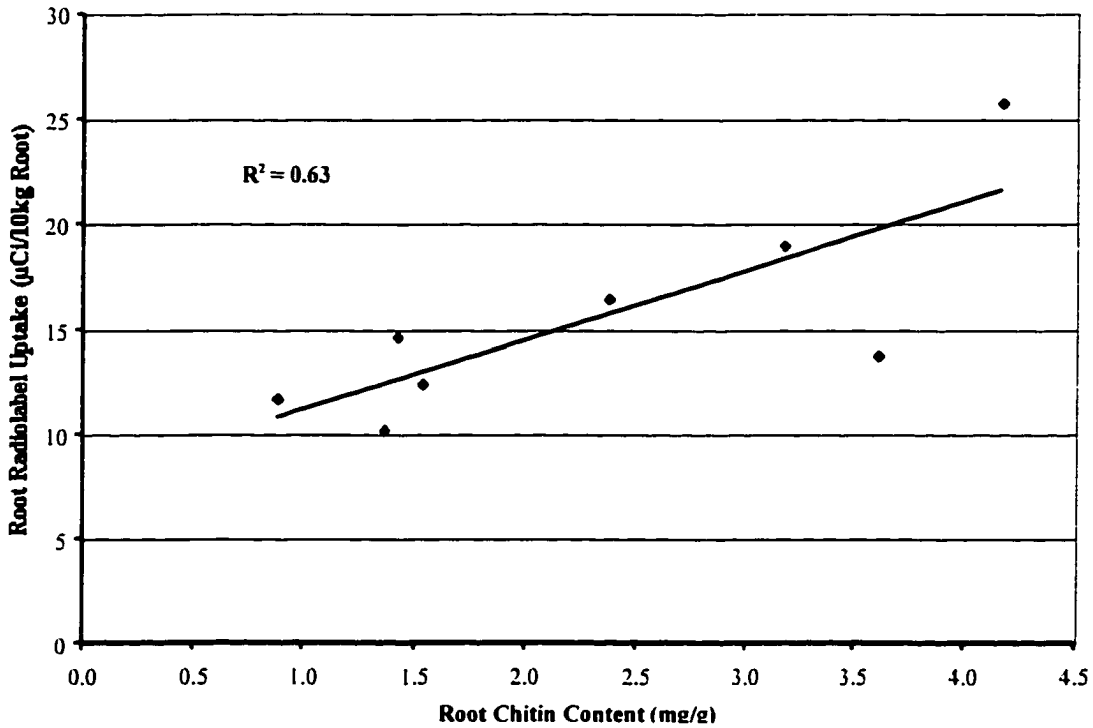

Figure 13: Chitin Content vs. Uptake in Willows in Aldrin Uptake Study

Root chitin content is plotted against root radiolabel uptake for the willow plants. Chitin content is a measure of mycorrhizal infection (≈ 85 mg chitin/g dry fungal mycelium).

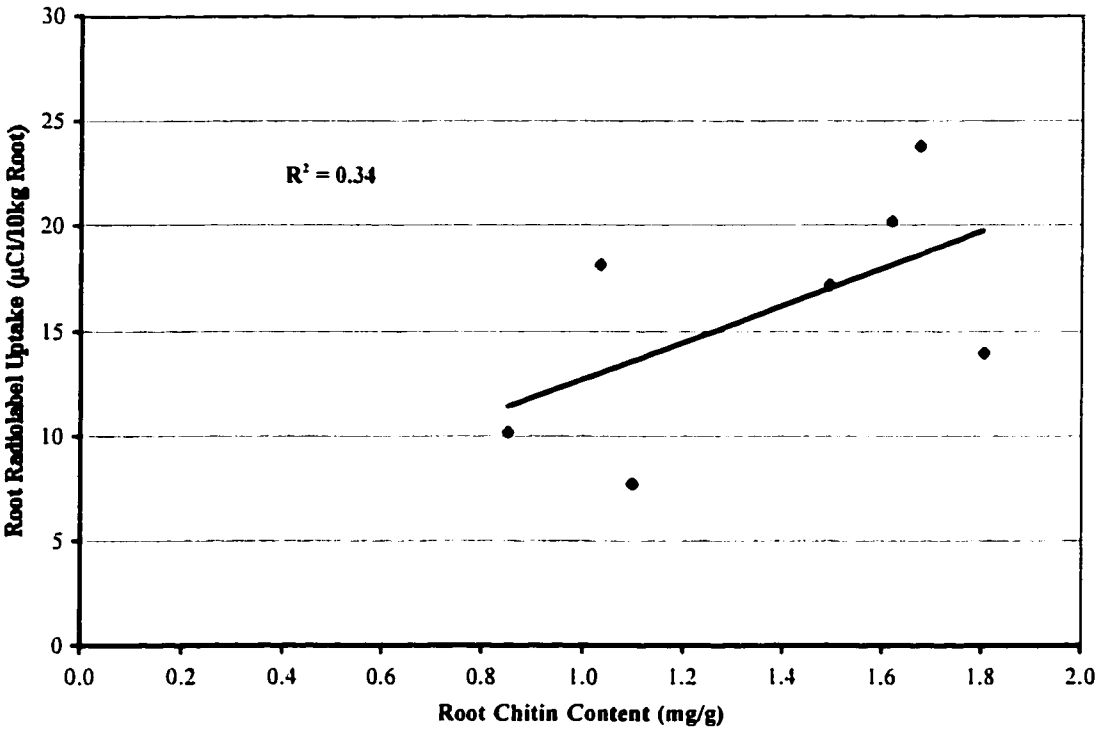

Figure 14: Chitin Content vs. Uptake in Poplars in Aldrin Uptake Study

Root chitin content is plotted against root radiolabel uptake for the poplar plants. Chitin content is a measure of mycorrhizal infection (≈ 85 mg chitin/g dry fungal mycelium).

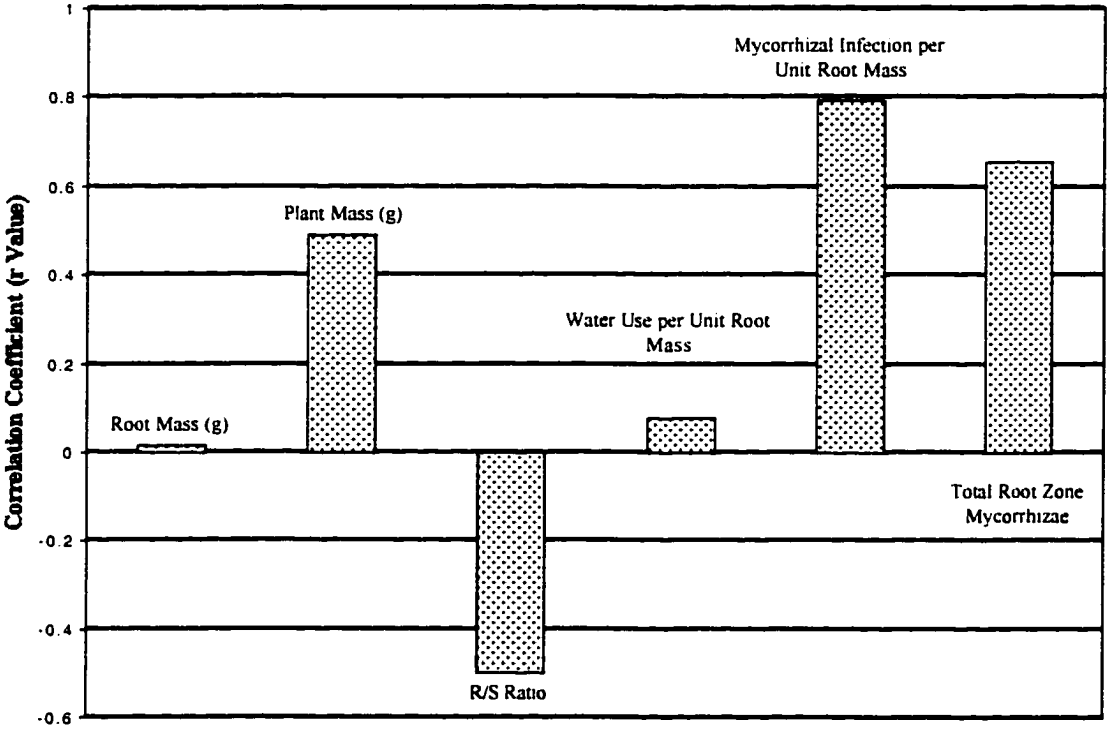

Figure 15: Willow Uptake Correlations in Aldrin Uptake Study

Final root radiolabel concentrations in *Salix alaxensis* are shown correlated to the listed vegetative and mycorrhizal parameters (n = 8). "R/S Ratio" refers to the dry mass of the roots divided by the dry mass of the shoots.

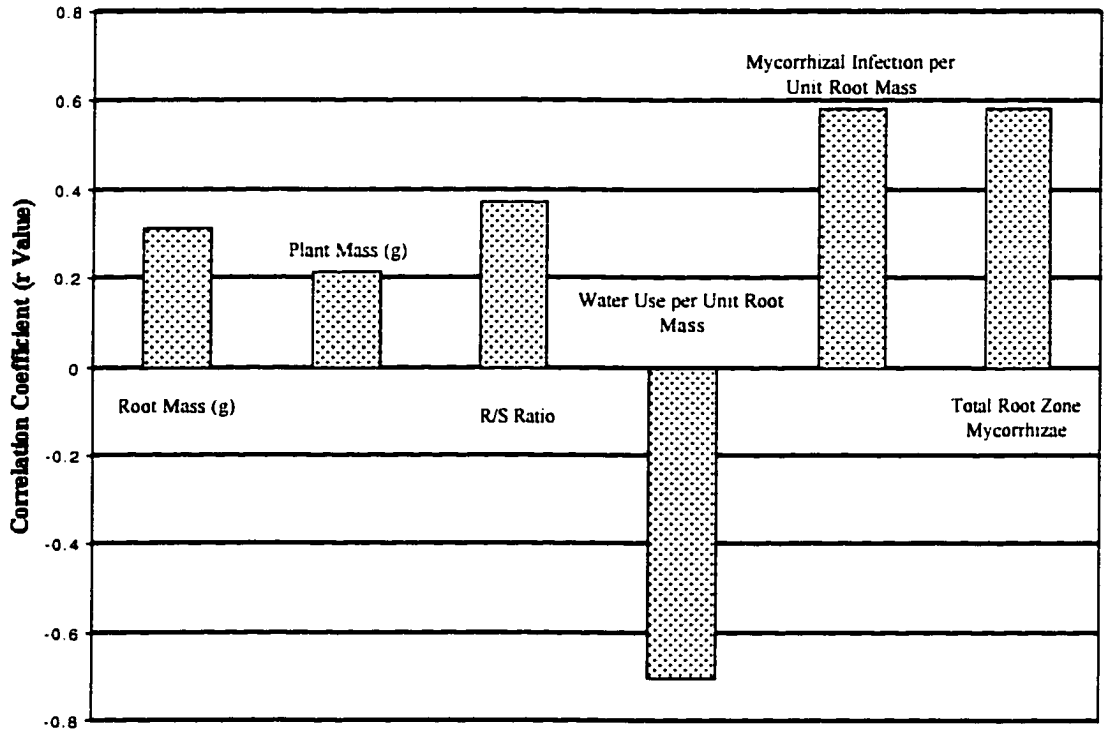

Figure 16: Poplar Uptake Correlations in Aldrin Uptake Study

Final root radiolabel concentrations in *Populus balsamifera* are shown correlated to the listed vegetative and mycorrhizal parameters (n = 7). "R/S Ratio" refers to the dry mass of the roots divided by the dry mass of the shoots.

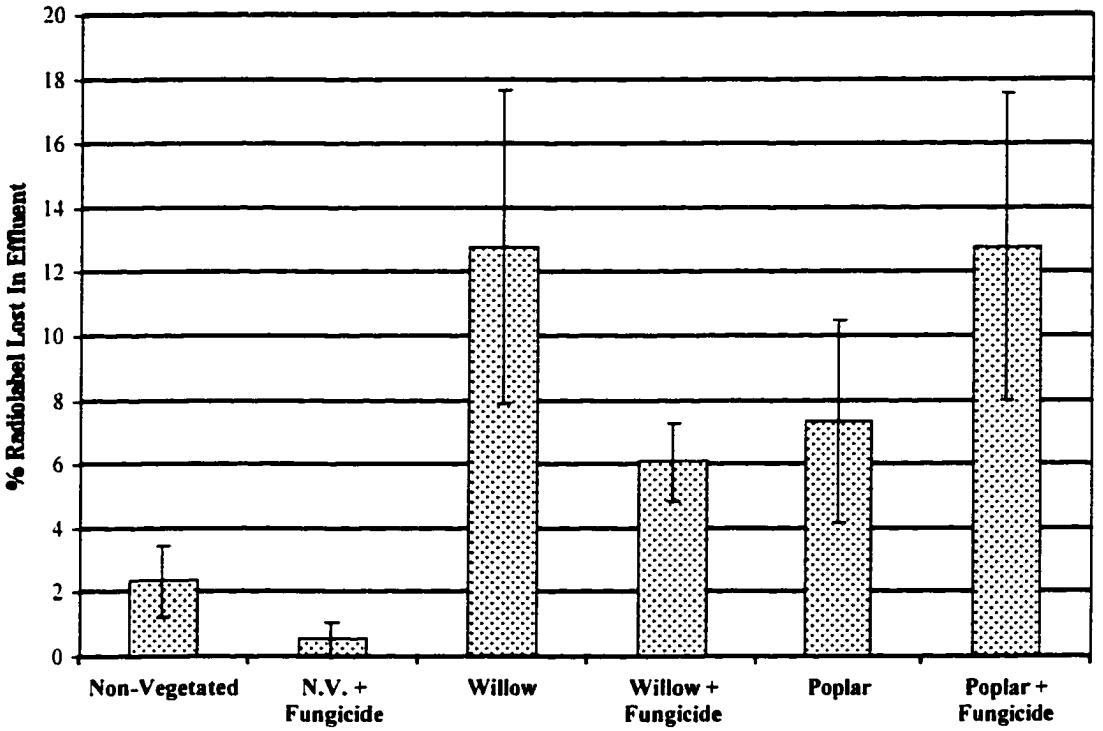

Figure 17: Estimated Loss in Effluent in Aldrin Uptake Study

Estimated total radiolabel loss via effluent water streams are shown for 6 phytoreactor systems. Estimates were based upon 3-4 measurements of effluent radiolabel over the experimental period. Effluent radiolabel was found to be a water-soluble transformation product of aldrin. Error bars represent ± 75% confidence interval.

Chapter 6: Unpublished Results

Over the course of this research, experimental results were obtained that were not deemed appropriate for publication in the *International Journal of Phytoremediation*. The following sections describe these results.

Section 6.1: Mycorrhizal Development Study

This section is a modified version of a report sent to our funding agency, the ENSR Corporation. The report was submitted in May, 1999.

6.1.1: Introduction

One of the basic tenets surrounding this research was that the plants supporting the healthiest populations of mycorrhizal fungi would be the most effective degraders of soil contaminants. It was important, therefore, to develop methods for manipulating mycorrhizal development in the experimental plants. Previous researchers demonstrated that ectomycorrhizal development was inhibited in black spruce and jack pine seedlings when the phosphorus fertilization regimen was increased from 1.5 mg/seedling to 7.2 mg/seedling (Browning and Whitney, 1992). Contrarily, other researchers have reported that the fertilization regimen had no effect upon mycorrhizal development in tamarack (Chakravarty and Chatarpaul, 1990). The purpose of this experiment was to determine the fertilization regimen under which feltleaf willow and balsam poplar would best

develop indigenous mycorrhizal associations. There was sufficient evidence to support the hypothesis that "high" nutrient levels would inhibit mycorrhizal development (Abbott, Robson and De Boer, 1984; Allen, 1991; Browning and Whitney, 1992), so we estimated a "nominal" concentration of nitrogen and phosphorus required for growth, and tested fertilization regimens at or below that level.

6.1.2: Procedure:

Feltleaf willow (*Salix alaxensis*) and balsam poplar (*Populus balsamifera*) were collected from natural stands in the Fairbanks area. Willows cuttings (≈ 30 cm) were collected from adult plants in a stand located in a grassy area near the junction of the Chena and Tanana rivers. Poplar cuttings (≈ 30 cm) were collected from adult plants located at two sites on the UAF campus. All cuttings were collected from growth that was at least one year old. After collection, cuttings were placed into plastic storage bags and stored in darkness at 4 °C until planting.

At planting time, cuttings were scrubbed with a stiff sponge, using a solution of 10% Clorox® Bleach (0.5% NaOCl) to remove dirt and fungal growth. The cuttings were then allowed to bathe in a 10% Clorox® solution for three minutes to kill any residual fungi. Cuttings were removed from the bleach bath, then rinsed thoroughly with RO water to remove any bleach solution. After rinsing, the cuttings were weighed, numbered, and placed into foam containers filled with 275 g of autoclaved sand. As a control, five plants of each type were placed into foam containers filled with autoclaved soil obtained from Ft. Wainwright and Ft. Richardson. Planted cuttings were then placed

into the UAF/IAB greenhouse to sprout under a 24-hour light cycle, with the temperature set at approximately 15-20 °C.

Within the first week of planting, the willows and poplars were placed under humidity tents to prevent desiccation while rooting. The plants were incubated under the humidity tents for a period of two weeks. To establish growth during the two-week tenting period, all plants were fertilized on four occasions with a 0.07% solution of 15-30-15 Miracle-Gro® (Scott's Miracle-Gro®, Inc.) commercial fertilizer (7.5 mM nitrogen, 0.6 mM phosphorus, 0.9 mM potassium).

One week after placement into the humidity tents, the plants were inoculated with a culture of ectomycorrhizal fungi. This culture was prepared from plate-grown fungi that had been originally collected from willow and poplar roots in the Fairbanks and Anchorage areas (Schnabel and White, 2000a).

Approximately three weeks after planting, the willows and poplars were segregated into treatment groups. Each treatment group initially contained ten plants per group. The Ft. Wainwright and Ft. Richardson control groups contained five plants per group. The plants were watered twice per day and fertilized once per day (50 ml of fertilizer solution) with a nutrient solution defined by the treatment group. All fertilizers used for the different treatment groups included nominal amounts of potassium and micronutrients, but varied in the amounts of nitrogen and phosphorus added. The various treatment groups are delineated in Table 9.

In addition to the nutrient concentrations, Table 9 also describes other treatment parameters. All plants were inoculated with mycorrhizal fungi except for those in Groups

J, K, and L, which were used as non-inoculated controls. Plants in Groups H and I were treated with 1 mg/L of surfactant solution to determine if the addition of nitrogen or phosphorus containing surfactants would affect growth. The surfactant containing nitrogen was Surfonic® T-20 (Hunstman Corporation). The surfactant containing phosphorus was Rexophos JV-05-015 (Huntsman Corporation). Approximately 15 g of leaf litter mulch were added to the soils of groups E, F, and G, to determine what effects added organic substrate would have on mycorrhizal formation.

All plants were harvested approximately three months after initial planting. During the harvest procedure, the new growth stems and leaves were first separated from the rest of the plants for later analysis. These portions were then stored for subsequent drying and weighing. The plant roots were then rinsed in a basin of tap water to remove soil particles. After rinsing, the roots were dried at 80 °C for two days, then weighed.

A chitin assay was performed on the root material to assess the amount of mycorrhizal formation (Vignon *et al.*, 1986). It has been demonstrated that fungal cell walls contain the glucosamine compound chitin, which is absent in plant cell walls (Bartnicki-Garcia, 1968). By measuring the amount of glucosamine in root material, a determination could be made concerning the amount of fungi contained within the root material. This assay was performed by first homogenizing the dried root material, then performing a colorimetric assay on the root homogenate. After the assay, the amount of chitin per unit root material could be determined, thus indicating the amount of fungi present.

6.1.3: Results/Discussion:

General results of this study are reported in Table 10 and Table 11. Parameters reported include the root chitin concentration, the total mass of chitin per plant, the dry mass of the root material, and the dry mass of the stems and leaves. The values for the lettered groups listed in the tables represent the average values for these parameters based upon the 8-10 viable plants per group at the end of the experiment. The Ft. Wainwright controls had 4-5 viable plants at the end of the experiment. The Ft. Richardson soil produced only one viable plant (discussed below) and was not included in the tables. Error is reported as a 90% confidence interval.

Chitin (i.e. mycorrhizae) Concentration Trends: The amount of chitin served as an indicator of the amount of fungi present. Preliminary experiments revealed that the mixed fungal culture used for this experiment contained 85 mg chitin/g dry fungal mass.

The highest chitin concentrations for both poplars and willows were associated with the plants fertilized with nominal concentrations of nitrogen and phosphorus (Group A). Chitin concentrations for the root systems at lower levels of nutrient addition were significantly lower than Group A, but were not significantly different from one another (Groups B, C, and D). Although there may have been a slight difference in actual fungal development (in all but one instance, the average chitin concentrations were consecutively lower at each stepwise nutrient deficiency), significance at the 90% confidence level could have been masked by the basal amino sugar concentration in the plant roots themselves.

The addition of leaf litter did not significantly increase the concentration of chitin in the roots. It was hypothesized that such an addition would increase the chitin concentration, but after a growth period of three months, the levels were not high enough to be qualified as statistically significant at the 90% confidence level. It should be noted, however, that in all six instances where this comparison was made (Groups E, F, and G versus A, B, and C respectively for both poplars and willows), the average chitin concentrations were higher in the leaf litter samples than in the samples without leaf litter. Thus, it appears likely that statistically significant differences could be delineated with higher replication.

The addition of nitrogen and phosphorus containing surfactants did not significantly affect the amount of chitin measured (Groups H and I versus B). This was not a surprising result, given that the concentration of nutrients contained in the surfactants themselves was negligible compared to the concentration of nutrients in the fertilizer solution. It is pertinent to note that the addition of surfactants did not appear to inhibit mycorrhizal formation, which would be an important consideration for the development of mycorrhizae-mediated phytoremediation systems amended with surfactants.

The non-inoculated controls contained the same concentrations of chitin as did the inoculated plants (Groups J, K, and L versus A, B, and D). Due to the need for consistent treatment, the controls were grown in the same growth trays as the inoculated groups. The root systems for most of the plants extended through the bottom of the containers and came into contact with the root systems of other plants. As ectomycorrhizal fungi

can propagate by mycelial contact, it is likely that the non-inoculated control plants became unintentionally infected with mycorrhizal fungi from the inoculated plants. The result of this occurrence is that there were no non-inoculated controls that could be aptly termed "non-inoculated."

The soils from the Ft. Wainwright site produced root masses with high concentrations of chitin. These concentrations were similar to the concentrations measured in the nominal fertilization treatment groups, even though the Ft. Wainwright plants were fertilized with zero nitrogen and phosphorus. This development was likely the result of nutrient pools located within the soil, which could have encouraged mycorrhizal development.

The soils from the Ft. Richardson site produced only one viable plant. The plants did not thrive in this soil most likely because the soil was observed to be extremely silty, which resulted in a high degree of compaction, poor aeration, and constant water saturation. In the field, this soil would probably support willow and poplar growth more effectively due to less-constrained movement of water through the soil.

Root Mass Trends: The mass of the dried root material served as an indication of the uptake capacity of the vegetative systems. With all other factors being equal, a plant with higher root mass would be able to remediate a higher amount of contamination in the soil.

As expected, the plants fed the highest amount of nutrients developed the largest root masses. The plants fed zero nitrogen and phosphorus developed the smallest root masses (Groups A and J versus Groups D and L). The addition of leaf litter did not

appear to have a significant impact upon root mass. For the poplar samples, the addition of detergents resulted in significantly lower root masses (Groups H and I versus Group B). Detergent addition to the willows did not significantly affect root mass. The root mass values for the Ft. Wainwright controls had a large amount of error (due to the silty media), and were therefore considered to be somewhat unreliable.

Stem/Leaf Trends: Although the foliar portion of plants do not intimately associate with contaminants in the soil, a measurement of the mass of this vegetative portion serves as an indicator of overall plant health, and thus uptake capacity.

As with the root masses, the stem and leaf masses were highest for the nominal nutrient groups (Groups A and J). Again, these values tended to decrease with a decreased amount of nutrients added. The addition of detergents or leaf litter did not appear to have a significant impact upon the development of the stems and leaves at the concentrations tested.

Poplars Vs. Willows: The poplars and willows developed similar amounts of chitin per unit root mass for most of the treatment groups. The willows consistently developed more extensive root masses, however, so the total amount of chitin measured in the willow root masses was higher than in the poplar root masses. Although these values weren't always statistically significant, it may be pertinent to note that the total chitin values for the willows were higher than those of the poplars in each of the thirteen treatment groups. The poplars, on the other hand, developed higher masses in the foliar portion of the plants than did the willows in every treatment group. Again, although the

actual differences weren't statistically significant in every instance, the fact that the values were higher for the poplars in every instance does indicate a trend.

Chitin Concentration vs. Total Plant Mass: The root chitin concentrations are plotted against the total plant dry weights in Figure 18 and Figure 19. Neither the Ft. Wainwright controls, nor the treatment groups receiving surfactant or leaf litter amendments were included in these figures. The original non-inoculated controls were included in these figures, as they were considered to be fully inoculated by the end of the experiment. As demonstrated in the figures, root chitin concentrations were correlated to the dry mass of the plants in both poplars and willows. In both figures, however, the two highest points represent treatment groups fed the nominal fertilizer regimen. If the high points were ignored, there would appear to be little correlation between chitin content and dry weight.

6.1.4: Conclusions

The results of this experiment indicate that the amount of nitrogen and phosphorus added was the most significant factor contributing to the development of root mass, stem and leaf mass, and mycorrhizal fungi for the poplars and willows tested. For the conditions given, a feed solution consisting of 100 mg/L nitrogen and 10 mg/L phosphorus (along with potassium and micronutrients) proved to be the most effective for total plant development. Although it is unfortunate that solutions containing higher concentrations of these nutrients were not tested, it is reasonable to assume based upon

previous studies that nutrients supplied at concentrations above those considered to be nominal would not yield a significantly higher amount of mycorrhizal development.

The benefits of leaf litter addition remain unclear. This experiment did not conclusively demonstrate that leaf litter enhanced fungal growth, but the data indicate that this is a possibility. On the other hand, addition of leaf litter to contaminated soils could provide a sorptive sink for hydrophobic contaminants, which could hinder remediation. As such, the relative benefits of leaf litter addition require further study.

The plants grown in Ft. Wainwright and Ft. Richardson soils were difficult to measure accurately. Both sets of soil were silty, which led to compaction and saturation problems. The Ft. Richardson soil produced only one viable plant, and the Ft. Wainwright soil stuck to the root masses of the plants that did grow. Further experiments using only these soils would likely yield larger margins of error than experiments using more controlled soils. It should be noted, however, that since these "actual" soils yielded high amounts of fungi on the plant roots, then they should be included as controls in subsequent experiments.

Finally, this experiment was developed to determine effective methods for manipulating ectomycorrhizal development on poplar and willow roots. It was assumed that plants with a higher degree of mycorrhizal infection would tend to serve as more effective remediators of recalcitrant organic contaminants than plants with a lesser degree of mycorrhizal development. The results of this experiment indicate that mycorrhizal development was not enhanced, but was instead inhibited under nutrient limiting conditions. Furthermore, the mycorrhizal inhibition was accompanied by the inhibition

of plant growth. Consequently, mycorrhizal manipulation via reduced fertilization would be an inappropriate strategy for uptake studies under similar conditions. Such a strategy would not allow researchers to decouple the effects of inhibited mycorrhizal development from the effects of inhibited plant growth. A more appropriate strategy would involve the use of fungicides that could inhibit mycorrhizal development while leaving plant growth unaffected. (Such was the strategy employed in the uptake studies described in Chapters 4 and 5.)

6.1.5: Acknowledgements

This work was supported by the ENSR Corporation and the U.S. Army (through the U.S. Army Corps of Engineers). Heather McIntyre and the staff at the Institute of Arctic Biology Greenhouse, Fairbanks, Alaska, provided additional support.

Section 6.2: Surfactant Studies

The notion of surfactant addition has been a recurring theme throughout the course of this research. The reports on the TCB and aldrin vegetative uptake studies, however, did not comprehensively address this issue. The results of surfactant addition in the TCB and aldrin uptake studies are described below.

6.2.1: Critical Micelle Concentration

Background: Surfactants, also called detergents, are long chained amphiphilic

molecules. As surfactant molecules have both hydrophobic and hydrophilic ends, they are able to serve as a barrier between polar and non-polar compounds. The critical micelle concentration (CMC) of a surfactant is that concentration at which the surfactant forms colloidal bubbles, or micelles in solution. It is at or above the CMC that surfactants are able to increase the effective solubility of hydrophobic compounds in aqueous solution (Guha and Jaffe, 1996).

CMC Determination: The method used to determine the CMC of the surfactants in this research was adapted from a previously reported method (Dominguez *et al.*, 1997). Five g/L of benzoylacetone were first dissolved into dioxane. This solution was then added to RO water at a dilution of 62.5:1. This stock solution was then added to aqueous, surfactant-containing solutions at a dilution of 7.5:1. The resulting solutions were then read at 250 and 312 nm on a Beckman DU 520 Spectrophotometer.

The results of three experimental runs are presented in Figure 20, Figure 21, and Figure 22. The CMC was determined by quantifying the relative amounts of benzoylacetone in the ketonic versus enolic tautomeric forms (Dominguez *et al.*, 1997). The ketonic form (read at 250 nm) dominated in aqueous solution. When the benzoylacetone was solubilized in surfactants above the CMC, the enolic form (read at 312 nm) dominated. As shown in Figure 20 and Figure 21, the measurements at 250 nm were variable and difficult to interpret. This result was attributed to the incompatibility of the available cuvettes to measurements at such short wavelengths. Consequently, the CMC value was determined on the basis of measurements at 312 nm alone, as shown in

Figure 22. Results of this experiment indicated that the CMC of a 50/50 solution of Surfonic® T-20, and Rexophos JV-05-015 was approximately 250 mg/L.

6.2.2: Surfactant Effects in Uptake Studies

Background: The surfactants Surfonic® T-20, and Rexophos JV-05-015 were added to the feedwater of selected phytoreactors in the TCB and aldrin uptake studies at a ratio of 50/50, in increasing concentrations throughout the course of the plant growth. The final surfactant concentration of 250 mg/L (125 mg/L Surfonic® T-20 + 125 mg/L Rexophos JV-05-015) was held constant for the final 60 days of growth. Due to the limited amount of replication, the findings concerning the effects of the added surfactants were inconclusive.

ANOVA Results: A two-factor analysis of the variance employing fungicide addition and surfactant addition as independent variables was performed for several parameters. The results of these analyses are detailed in Table 12. At least three replicates were required for such an analysis, and although there were three replicates of each treatment group at the beginning of the study, it was necessary to disregard several specimens due to disease and/or mortality.

The first analysis, the effects of surfactant and fungicide upon vegetative dry weight, required the assumption that neither TCB nor aldrin had an impact upon vegetative dry weight under the experimental conditions. Such an assumption was reasonable, given the findings of other researchers (Pal *et al.*, 1980; WHO, 1991). As the f-values for both fungicide and surfactant addition were below the f-critical values in

both the willows and the poplars, the amendments did not have a significant effect at the 95% confidence level.

The willows in the TCB uptake study comprised the only treatment group in which all the plants remained viable at the end of the study. Consequently, this group was the only one of four for which a two-way ANOVA could be performed for radiolabel uptake. For this treatment group, it was found that fungicide addition had a significant impact upon radiolabel uptake, whereas surfactant addition did not (Table 12).

Finally, a two-way ANOVA was performed for the samples in the aldrin study that leached radiolabel in the effluent streams. The replication in this analysis arose not from replicate phytoreactors, but from repeated samples obtained from the same phytoreactors at different times. The results of this analysis reveal that neither fungicide addition or surfactant addition had a significant impact upon the amount of soluble radiolabel leached from willow or poplar phytoreactors.

Conclusions: The results of these analyses led to the conclusion that the surfactant solution used did not have a measurable impact upon any of the vegetative parameters tested. Furthermore, the finding that no detectable radiolabel was sorbed to the effluent carbon trap of any phytoreactor led to the conclusion that the surfactants did not desorb the parent product from the soil carbon to any measurable extent. As the actual parameter being studied was contaminant mobilization rather than simple surfactant addition, the effects of contaminant mobilization via surfactant addition were not tested.

Table 9: Mycorrhizal Development Study Experimental Design

Group	Mycorr. Inoc.	Nutrient Conc.*	N-Det (1ppm)	P-Det (1 ppm)	Leaf Litter
A	+	N	0	0	0
B	+	L	0	0	0
C	+	VL	0	0	0
D	+	0	0	0	0
E	+	L	0	0	+
F	+	VL	0	0	+
G	+	0	0	0	+
H	+	L	+	0	0
I	+	L	0	+	0
J	0	N	0	0	0
K	0	L	0	0	0
L	0	0	0	0	0
FW Cont.	+	0	0	0	0
FR Cont.	+	0	0	0	0

Experimental setup for the Mycorrhizal Development Study is shown. Each group contained 10 replicates at the beginning of the experiment, except for the Ft. Wainwright (FW Cont.) and Ft. Richardson (FR Cont.) controls, which contained 5 replicates. "Mycorr. Inoc." denotes the addition of mixed-culture mycorrhizal fungal inoculum. "N-Det" represents the surfactant Surfonic® T-20; "P-Det" represents Rexophos JV-05-015. "Leaf Litter" denotes the addition of 15 g of leaf litter to soils.

*Nutrient Concentrations:
N= nominal = 100 mg/L nitrogen; 10 mg/L phosphorus
L= low = 10 mg/L nitrogen; 1 mg/L phosphorus
VL= very low = 1 mg/L nitrogen; 0.1 mg/L phosphorus
0= 0 mg/L nitrogen; 0 mg/L phosphorus

Table 10: Mycorrhizal Development Study Poplar Growth Results

Group	mg Chitin /g Root	Total Chitin (mg)	Root Dry Weight (g)	Stems/Leaves Dry Weight (g)
A	5.4 ± 0.8	3.1 ± 0.8	0.58 ± 0.14	5.9 ± 1.6
B	1.8 ± 0.3	0.8 ± .01	0.46 ± 0.03	4.0 ± 0.5
C	1.6 ± 0.3	0.5 ± 0.1	0.29 ± 0.05	2.4 ± 0.4
D	1.5 ± 0.5	0.4 ± 0.1	0.28 ± 0.04	2.6 ± 0.6
E	2.0 ± 0.3	0.9 ± 0.2	0.44 ± 0.08	3.3 ± 0.6
F	1.8 ± 0.2	0.5 ± 0.2	0.30 ± 0.09	2.5 ± 0.7
G	1.8 ± 0.2	0.7 ± 0.2	0.37 ± 0.08	2.7 ± 0.6
H	1.6 ± 0.3	0.5 ± 0.1	0.32 ± 0.04	3.4 ± 0.5
I	1.8 ± 0.3	0.6 ± 0.1	0.33 ± 0.06	3.4 ± 0.8
J	4.6 ± 0.8	3.2 ± 0.7	0.73 ± 0.15	7.0 ± 1.4
K	1.7 ± 0.3	0.7 ± 0.1	0.46 ± 0.05	4.0 ± 0.9
L	0.7 ± 0.2	0.2 ± 0.1	0.29 ± 0.04	2.3 ± 0.6
FW Controls	6.1 ± 1.2	3.3 ± 1.4	0.54 ± 0.21	1.8 ± 0.7

Chitin content is a measure of the amount of mycorrhizal development (≈ 85 mg chitin/g dry fungal mycelium). Error for lettered groups is reported as a 90% confidence interval about the mean of 8-10 replicates. Error for Ft. Wainwright controls is reported as 90% confidence interval about the mean of 5 replicates.

Table 11: Mycorrhizal Development Study Willow Growth Results

Group	mg Chitin /g Root	Total Chitin (mg)	Root Dry Weight (g)	Stems/Leaves Dry Weight (g)
A	4.2 ± 1.0	4.1 ± 1.7	0.91 ± 0.24	4.5 ± 1.2
B	2.4 ± 0.6	1.3 ± 0.4	0.67 ± .018	2.1 ± 0.3
C	1.6 ± 1.0	0.6 ± 0.3	0.47 ± 0.11	1.7 ± 0.4
D	2.5 ± 0.5	0.8 ± 0.3	0.32 ± 0.07	1.6 ± 0.3
E	3.4 ± 0.6	1.5 ± 0.5	0.48 ± 0.15	1.8 ± 0.4
F	2.8 ± 1.0	0.8 ± 0.6	0.47 ± 0.22	1.4 ± 0.4
G	2.5 ± 0.6	0.9 ± 0.2	0.38 ± 0.08	1.9 ± 0.6
H	2.3 ± 0.6	1.5 ± 0.3	0.75 ± 0.19	2.7 ± 0.7
I	2.5 ± 0.5	1.6 ± 0.5	0.69 ± 0.17	2.3 ± 0.4
J	3.9 ± 0.8	5.00 ± 2.1	1.26 ± 0.45	5.0 ± 1.5
K	2.8 ± 1.0	1.4 ± 0.5	0.57 ± 0.15	2.5 ± 0.7
L	2.5 ± 0.9	0.8 ± .01	0.42 ± 0.19	1.8 ± 0.4
FW Controls	8.3 ± 1.5	7.7 ± 5.8	0.84 ± 0.47	1.4 ± 0.2

Chitin content is a measure of the amount of mycorrhizal development (≈ 85 mg chitin/g dry fungal mycelium). Error for lettered groups is reported as a 90% confidence interval about the mean of 8-10 replicates. Error for Ft. Wainwright controls is reported as 90% confidence interval about the mean of 4 replicates.

Table 12: Two-Factor ANOVA Results

Parameter	n	f-Critical	f-Fungicide	f-Surfactant
Willow Dry Weight	4	4.75	2.14	0.22
Poplar Dry Weight	3	5.32	0.19	0.42
Willow TCB Uptake	3	5.32	9.81	0.02
Willow Eff. Radiolabel	3	5.32	0.24	1.09
Poplar Eff. Radiolabel	4	4.75	2.07	0.03

Results from two-factor analyses of the variance are summarized. Analyses were performed at the 95% confidence level. The f-values for fungicide and surfactant indicate the effects of amending vegetative feedwater with these amendments. Values above f-Critical indicate significant difference. "Effluent radiolabel" refers to the amount of effluent radiolabel leached from selected phytoreactors in the aldrin uptake study sampled at various (n) intervals.

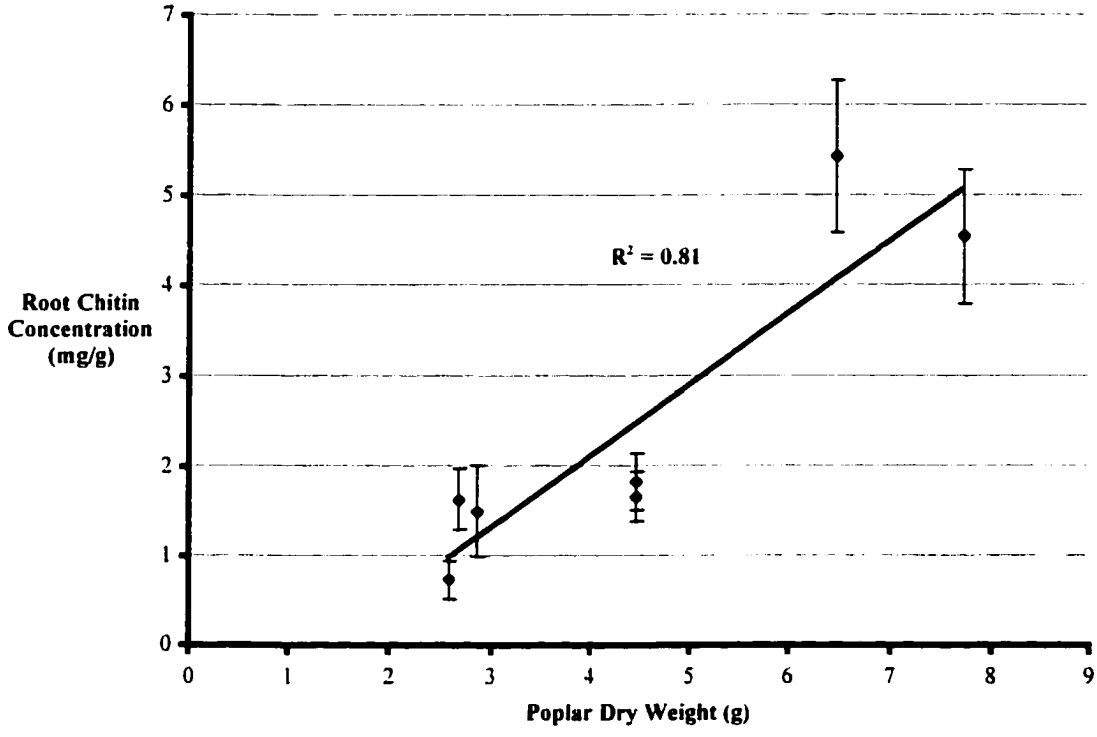

Figure 18: Poplar Dry Weight vs. Root Chitin Concentration

Chitin concentration in dried root material is plotted against total dry weight of the poplars. Chitin content is a measure of the amount of mycorrhizal development (≈ 85 mg chitin/g dry fungal mycelium). Only samples not amended with surfactant or leaf litter are shown. Error bars represent 90% confidence interval about the mean of 8-10 replicates.

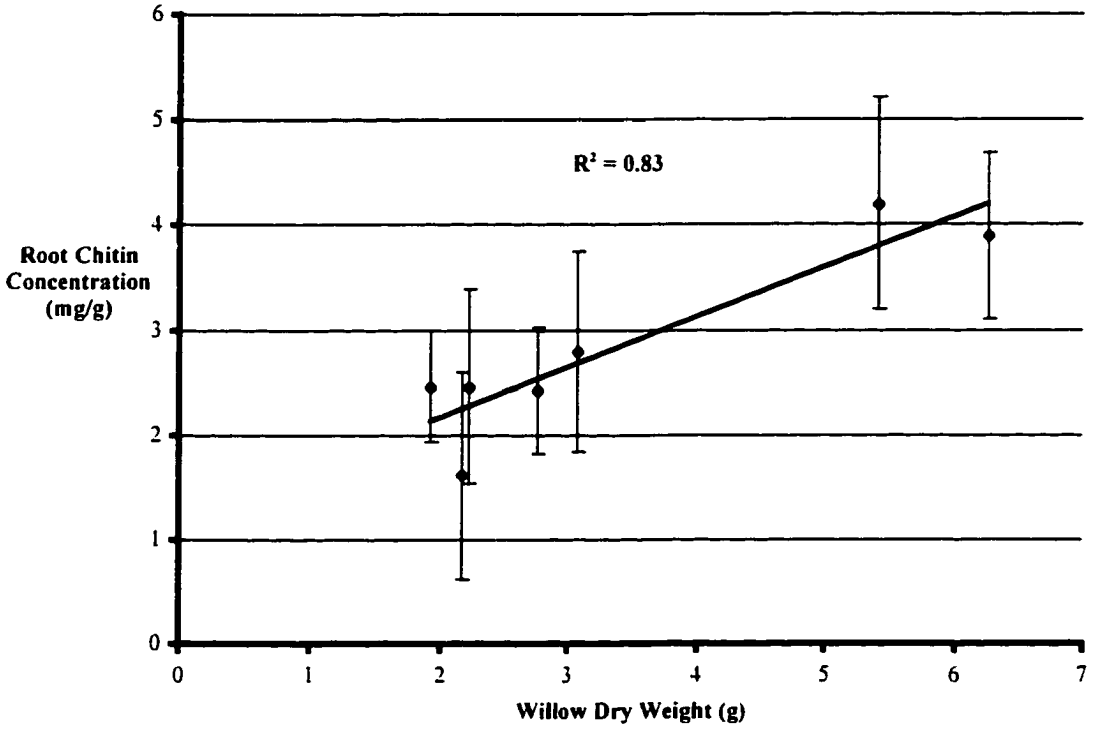

Figure 19: Willow Dry Weight vs. Root Chitin Concentration

Chitin concentration in dried root material is plotted against total dry weight of the willows. Chitin content is a measure of the amount of mycorrhizal development (≈ 85 mg chitin/g dry fungal mycelium). Only samples not amended with surfactant or leaf litter are shown. Error bars represent 90% confidence interval about the mean of 8-10 replicates.

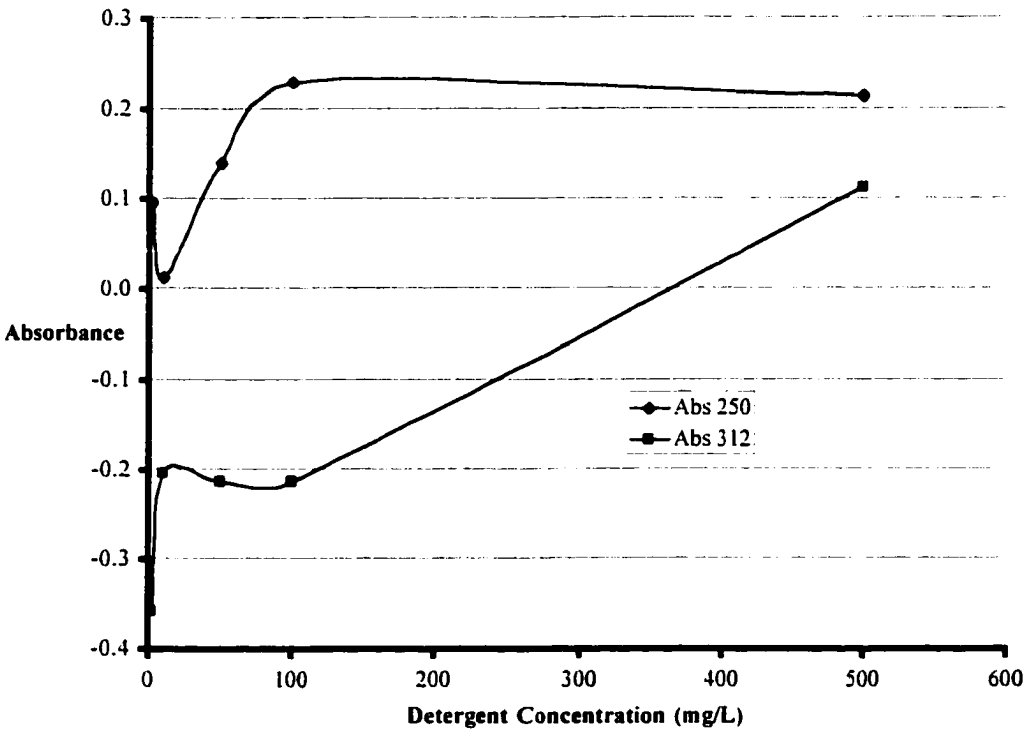

Figure 20: CMC Determination, Run #1

Absorbance values of benzoylacetone at 250 and 312 nm are plotted as a function of surfactant concentration.

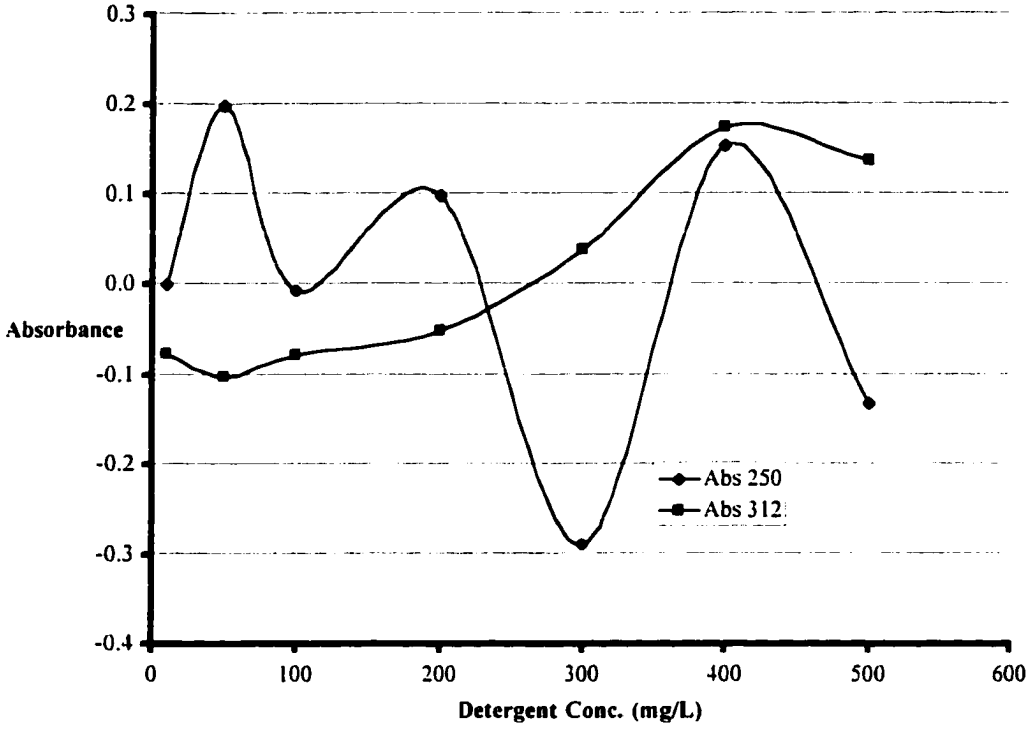

Figure 21: CMC Determination, Run #2

Absorbance values of benzoylacetone at 250 and 312 nm are plotted as a function of surfactant concentration.

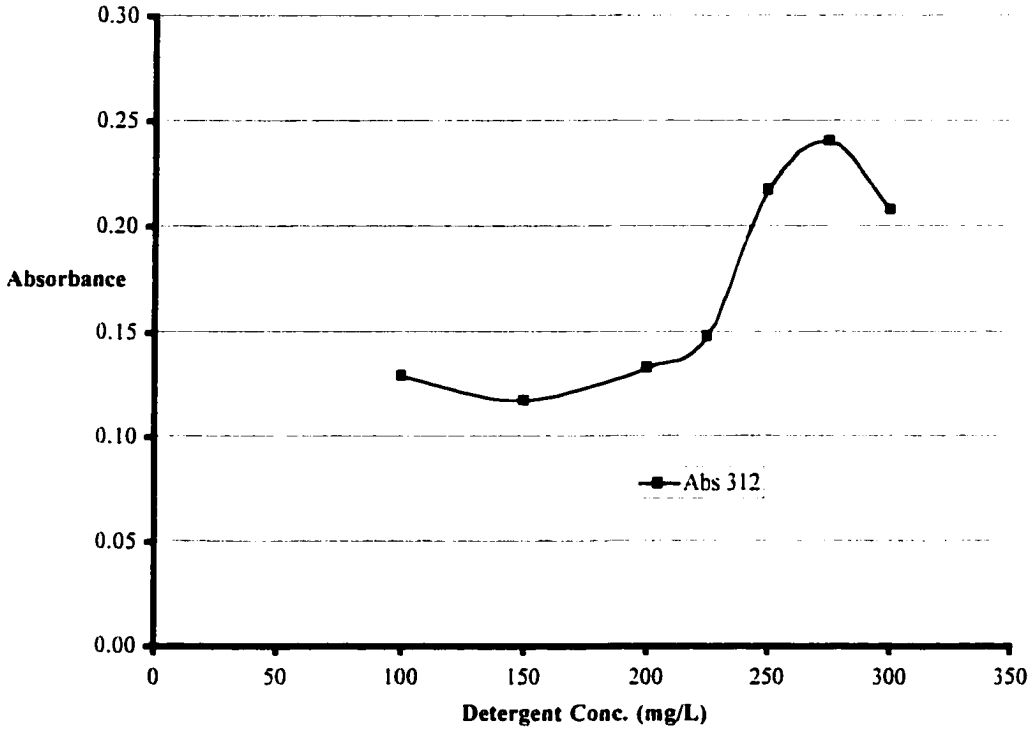

Figure 22: CMC Determination, Run #3

Absorbance values of benzoylacetone at 312 nm are plotted as a function of surfactant concentration.

Chapter 7: Conclusions

Section 7.1: Experimental Conclusions

7.1.1: *In Vitro* Conclusions

1) **A mixed culture of ectomycorrhizal fungi, grown in vitro, was able to take up radiolabelled aldrin and PCB's from aqueous solution.** This finding was considered to be an essential plank in the overall research design. Although mixed cultures grown in vitro would undoubtedly differ in constitution and activity from the same cultures grown in situ, this result indicated that the culture had the potential to enhance field biotransformations.

2) **The mixed culture of ectomycorrhizal fungi grown in vitro had the capacity to use at least two of the three tested surfactants as a carbon source.** It was hypothesized that if the fungi were able to biodegrade the surfactants, then such activity might increase the transformation potential of contaminants associated with the surfactants.

3) **Surfactant addition increased the ability of mixed culture ectomycorrhizae grown in vitro to take up radiolabelled aldrin and PCB's from aqueous solution.** It was not determined whether the surfactants acted to increase the solubility of the contaminants in aqueous solution, or acted to increase the hyphal membrane

permeability to the contaminants. It is likely that both mechanisms acted to some extent. These results led to further studies employing two of the three surfactants (Surfonic® T-20 and Rexophos JV-05-015).

4) **Surfactant addition increased the amount of aldrin and PCB uptake by a mixed culture of ectomycorrhizal fungi during a period of low growth.** Aldrin and PCB uptake was shown to be relatively independent of hyphal growth. Furthermore, surfactant addition increased hyphal uptake regardless of the growth rate. These results indicated that surfactant addition had the potential to be effective during the entire vegetative life cycle.

7.1.2: Mycorrhizal Development Conclusions

1) **Mycorrhizal development in feltleaf willow and balsam poplar was inhibited under nutrient-limiting conditions.** Mycorrhizal development was coupled to vegetative development in the sandy soil used for this experiment. This finding demonstrated that it would be ineffective in later studies to attempt to enhance mycorrhizal development through nutrient limitation. It is unfortunate that nutrient concentrations above those considered to be nominal were not tested, however, there was little evidence in the literature to indicate that high nutrient regimens would yield exaggerated mycorrhizal development.

2) **The sandy soil matrix used in the mycorrhizal development study was optimal for experimental control, but reduced the applicability to most field soils.** The large particle size and low organic content of the sand allowed for the root material to be easily separated from the soil with mycorrhizal hyphae intact. This led to accurate measurements of both root mass and mycorrhizal development. Furthermore, the sand allowed adequate drainage through the cups, thus avoiding altered development as a result of water saturation. Finally, as the sand was all of a similar mesh, was autoclaved and had negligible organic content, it could be assumed that the soil was homogeneous over all the samples at the outset of the experiment. A detrimental effect of using the sand as opposed to field soils was that the sand had few sorptive sites for the exchange of mineral or organic nutrients. Consequently, the mineral nutrients fed to the systems likely remained in the soil solution or drained out of the bottom of the cups. As a result, the nutrient depletion zones into which mycorrhizal hyphae commonly develop, were never formed. Hence, mycorrhizal development was likely inhibited under nutrient-limiting conditions because there were few nutrients available to mycorrhizal fungi that were not available to the plant roots as well. Such an impact could possibly have been avoided through the use of field soils with higher cation exchange capacities and organic content.

3) **Soils inoculated with mixed-culture ectomycorrhizal fungi produced the same amount of mycorrhizal development in the vegetation as non-inoculated soils.** The cross contamination was theorized to have resulted from hyphal contact between

plants cultivated in the same growth pallets. It is possible, however, that the original cuttings were not adequately sterilized at the outset of the experiment. The most effective method for ensuring that the samples were non-mycorrhizal would have been to cultivate samples in sealed containers, in sterilized soil, from sterilized seed. Contrary to the original experimental design, there were no true non-inoculated controls. Consequently, mycorrhizal manipulation through differential inoculation was ruled out as a viable strategy in further studies.

4) **Fungicide addition was determined to be the most viable option for manipulating mycorrhizal development in further studies under similar conditions.** Fungicide addition would theoretically reduce mycorrhizal development without directly affecting plant growth. Fungicide addition would also likely affect the bacterial consortia as well, however, so its use was not optimal. In addition, although it was desired to investigate the effects of mycorrhizal enhancement, fungicide addition would actually result in the investigation of mycorrhizal inhibition.

7.1.3: Uptake Study Conclusions

1) **Mycorrhizal infection was highly correlated with radiolabel uptake in the willow plants in both the TCB and aldrin studies.** Although a causative relationship was implied, this relationship was not directly shown. With respect to other degradative processes, however, mycorrhizal infection appeared to be the most probable mechanism for the enhancement of vegetative uptake. Mycorrhizal infection could

have enhanced uptake via increased extracellular degradation, increased membrane permeability to parent or transformation products, and/or increased root surface area and reach.

2) **Mycorrhizal infection was somewhat correlated with radiolabel uptake in the poplar plants in both the TCB and aldrin studies.** Poplar mycorrhizal development was not strongly affected by the addition of fungicides in either experiment. Consequently, linear correlations between mycorrhizal development and radiolabel uptake were not as apparent as in the willow systems because in the poplar systems, mycorrhizal growth was relatively constant throughout the treatment groups.

3) **Most of the radiolabelled residues detected in the vegetative material in both uptake studies took the form of bound transformation product.** Consequently, mechanisms that would increase uptake would likely increase contaminant transformation as well. While the toxicity of the bound residue remains unclear, these products would likely be non-detectable in the absence of the ^{14}C radiolabel.

4) **Some amount of TCB was taken up as parent product by the willows and poplars, and roughly 13-34% of the total radiolabel taken up remained in the root material as extractable TCB.** This finding demonstrated that the degradative processes were not 100% efficient. As all of the detectable radiolabel was

sequestered in the root systems, however, neither the TCB nor the transformation residues were xylem mobile.

5) **The willows and poplars took up some amount of aldrin and/or dieldrin, and roughly 11-21% of the total radiolabel taken up remained in the root material as extractable dieldrin.** Again, this finding demonstrated that the degradative mechanisms were not 100% efficient over the experimental period.

6) **Dieldrin and/or its residues were found to be xylem mobile in the willows and poplars.** Leaf radiolabel concentrations were found to be roughly one order of magnitude lower that root radiolabel concentrations for both the willows and poplars. In the willow plants, dieldrin accumulated in the leaves to approximately 5 μg/kg, or less than 1% of the total xylem mobile radiolabel, and less than 0.1% of the total radiolabel taken up by the roots. Although the poplars translocated approximately the same amount of radiolabel to the leaves as did the willows, the poplars did not accumulate detectable dieldrin in the leaves (< 1 μg/kg). All other factors being equal, the poplars would be more appropriate for the phytoremediation of aldrin because they would be less likely than willows to disperse dieldrin offsite.

7) **Vegetation enhanced the epoxidation of aldrin in the soils; fungicide inhibited the epoxidation of aldrin.** While the formation of dieldrin was not considered to be a remediative process, this transformation did serve as an index of bioavailability

and/or bioactivity. Consequently, the presence of vegetation was shown to increase the general level of bioactivity. The finding that fungicide addition decreased epoxidation in vegetated soils to non-detectable levels indicated that plant-associated soil fungi potentially effected a large proportion of the aldrin epoxidation.

8) **A water-soluble transformation product of aldrin was detected in the effluent streams of willow, poplar, and non-vegetated phytoreactors.** This transformation product(s) was estimated to account for 6-12% of the total aldrin added to the vegetated systems, and 1-2% of the aldrin added to the non-vegetated systems. Although the water-soluble product(s) was not identified, dihydrochlordene dicarboxylic acid has been reported as a major hydrophilic metabolite of aldrin (Stewart and Gaul, 1977). As a water-soluble metabolite would likely migrate off of an uncontained phytoremediation site, further investigation into the identity and toxicity of the water-soluble product(s) is encouraged.

9) **The mixture of 125 mg/L Surfonic® T-20 and 125 mg/L Rexophos JV-05-015 was ineffective for the mobilization of TCB or aldrin in the phytoreactors.** Although surfactant addition was shown to enhance hyphal uptake in the *in vitro* studies, the surfactants did not increase uptake in the bench-scale uptake studies. Furthermore, in the uptake studies, the surfactants were added at their measured CMC, or the concentration at which contaminant-mobilizing micelles are formed. In the *in vitro* studies, the surfactants were added at much lower concentrations. The *in vitro* studies

were conducted in the presence of erythromycin and tetracycline to inhibit bacterial activity. In the vegetative uptake studies, no bactericides were used. It is plausible, then that the bacteria present in the phytoreactor soils severely restricted the activity of the biodegradable surfactants. Regardless, as no detectable radiolabel was found in the effluent carbon traps of the TCB or aldrin phytoreactors, the surfactants did not mobilize the contaminants through the soils. Furthermore, as contaminant mobilization rather than surfactant addition was the actual parameter being considered, the effects on uptake of contaminant mobilization via surfactant addition remain unstudied. As the desorption of contaminant from the soil was regarded to be the rate-limiting step in the transformation processes, further studies with other surfactants or different concentrations are encouraged.

10) **Both aldrin and TCB appeared to be amenable to phytoremediation using willows and/or poplars.** In the TCB systems, both species were thought to take up all of the contaminant and/or residues available in solution. Addition of the *appropriate* surfactants could potentially enhance uptake and transformation by increasing the concentration of TCB in solution. In the aldrin systems, the addition of vegetation increased the formation of water-soluble metabolite(s). Although the identity and toxicity characteristics of the metabolite(s) require further investigation, the rate of formation indicates that this may be a plausible phytoremediation strategy.

Section 7.2: Field Applicability

The bulk of this research was conducted in laboratories and greenhouses using individual species and contaminants. One project goal, however, was to generate general conclusions and ideas for use in phytoremediation field projects. Following is a list of generalized conclusions and recommendations:

7.2.1: Field Conclusions

1) **In general, plants having a more robust population of mycorrhizal fungi will be better able to take up and/or transform recalcitrant chlorinated organics.** Although the mycorrhizal consortia will differ according to field conditions and vegetative species, a wide variety of mycorrhizal fungal species have been shown to degrade recalcitrant organics (Donnelly *et al.*, 1993; Donnelly and Fletcher, 1995; Meharg *et al.*, 1997a; Meharg *et al.*, 1997b; Trojanowski *et al.*, 1984). This conclusion was supported in the present research, where the effects of the general mycorrhizal population were studied. Although this study focused on the activity of ectomycorrhizal fungi; vesicular-arbuscular, ericoid, and orchid mycorrhizae have also been reported to be capable of degrading organic soil constituents (Allen, 1991).

2) **Although 100 mg/L-N and 10 mg/L-P was determined to be optimal for mycorrhizal development in the sands used for this study, such may not be the**

case under field conditions. As discussed previously, field soils often exhibit a higher CEC and contain a higher level of organic matter than the clean sand used for the mycorrhizal development study. Consequently, it is likely that mycorrhizal fungi would be more apt to proliferate in field soils where the retardation of nutrient mobility results in the formation of depletion zones. As an example, the Ft. Wainwright control soils used in the mycorrhizal development study resulted in mycorrhizal concentrations at or above the levels achieved in the optimal-nutrient sands. The Ft. Wainwright soils, however, received no mineral nutrients. Soil analysis revealed that the Ft. Wainwright soils originally contained approximately 10 mg/kg inorganic nitrogen, and 2 mg/kg extractable phosphorus. The available nutrients were lower in the field soils than in the sands, yet mycorrhizal development was not inhibited. These results demonstrate that mycorrhizal development under field conditions may require site-specific characterization.

3) **Although fungicide addition was considered to be the most viable option available for mycorrhizal manipulation in this study, the exploration of other options is encouraged.** As previously stated, fungicide addition *inhibited* mycorrhizal development, whereas in the field, mycorrhizal *enhancement* would be desired. As discussed above, it is possible that under field conditions, mycorrhizal development could be enhanced through the depletion of mineral nutrients. Addition of organic nutrients such as manure have the potential to increase the activity of organic-degrading mycorrhizal fungi. Also, development of contaminant-degrading

mycorrhizal fungi could potentially be brought about by the selective inoculation of degradative species (e.g., *Hysterangium gardneri, Radiigera atrogleba, Paxillus involutus, Suillus variegatus*). Pure cultures of ectomycorrhizal fungi can be obtained from the USDA Forest Service, Pacific Northwest Forest Research Station, Corvallis, OR.

4) **Surfactant addition has been proven effective for the mobilization of PCB's and other hydrophobic compounds in the field.** A recent study demonstrated that polyoxyethylene 10 lauryl ether (POL), added at approximately 1300 mg/L, mobilized over 80% of the PCB's in an aged soil contaminated with mixed PCB congeners (Layton *et al.*, 1998). This concentration was more than 200 times the given critical micelle concentration for POL. Given that the surfactants employed in the uptake study were added at the measured CMC, it is possible that Surfonic® T-20 and/or Rexophos JV-05-015 would be more effective mobilizers at higher concentrations. Triton X100, Triton N101, Brij 35, and Brij 30 also have potential for further study (Guha and Jaffe, 1996). In a phytoremediation field site, however, the addition of high surfactant concentrations could result in the offsite migration of product. A balance would be required, therefore, to ensure that enough surfactant was added to enhance biological transformations, but that not enough was added to promote the offsite migration of contaminant. An optimal surfactant concentration would have to be found for each site individually, taking into account the organic content of the soil, the concentration and solubility of the contaminants, the

hydrogeologic regimen of the site, the transpiration rate of the vegetation, and the biodegradability of the surfactant. Column studies employing site soils and a range of surfactant concentrations would be the logical first step in the determination of optimal surfactant concentrations.

5) **The effectiveness of a mycorrhizal-enhanced phytoremediation strategy involving surfactant addition would be largely dependant upon the species used, the concentration and characteristics of the contaminant(s), and the conditions at the site.** Fast growing woody species such as willows and poplars create large amounts of biomass, have extensive root systems, and transpire relatively large amounts of water. Such characteristics are often desirable for phytoremediation strategies. Other species, such as ericaceous and orchid species, support mycorrhizae well known for the ability to degrade organic soil constituents (Allen, 1991), but are often slow growing and support smaller root systems. Toxic contaminants are generally slower to biodegrade than less toxic contaminants with similar chemical structures (Bandala *et al.*, 1998). Furthermore, hydrophobicity affects bioavailability, which in turn affects biodegradability. Aged PCB sites, for example, generally retain the more hydrophobic highly chlorinated congeners much longer than they retain congeners with a low degree of chlorine substitution (Pal *et al.*, 1980). Additionally, higher temperatures and a lower soil organic content would be expected to increase the effectiveness of a phytoremediation strategy for hydrophobic chlorinated organic contaminants (Goodman *et al.*, 1992). Given the array of variables affecting the

uptake and/or transformation processes, each phytoremediation site will require individual consideration.

7.2.2: Suggested Field Strategy

The employment of mycorrhizal fungi and surfactant addition has the potential to enhance the phytoremediation of recalcitrant chlorinated organics. Below is a recommended strategy for further field trials:

- **Determine the expectations of the responsible party.** Phytoremediation is inherently slower than other remediation strategies such as excavation and incineration. Consequently, phytoremediation is not appropriate for all applications.

- **Determine the identity and concentration of all the contaminants of concern.** Research the relative toxicity and solubility of the contaminant(s) on the site. Ensure that the vegetative species planned are able to prosper in soils contaminated at the levels determined.

- **Examine the soil properties.** Determine the organic content, CEC, and nutrient concentrations. Evaluate the groundwater and surface water flow regimens. Examine the vegetation growing at or near the site for obvious signs of disease, inhibited growth, or nutrient depravation.

- **Perform column studies employing site soils and various concentrations of surfactants.** Determine the optimal surfactant concentration to add, taking into consideration the groundwater flow regimen, the transpiration rates of the vegetation, the hydrophobicity of the contaminants, and the organic content of the soil.

- **Fertilize, if necessary.** Organic fertilizers such as manure are recommended, as organic fertilizers would be less likely to inhibit mycorrhizal development. If organic materials are added, ensure that the surfactant concentrations are appropriate for the fertilized soils.

- **Plant the vegetation.** Select a species appropriate for growth at the site and demonstrating the growth characteristics desired. Hybrid poplars (*Populus deltoides nigra*) are widely used for phytoremediation, but myriad other species are discussed in the literature as well.

- **Inoculate with mycorrhizal fungi.** Research the scientific literature for clues concerning which species to use. Consult with experienced researchers concerning the field viability of prospective species. As stated previously, mycorrhizal fungal inoculum can be obtained from the USDA Forest Service.

- **Add the surfactants to the system.** Restrict the offsite migration of soil solution if possible. If studying an uncontained site, ensure that surfactant addition does not

mobilize parent product into the groundwater or wash contaminants into the surface runoff.

- **Set up control plots.** Create controls for vegetation, mycorrhizal inoculation, and surfactant addition.

- **Evaluate the system at regular intervals.** Determine the contaminant concentrations in the soils and vegetation with respect to the control plots. Analyze the foliar tissue to determine whether contaminants could migrate offsite in wind blown leaves. Assay the soil and vegetation for reported metabolites.

- **Modify the experimental design to enhance successful strategies.** As phytoremediation projects typically require years for completion, it may be desirable to alter the experimental plan to emphasize the strategies demonstrating the most promising early results. It is expected that as more and more information is gathered from the system under study, the researchers will gain an intuition concerning which mechanisms present the most potential. It is reasonable, therefore, to alter the experimental design in an attempt to further the degradative capabilities of the system.

Section 7.3: Final Considerations

This study has presented the beginnings of a new phytoremediation strategy. The strategy has the potential to effect the *in situ* transformation of recalcitrant chlorinated organic contaminants. Although such contaminants are not now generally considered to be amenable to phytoremediation, further research into this area could result in the development of a relatively inexpensive, effective remediation strategy. Consequently, the use of such a strategy could allow for the remediation of sites that would otherwise remain contaminated, useless, and ignored.

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Appendix A: *In Vitro* Raw Data

Table 13: Non-Radiolabelled Experiment Raw Data

Amendment	Media	Hyphal wt. (mg)	Media	Hyphal wt. (mg)
None	Control	3.26	C-Limiting	0.23
None	Control	3.69	C-Limiting	0.39
None	Control	4.00	C-Limiting	0.29
Aldrin	Control	3.39	C-Limiting	0.33
Aldrin	Control	3.11	C-Limiting	0.39
Aldrin	Control	3.31	C-Limiting	0.43
Biphenyl	Control	2.51	C-Limiting	0.40
Biphenyl	Control	2.42	C-Limiting	0.29
Biphenyl	Control	2.62	C-Limiting	0.29
2,2'-DCB	Control	1.91	C-Limiting	0.41
2,2'-DCB	Control	2.31	C-Limiting	0.14
2,2'-DCB	Control	1.29	C-Limiting	0.40
3,3',4,4'-TCB	Control	2.95	C-Limiting	0.34
3,3',4,4'-TCB	Control	5.31	C-Limiting	0.36
3,3',4,4'-TCB	Control	3.27	C-Limiting	0.27
2',3,3',4,5-PtCB	Control	2.92	C-Limiting	0.33
2',3,3',4,5-PtCB	Control	3.29	C-Limiting	0.31
2',3,3',4,5-PtCB	Control	3.39	C-Limiting	0.50
Surfonic T-20	Control	3.31	C-Limiting	0.48
Surfonic T-20	Control	3.30	C-Limiting	0.41
Surfonic T-20	Control	2.88	C-Limiting	0.48
Rexophos	Control	0.30	C-Limiting	0.20
Rexophos	Control	0.28	C-Limiting	0.31
Rexophos	Control	0.29	C-Limiting	0.34
Surfornic L24-9	Control	2.98	C-Limiting	0.75
Surfornic L24-9	Control	2.86	C-Limiting	0.67
Surfornic L24-9	Control	3.26	C-Limiting	0.72

Table 14: Surfactant Enhanced Uptake Raw Data

Amendment	TCB % Uptake	PtCB % Uptake	Aldrin % Uptake
none	n/a	0.80	n/a
none	2.33	0.04	0.27
none	2.67	1.04	0.05
Surfornic L24-9	9.09	0.33	0.53
Surfornic L24-9	0.85	0.00	0.67
Surfornic L24-9	0.41	0.47	0.46
Surfonic T-20	7.78	2.37	0.07
Surfonic T-20	7.10	0.37	0.78
Surfonic T-20	0.38	1.96	0.02
Rexophos	6.04	1.26	0.06
Rexophos	12.45	0.02	0.15
Rexophos	11.49	1.44	0.04

Table 15: Uptake Over 10 and 30-Day Periods

Contaminant	Amendment	Hyp. wt. (mg) 10-Days	% Uptake/mass 10-Days	Hyp. wt. (mg) 30-Days	% Uptake/mass 30-Days
Aldrin	none	3.67	n/a	2.2	0.35
Aldrin	none	2.22	0.12	3.81	0.22
Aldrin	none	2.68	0.02	2.01	0.53
Aldrin	Surfornic L24-9	3.19	0.17	2.58	0.30
Aldrin	Surfornic L24-9	3.66	0.18	1.68	7.55
Aldrin	Surfornic L24-9	3.09	0.15	2.69	1.69
Aldrin	Surfonic T-20	2.95	0.02	2.09	1.25
Aldrin	Surfonic T-20	3.18	0.24	1.67	0.38
Aldrin	Surfonic T-20	2.52	0.01	1.88	0.31
Aldrin	Rexophos	3.49	0.02	1.36	0.06
Aldrin	Rexophos	2.98	0.05	3.07	0.22
Aldrin	Rexophos	3.2	0.01	2.08	0.10
PtCB	none	4.13	0.19	2.9	0.69
PtCB	none	2.35	0.02	2.76	0.90
PtCB	none	2.9	0.36	3.38	0.06
PtCB	Surfornic L24-9	3.04	0.11	4.35	0.25
PtCB	Surfornic L24-9	2.13	-0.01	2.43	0.12
PtCB	Surfornic L24-9	1.48	0.32	3.32	0.39
PtCB	Surfonic T-20	3.35	0.71	2.65	1.78
PtCB	Surfonic T-20	3.05	0.12	3.41	0.01
PtCB	Surfonic T-20	2.25	0.87	1.28	1.30
PtCB	Rexophos	3.41	0.37	2.55	4.61
PtCB	Rexophos	2.22	0.01	1.47	0.30
PtCB	Rexophos	2.28	0.63	2.95	1.14

Appendix B: Vegetative Uptake Study Raw Data

Table 16: Plant Harvest Weights (Group 1)

Samp	Plant	Cont.	Fungicide	Surfactant	Root FW (g)	Leaf FW (g)	Stem FW (g)
1	non-veg.	ald	+	0	n/a	n/a	n/a
2	non-veg.	ald	0	+	n/a	n/a	n/a
3	non-veg.	TCB	+	0	n/a	n/a	n/a
4	non-veg.	TCB	0	+	n/a	n/a	n/a
5	willow	ald	+	0	17.5	13.1	10.9
6	willow	ald	0	0	14.4	20.5	13.3
7	willow	ald	+	+	9.3	15.1	8.5
8	willow	ald	0	+	11.6	16.3	14.6
9	willow	TCB	+	0	18.2	14.6	11.5
10	willow	TCB	0	0	15.8	14.2	8.9
11	willow	TCB	+	+	14.2	15.9	12.8
12	willow	TCB	0	+	12.0	16.5	11.2
13	poplar	ald	+	0	11.3	8.5	6.1
14	poplar	ald	0	0	6.6	9.6	8.9
16	poplar	ald	0	+	3.8	5.7	1.8
19	poplar	TCB	+	+	11.8	6.4	5.3
20	poplar	TCB	0	+	13.0	12.0	7.9

Shaded areas represent plants dead or dying at the end of the experimental period.

Table 17: Measured Parameters (Group 1)

Samp	Plant DW (g)	Uptake (μCi/10kg Rt)	Chitin (mg/g Rt)	% Rec. Soil	% Rec. Roots	% Rec. Leaves	% Rec. Effluent
1	n/a	n/a	n/a	40.7	n/a	n/a	n/a
2	n/a	n/a	n/a	53.1	n/a	n/a	n/a
3	n/a	n/a	n/a	73.7	n/a	n/a	n/a
4	n/a	n/a	n/a	74.5	n/a	n/a	n/a
5	12.1	10.2	1.4	32.6	2.0	0.07	n/a
6	13.7	19.0	3.2	28.3	2.6	0.17	n/a
7	10.0	11.7	0.9	41.6	1.5	0.21	n/a
8	14.2	13.7	3.6	35.9	2.6	0.16	n/a
9	12.6	3.0	n/a	77.7	0.6	n/a	n/a
10	10.6	11.3	n/a	70.4	1.7	n/a	n/a
11	12.8	4.0	n/a	82.5	0.6	n/a	n/a
12	12.7	5.4	n/a	76.8	0.8	n/a	n/a
13	8.5	12.6	n/a	37.3	1.6	0.06	n/a
14	8.9	14.4	n/a	27.1	1.1	0.41	n/a
16	5.2	7.7	1.1	40.6	0.4	0.15	n/a
19	8.6	5.1	n/a	87.5	0.6	n/a	n/a
20	10.5	4.6	n/a	88.2	0.6	n/a	n/a

Shaded areas represent plants dead or dying at the end of the experimental period.

Table 18: Plant Harvest Weights (Group 2)

Samp	Plant	Cont.	Fungicide	Surfactant	Root FW (g)	Leaf FW (g)	Stem FW (g)
1	non-veg.	ald	+	0	n/a	n/a	n/a
2	non-veg.	ald	0	+	n/a	n/a	n/a
3	non-veg.	TCB	+	0	n/a	n/a	n/a
4	non-veg.	TCB	0	+	n/a	n/a	n/a
5	willow	ald	+	0	7.2	7.8	4.0
6	willow	ald	0	0	12.3	14.8	12.3
7	willow	ald	+	+	17.3	13.4	10.8
9	willow	TCB	+	0	14.6	15.0	15.2
10	willow	TCB	0	0	10.3	12.4	2.1
11	willow	TCB	+	+	8.0	8.5	5.8
12	willow	TCB	0	+	14.7	16.1	14.3
13	poplar	ald	+	0	16.8	9.7	8.9
14	poplar	ald	0	0	14.8	7.9	5.8
15	poplar	ald	+	+	7.5	7.9	4.8
16	poplar	ald	0	+	10.0	9.4	6.0
17	poplar	TCB	+	0	8.2	7.6	4.6
18	poplar	TCB	0	0	11.7	7.9	4.4
19	poplar	TCB	+	+	14.7	8.6	8.3
20	poplar	TCB	0	+	11.7	8.1	5.9

Darkly shaded areas represent plants dead or dying at the end of the experimental period. Lightly shaded areas represent plants recovering from water stress at the end of the experimental period.

Table 19: Measured Parameters (Group 2)

Samp	Plant DW (g)	Uptake (μCi/10kg Rt)	Chitin (mg/g Rt)	% Rec. Soil	% Rec. Roots	% Rec. Leaves	% Rec. Effluent
1	n/a	n/a	n/a	44.0	n/a	n/a	n/a
2	n/a	n/a	n/a	48.6	n/a	n/a	n/a
3	n/a	n/a	n/a	62.6	n/a	n/a	n/a
4	n/a	n/a	n/a	73.5	n/a	n/a	n/a
5	7.2	12.4	1.5	28.8	1.1	0.07	n/a
6	14.1	25.8	4.2	33.3	4.4	0.29	n/a
7	12.6	14.6	1.4	45.2	2.8	0.11	n/a
9	14.3	3.4	1.0	80.0	0.7	n/a	n/a
10	11.7	6.2	4.1	83.7	0.9	n/a	n/a
11	7.5	4.3	1.8	72.6	0.4	n/a	n/a
12	14.1	8.8	3.4	74.5	1.5	n/a	n/a
13	12.6	10.2	0.9	39.7	2.0	0.43	n/a
14	10.9	23.8	1.7	29.6	4.9	0.35	n/a
15	6.5	20.2	1.6	43.6	1.3	0.04	n/a
16	9.5	18.2	1.0	30.4	2.3	0.36	n/a
17	6.5	13.9	2.6	92.8	1.1	n/a	n/a
18	9.9	7.7	0.9	68.6	1.5	n/a	n/a
19	10.9	3.3	0.9	87.2	0.5	n/a	n/a
20	9.1	4.6	1.7	85.5	0.7	n/a	n/a

Darkly shaded areas represent plants dead or dying at the end of the experimental period. Lightly shaded areas represent plants recovering from water stress at the end of the experimental period.

Table 20: Plant Harvest Weights (Group 3)

Samp	Plant	Cont.	Fungicide	Surfactant	Root FW (g)	Leaf FW (g)	Stem FW (g)
1	non-veg.	ald	+	0	n/a	n/a	n/a
2	non-veg.	ald	0	+	n/a	n/a	n/a
3	non-veg.	TCB	+	0	n/a	n/a	n/a
4	non-veg.	TCB	0	+	n/a	n/a	n/a
5	willow	ald	+	0	16.1	13.6	10.0
6	willow	ald	0	0	12.7	17.3	12.8
7	willow	ald	+	+	16.1	13.0	10.1
8	**willow**	**ald**	**0**	**+**	**7.1**	**10.6**	**7.6**
9	willow	TCB	+	0	17.1	12.0	12.6
10	**willow**	**TCB**	**0**	**0**	**6.1**	**19.6**	**11.4**
11	**willow**	**TCB**	**+**	**+**	**13.1**	**13.2**	**11.6**
12	**willow**	**TCB**	**0**	**+**	**13.7**	**14.1**	**11.2**
13	poplar	ald	+	0	10.4	7.2	7.3
14	poplar	ald	0	0	5.3	6.5	3.9
15	poplar	ald	+	+	21.3	9.1	4.5
16	poplar	ald	0	+	2.6	5.1	2.6
17	poplar	TCB	+	0	11.4	8.5	8.2
18	poplar	TCB	0	0	8.1	8.3	5.0
19	poplar	TCB	+	+	8.3	8.0	6.0
20	poplar	TCB	0	+	7.1	8.5	7.8

Shaded areas represent plants recovering from water stress at the end of the experimental period.

Table 21: Measured Parameters (Group 3)

Samp	Plant DW (g)	Uptake (μCi/10kg Rt)	Chitin (mg/g Rt)	% Rec. Soil	% Rec. Roots	% Rec. Leaves	% Rec. Effluent
1	n/a	n/a	n/a	58.0	n/a	n/a	0.6
2	n/a	n/a	n/a	55.7	n/a	n/a	2.4
3	n/a	n/a	n/a	68.9	n/a	n/a	n/a
4	n/a	n/a	n/a	70.4	n/a	n/a	n/a
5	12.5	22.3	n/a	39.7	4.0	0.17	34.2
6	14.2	24.1	n/a	28.5	4.1	0.62	13.9
7	12.8	20.9	n/a	40.8	4.3	0.18	5.8
8	9.8	16.5	2.4	37.2	1.4	0.12	11.7
9	12.9	5.5	1.1	86.8	1.2	n/a	n/a
10	11.6	10.3	5.5	86.4	0.6	n/a	n/a
11	12.2	5.9	1.6	80.9	0.9	n/a	n/a
12	12.9	12.3	4.5	86.3	2.3	n/a	n/a
13	9.3	14.0	1.8	34.7	1.5	0.05	12.1
14	6.1	18.5	n/a	33.3	1.4	0.04	8.7
15	12.2	17.3	1.5	41.5	5.0	0.13	13.5
16	4.3	37.3	n/a	54.4	1.2	0.22	6.0
17	9.7	4.1	1.7	93.4	0.5	n/a	n/a
18	7.8	3.4	2.8	85.5	0.3	n/a	n/a
19	7.3	5.7	1.9	92.5	0.5	n/a	n/a
20	9.5	7.7	1.3	88.3	0.7	n/a	n/a

Shaded areas represent plants recovering from water stress at the end of the experimental period.