RHIZOREMEDIATION OF DIESEL CONTAMINATED

SOIL USING *SALIX ALAXENSIS*

By

Kelly M. McFarlin

RECOMMENDED:

Advisory Committee Co-Chair

Advisory Committee Co-Chair

Chair, Department of Civil and Environmental Engineering

APPROVED:

Dean, College of Engineering and Mines

Dean of the Graduate School

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A

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Kelly Marie McFarlin, B.S.

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Abstract

An outdoor pot study and a microcosm study were conducted to evaluate the potential for *Salix alaxensis* (felt leaf willow) to rhizoremediate diesel-contaminated soil. The pot study was conducted for 96 days during an Alaskan interior summer with *S. alaxensis* grown in soil contaminated with diesel fuel oil #2. The concentration of diesel range organics (DRO) and the most probable number (MPN) of diesel degrading microorganisms in the rhizosphere were measured initially and compared to final values. A microcosm study was also performed with crushed willow roots to simulate root turnover, in which the abundance of diesel degrading microorganisms was also determined. It was hypothesized that treatments containing willow and fertilizer would foster the greatest abundance of diesel degrading microorganisms and thus would provide the largest decrease of diesel range organics. In the pot study, growth of *S. alaxensis* resulted in the largest decrease of DROs, although treatments amended with fertilizer contributed to a significant increase in MPN of diesel degrading microorganisms. The microcosm study indicated that the addition of crushed willow roots to contaminated soil produced a similar abundance of diesel degrading microorganisms as the addition of salicylic acid. The findings suggest that *S. alaxensis* can be a useful plant for rhizoremediation of diesel-contaminated soil.

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Chapter 1 Introduction

1.1 Petroleum contamination

Petroleum is the primary source of fuel in urban cities and in rural Alaska. Diesel fuel is mainly produced from petroleum, but includes a variety of additives. Diesel fuel predominantly contains a mixture of hydrocarbons that typically include between 8 and 28 carbon atoms per molecule. These hydrocarbons are mainly composed of alkanes (40 to 70 mass %), cycloalkanes (10 to 25 mass %), alkenes (up to 5%) and aromatics (10 to 30%) (ATSDR, 1995; Trapp *et al.*, 2001). Petroleum hydrocarbons are of interest because many of them are ubiquitous contaminants and chemical carcinogens.

Diesel fuels are used in internal combustion engines and are different from fuel oils, which are used in lamps and heaters. Fuel oil is classified into six classes, numbered 1 through 6, according to its boiling point, composition and purpose. Diesel fuel #2 is similar in chemical composition to fuel oil #2, except diesel fuel #2 is amended with chemical additives (ATSDR, 1995).

The burning of fuel oil is very common in Alaska as a heating source. In 2007, the residential and industrial consumption of fuel oil #2 in Alaska averaged about 30 million gallons (DOE, 2007). Major causes of diesel spills include leaking storage tanks, land disposal of petroleum waste and accidental or intentional spills (Bossert and Bartha, 1984). Regulatory agencies require that contaminated sites be remediated and impose fines upon responsible parties for noncompliance.

Water soluble aromatics such as benzene, toluene, ethylbenzene and the xylene isomers (BTEX), are contaminants of the greatest concern as they are toxic and/or confirmed carcinogens (Dean, 1985). Polycyclic aromatic hydrocarbons (PAHs) are widespread soil contaminants usually found as mixtures of low molecular weight (two to

three fused aromatic rings) and high molecular weight (four or more rings) compounds. PAHs are compounds of intense public concern owing to their persistence in the environment and toxic effects on human health (Kanaly and Harayama, 2000).

Acute PAH toxicity is rarely reported in humans, fish, or wildlife (ATSDR, 1995). Instead, PAHs in general are more frequently associated with chronic effects, such as changes in the liver and harmful effects on the kidneys, heart, lungs, and the nervous system (ATSDR, 1995). Studies have also shown that mammals oxidize PAHs to reactive electrophilic intermediates (Thakker, 1985). Increased occurrence of cancer and other immunological, reproductive, fetotoxic, and genotoxic effects have also been associated with some of the compounds found in fuel oils, and are often considered the result of exposure to complex mixtures of chronic-risk aromatics, rather than low levels of a single compound (ATSDR, 1995). As of 1999, polycyclic aromatic hydrocarbons were present at nearly 50 percent of the 1,430 National Priority List (NPL) sites in the United States (McCutcheon and Schnoor, 2003).

In general, low molecular weight PAHs biodegrade prior to high molecular weight PAHs, which are more resistant to microbial degradation (Shiaris, 1989; Wilson and Jones, 1993). Four and five-ring PAHs are more recalcitrant than lower molecular weight alkanes and aromatics because of the resonance energies of their structures and their low water solubilities (Klevens, 1950; Heitkamp *et al.*, 1988). The chemical structures of high molecular weight PAHs also cause low bioavailability due to their partitioning into clay and organic matter (Yi and Crowley, 2007). This is a current obstacle in bioremediation since the higher molecular weight recalcitrant PAHs are also more carcinogenic.

1.2 PAH biodegradation

PAH contaminated soil is primarily remediated with soil bacteria (Schnoor *et al.*, 1995; Yi and Crowley, 2007; Alexander, 1994; Burken and Schnoor, 1996; Nichols *et al.*,

1997; Siciliano *et al.*, 2003; Shabad and Cohan, 1972). Aerobic biodegradation of petroleum hydrocarbons has been well studied in both laboratory and field settings (Leahy and Colwell, 1990; Prince, 1993). Published mechanisms for PAH biodegradation are mainly focused on low molecular weight PAHs (Kanaly and Harayama, 2000; Pothuluri and Cerniglia, 1994; Sutherland *et al.*, 1995). Typically, the aerobic biodegradation of aromatics starts with a monooxygenase or dioxygenase enzyme adding two adjacent -OH groups from O_2 to form a diol (Martinko *et al.*, 2005; Gibson *et al.*, 1975; Cerniglia and Heitkamp, 1989). A ring cleavage dioxygenase enzyme then adds oxygen once again to cleave the ring in the ortho or meta position (Barnsley, 1976). In order to break down the aromatic ring into more common cellular metabolites, the ring cleavage product is further hydroxylated into two common intermediates, catechol and protocatechuate (Harayama *et al.*, 1987). These intermediates funnel a wide variety of PAHs through the β-ketodipate pathway, which produces acetyl-CoA and succinyl-CoA as end products (Harwood and Parales, 1996). Acetyl-CoA is then used in the Krebs Cycle to provide energy for the microorganisms degrading the PAH compound (Martinko *et al.*, 2005). Other metabolites are also formed such as pyruvate, acetylaldehyde and fumarate that are used by microorganisms for protein synthesis (Sims and Overcash, 1983; Yen and Serdar, 1988).

Far less research has been conducted on the biodegradation of high molecular weight PAHs by aerobic bacteria, but research has shown that it may follow the same mechanisms as lower molecular weight PAH aerobic degradation. The microbial biodegradation of high molecular weight compounds is complicated by the bioavailability of these large molecules and their capability of supporting microbial growth. The biodegradation of pyrene, chrysene, benzo(a)pyrene, and other high molecular weight PAHs have been demonstrated in a few microbial species (Chen and Aitken, 1999; Heitkamp *et al.*, 1988; Mahaffey *et al.*, 1988).

Several petroleum degrading bacterial species have been identified that exist naturally in the environment. Soil bacteria in the genera *Pseudomonas, Mycobacterium, Flavobacterium, Rhodococus, Burkholderia*, *Arthrobacter* and *Sphingomonas* are the most studied. Wackett and Hershberger (2001), identified species of hydrocarbon degraders and separated them into microbial-enzyme families based on signature sequences. *Pseudomonas fluorescence, Streptomyces purpurascens, Flavobacterium species, Pseudomonas putida* and *Trichosporon cutaneum* all contain the flavoprotein monooxygenase enzyme that performs aromatic-ring hydroxylation (Wackett and Hershberger, 2001). More specifically, *P. putida* has been shown to possess the salicylate hydroxylase enzyme, an essential enzyme for the mineralization of naphthalene (Yen and Serdar, 1988; Wackett and Hershberger, 2001).

1.3 Genetics and gene regulation

Of the organisms that have been shown to degrade hydrocarbons, pseudomonads are the most studied. Research has shown that petroleum degradative genes are located on plasmids (Yen and Serdar, 1988). Enzymes encoded on these plasmids are known to break down complex hydrocarbon molecules. This allows their host to utilize a given hydrocarbon or one of its close derivatives as an energy and carbon source. Most pseudomonads contain the naphthalene catabolic plasmid identified as NAH7, which is the first recognized and most studied degradative plasmid (Yen and Serdar, 1988).

The NAH7 plasmid encodes for two pathways: an upper pathway that signals naphthalene degradation and the lower pathway for salicylate degradation (Yen and Serdar, 1988). The genes located on the NAH7 plasmid encode for the enzymes that perform the first 11 steps of naphthalene oxidation (Yen and Serdar, 1988; Schell, 1985). The upper pathway is found on the first operon and includes the genes *nah*ABCDEF. The upper pathway encodes for the degradation of naphthalene to salicylate. The lower pathway is located on the second operon and includes the genes *nah*GHIJK. This pathway encodes for the oxidation of salicylate via the catechol meta-cleavage pathway

to acetylaldehyde and pyruvate (Yen and Serdar, 1988; Schell, 1985). The overall degradation performed by the expression of the NAH7 plasmid results in the cleavage of one of the two benzene rings that make up naphthalene to ultimately generate pyruvate as an end product.

Activation of the *nah* operons from NAH7 requires an inducer (Yen and Serdar, 1988). All of the naphthalene oxidation enzymes encoded by the two *nah* operons of NAH7 can be induced by salicylate (Yen and Gunsalus, 1982; Barnsley, 1975; Schell, 1985). Salicylate induces naphthalene degradation by increasing the synthesis of mRNA from the two *nah* operons (Schell, 1985). Measurements of *nah* mRNA levels showed that induction by salicylate results in an approximately 30-fold increase in the levels of RNA encoding the structural genes of the upper and lower pathways (Schell, 1985). Induction of naphthalene dioxygenase enzymes by salicylate therefore acts at the transcriptional level (Yen and Serdar, 1988; Schell, 1985).

Naphthalene itself is not an inducer of the NAH7-encoded naphthalene oxidation pathway even though both *nah* operons are induced in its presence. Research has shown that induction occurs when the upper pathway (*nah*ABCDEF) is expressed in the absence of expression of the lower pathway. Yen and Serdar (1988) completely blocked induction with naphthalene by placing a Tn5 insertion in the *nah*A, *nah*B, *nah*C, or *nah*D gene in the first operon, and the same insertion in the *nah*G or *nah*I gene located in the second operon produced no effect. These results indicate that expression of the first *nah* operon produces the inducer, salicylate. The same conclusion was also reached for the naphthalene catabolic pathway specified by *Pseudomonas* species ATCC 17483 (Connors and Barnsley, 1980). Overall, it is believed that the first and second operons of the NAH7 plasmid are both induced by the first operons' end product, salicylate (Schell, 1985; Peters and Verma, 1990).

1.4 Cometabolism

Many aerobic PAH-degrading bacteria have been shown to degrade a range of PAHs, and when grown on one PAH, these organisms are typically able to degrade others, including high molecular weight PAHs (Chen and Aitken, 1999; Sutherland *et al.*, 1995). In some cases, the degradation of high molecular weight PAHs has been attributed to cometabolism by cells growing on low molecular weight PAHs and phenolics (Heitkamp *et al.*, 1988; Chen and Aitken, 1999; Leigh *et al.*, 2002; Anderson *et al.*, 1993). For example, the presence of a low molecular weight PAH such as naphthalene has been shown to induce the oxygenase responsible for the initial attack on the high molecular weight PAH molecules (Chen and Aitken, 1999). White *et al.* (2005) also found greater degradation of larger three-ring alkylated phenanthrenes, anthracenes and dibenzothiopenes in vegetated fertilized plots compared to non-vegetated non-fertilized plots, while the alkylated two-ring naphthalenes were degraded equally in all treatments. In this field experiment, the numbers of total bacterial, fungal and PAH degraders increased as the rhizosphere volume increased and most likely resulted in the increase of recalcitrant PAH biodegradation. These studies suggest the importance of low molecular weight aromatic compounds in stimulating indigenous microorganisms to degrade higher molecular weight PAHs.

1.5 Phytoremediation and rhizoremediation

Phytoremediation has great potential to treat petroleum hydrocarbon contaminated soil. Phytoremediation is the use of plants to remove, degrade, or contain chemical contaminants located in the soil, sediments, groundwater, surface water and even the atmosphere (McCutcheon and Schnoor, 2003; Cofield *et al.*, 2007; Cunningham *et al.*, 1996). Different types of phytoremediation systems exist that specialize in specific mechanisms to remediate a variety of chemical contaminants. The rhizosphere is described as the zone of soil affected by roots; therefore rhizoremediation is a type of phytoremediation technology that relies upon rhizospheric microorganisms to degrade

contaminants in the surrounding soil and/or groundwater. For hydrophobic contaminants such as diesel range organics, the main phytoremediation process contributing to degradation in contaminated soil is rhizoremediation (Schnoor *et al.*, 1995; Yi and Crowley, 2007; Alexander, 1994; Burken and Schnoor, 1996; Nichols *et al.*, 1997; Siciliano *et al.*, 2003). A major advantage of phytoremediation over other conventional treatment strategies is its low cost. Phytoremediation has been estimated to cost approximately $162 per cubic meter of petroleum-contaminated soil compared to approximately $810 per cubic meter for excavation and incineration (Rock and Sayer, 1998).

The uptake of PAHs by plants used in phytoremediation is an important issue to consider, since it can become a possible channel for contaminants to enter the food chain. Most PAHs are not water soluble and it is accepted that they are not taken up by plants in substantial quantities (Kolb and Harms, 2000; Cofield *et al.*, 2007; Reilley *et al.*, 1996). The uptake of large hydrophobic compounds into plant tissue is dependent upon the pollutant's water solubility and octanol-water partitioning coefficient, K_{ow} (Olson *et al.*, 2003). Most DROs (C_8 to C_{28}) have a K_{ow} greater than 3.0 and are unlikely to be taken up by plants (Schnoor *et al.*, 1995). Naphthalene, a medium molecular weight PAH (128 $g \cdot mole^{-1}$) with a water solubility of 30 $mg \cdot L^{-1}$ and a log K_{ow} of 3.37 (Lide, 1991) is unlikely to be significantly translocated within a plant. Other research has shown that benzene (log $K_{ow} = 2.13$), a monoaromatic, can sorb onto roots in small quantities (5-9 $mg \cdot L^{-1}$), while translocation and biodegradation still remain the mechanisms responsible for degradation (Corseuil and Moreno, 2001). This indicates that small aromatics are capable of plant translocation, but not in considerable quantities.

Experimental data on the uptake of alkanes into higher plants is not available, but simulations with a plant uptake model (Trapp *et al.*, 2001) indicate the potential uptake of long-chain (heavy) alkanes into fine roots and a slow translocation into stems and leaves. The slow translocation of heavy alkanes is probably due to their extremely low water

solubility and high sorption to roots (Grosser *et al.*, 2000). Short-chain (light) alkanes are more mobile than long-chain alkanes and have a higher vapor pressure (Trapp *et al.*, 2001). Similar to other research, the model predicts that light alkanes may be taken up into tree tissue but they are either quickly metabolized or volatilized. Monoaromatics such as benzene behave similarly to light alkanes and PAHs behave more like heavy alkanes (Trapp *et al.*, 2001).

1.6 The rhizosphere

The term 'rhizodeposition' describes the loss of carbon from the roots into the rhizosphere and includes the exudation of soluble plant products plus turnover from root die back. As roots grow and die-back due to fluctuating water tables, soil moisture, and fall turnover; significant amounts of organic carbon, nutrients, and secondary metabolites are released into the surrounding soil (Yi and Crowley, 2007; Kapulnik, 1996; Leigh *et al.*, 2002). Organic substances released by roots vary along the length of the root and vary in concentration depending on the nature of the compound (e.g. water soluble or volatile), soil moisture, and the rate of metabolism by microorganisms (Bowen and Rovira, 1991). The quantities and characteristics of root-released organic compounds are also dependent upon the plant species, the plant's age (Hedge and Fletcher, 1996), the season (Leigh *et al.*, 2002; Hedge and Fletcher, 1996), distance from the root tip (Jaeger *et al.*, 1999; Paul and Clark, 1989) and health of the plant (Gaffney *et al.*, 1993). Roots generally consist of sugars (15-65% total organic carbon, TOC), organic acids (9-33% TOC), amino acids (2-31%) and phenolics (0.3-4 mg of carbon per gram of root material) (Dutta and Appelqvist, 1991; Fauconnier *et al.*, 2003; Lynch and Whipps, 1990).

Quantities of organic compounds released into the rhizosphere vary among plants (Lynch and Whipps, 1990), but are generally estimated to be between 10 and 100 mg of carbon per gram of root material (Qui *et al.*, 2002). Overall, between 25-90% of the total carbon photosynthesized by plants is released from roots into the surrounding soil (Martin, 1976; Barber and Martin, 1976; Warembourg and Billes, 1979; Whipps and

Lynch, 1985). Organic substrates released into the rhizosphere are the products of photosynthesis and are thus the result of primary production (Whipps and Lynch, 1985). Annual plants lose approximately 40 to 90 percent of the net fixed carbon to rhizodeposition (McCutcheon and Schnoor, 2003). Perennial plants however, have a cyclic rhizodeposition as their roots grow and die back over each growing season, allowing them to release less fixed carbon than annual plants, roughly 25 to 80 percent (McCutcheon and Schnoor, 2003; Lynch and Whipps, 1990; Shimp *et al.*, 1993). The rhizosphere microbial community metabolizes influxes of carbon and other compounds released by roots into the surrounding soil.

Many different types of plant-derived organic compounds are released into the terrestrial environment through rhizodeposition (Kuzyakov and Domanski, 2000; Lynch and Whipps, 1990). Four general classes of organic compounds have been identified as rhizodeposits: exudates, secretions, mucilages, and root lysates (Rovira *et al.*, 1976; Whipps and Lynch, 1985). Exudates are low-molecular-weight compounds, including water soluble and volatile compounds such as sugars, amino acids, organic acids, hormones and vitamins which leak passively from root cells; requiring no metabolic energy (Whipps and Lynch, 1985; Rovira, 1956; Rovira, 1969; Rovira, 1973; Pearson and Parkinson, 1961). Secretions are compounds of low or high molecular weight, such as polymeric carbohydrates and enzymes that require metabolic processes for their release (Bowen and Rovira, 1991; Olson *et al.*, 2003; Whipps and Lynch, 1985). Plant mucilages are gelatinous materials secreted by root cap cells, epidermal cells, and include sloughed off cells. Root lysates are compounds released into the rhizosphere by the lysis of root cells including cell walls and with time the whole root (Bowen and Rovira, 1991; Olson *et al.*, 2003; Whipps and Lynch, 1985). Root lysates may include many different structures and chemical compounds and are dependent upon the type of plant. As the root cells break open, compounds from vacuoles (organelles that store secondary plant metabolites), cytoplasmic content, cell membranes, and cell wall material may be introduced into the rhizosphere.

The abiotic and biotic conditions of the rhizosphere have been shown to differ greatly from the bulk soil (Bowen and Rovira, 1999; Curl and Truelove, 1986; Klein, 2000). Compounds such as amino acids, carboxylic acids, carbohydrates, nucleic acid derivatives, growth factors, enzymes, and other related primary and secondary plant metabolites are excreted from the roots and influence a diverse microbial community (Alexander, 1977; McCutcheon and Schnoor, 2003; Schnoor *et al.*, 1995; Siciliano and Germida, 1998; Yi and Crowley, 2007; Whipps and Lynch, 1985). The influx of organic compounds into the rhizosphere significantly contributes to increasing the microbial abundance in the rhizosphere (Barber and Lynch, 1977; Bowen and Rovira, 1999; Bowen and Rovira, 1976; Curl and Truelove, 1986; Lynch and Whipps, 1990; Kamath *et al.*, 2004; Whipps and Lynch, 1985). The microbial density in the rhizosphere soil may be at least 10 to 100 times higher than the bulk soil (Siciliano and Germida, 1998; Bowen and Rovira, 1999; Foster and Rovira, 1978; Paul and Clark, 1989; Reilley *et al.*, 1996; Miya and Firestone, 2000; Miya and Firestone, 2001; Nichols *et al.*, 1997). This increased microbial biomass due to root released compounds has been described by researchers as a "general rhizosphere effect". The major driving force of the rhizosphere effect appears to be the continuous input of plant-derived substrates deposited as exudates and lysates from root turnover (Leigh *et al.*, 2002). A diverse microbial community is beneficial to plants because rhizosphere microorganisms have been shown to promote plant growth, enhance mineral and water uptake, produce antibiotics to inhibit soil pathogens, and promote plant regulators (Kapulnik, 1996).

It is also suggested that the presence of rhizospheric microorganisms increases root exudation (Curl and Trulove, 1986; Barber and Martin, 1976), although the mechanisms are not entirely clear. This effect could be due to the release of metabolites from rhizospheric microorganisms that affect root physiology and lead to increased exudation. Exudates are primarily synthesized in the shoot system during the process of photosynthesis and then translocated to the root system (Curl and Truelove, 1986). Some microorganisms are believed to release chemicals that influence plant growth and

therefore the exudation of photosynthesized exudates; this could have an effect on the quality and quantity of materials released by the roots.

1.7 Rhizodegradation of petroleum

The presence of roots is thought to offer an advantageous environment for the growth of microbial communities and thus facilitate an increase in biodegradation. Vegetation enhances the bioremediation of PAHs by increasing the microbial biomass through facilitating the movement of gases, nutrients and water throughout the rhizosphere (Fick *et al.*, 1999). Plants have been shown to enhance the biodegradation of many diesel range organics (Aprill and Sims, 1990; Reilley *et al.*, 1996; Banks *et al.*, 1999; Binet *et al.*, 2000; Liste and Alexander, 1999; Miya and Firestone, 2000; Miya and Firestone, 2001; Kanaly and Harayama, 2000; Kanaly *et al.*, 1997). Increased biodegradation of diesel range organics is most likely the result of microbial degradation due to an increase in microbial numbers and is not related to plant uptake and metabolism (Schnoor *et al.*, 1995; Yi and Crowley, 2007; Alexander, 1994; Burken and Schnoor, 1996; Nichols *et al.*, 1997; Siciliano *et al.*, 2003). Bacterial numbers in DRO-contaminated soil have been shown to be 70-200 times greater in the presence of plants than in their absence (Schwab and Banks, 1994). It is understood that rhizospheric bacteria are located within 5 mm of the surface of the root and their numbers decrease with increasing distance from the root (Paul and Clark, 1989). Reynolds *et al.* (1998) has shown that TPH degrading bacteria in field treatments containing plants and nutrients resulted in a significant 287-fold increase of degrading microorganisms over the non-planted control. The significantly lower TPH concentrations in the planted treatments amended with nutrients suggests the importance of plant-microbe interactions in successful bioremediation strategies.

1.8 The role of plant secondary compounds in rhizoremediation of aromatics

Secondary plant metabolites are an important class of root released compounds because they have similar structures to anthropogenic pollutants. Secondary plant metabolites such as phenolic compounds have also been shown to promote the growth of

certain soil bacteria that degrade organic compounds such as PAHs (Anderson *et al.*, 1993; Jordahl *et al.*, 1997; Nichols *et al.*, 1997). It is suggested that these metabolites stimulate rhizospheric microorganisms to metabolize aromatic pollutants.

A variety of aromatic-phenolic compounds, such as salicylic acid are found naturally in the rhizosphere. Certain microbes have evolved specific catabolic pathways enabling them to use these compounds as carbon or energy sources (Peters and Verma, 1990). For example, the beta-ketoadipate and the naphthalene pathway both reside on transferable plasmids. The beta-ketoadipate pathway degrades monocyclic compounds with genes that reside on the TOL plasmid and the naphthalene pathway degrades dicyclic compounds with genes that reside on the NAH7 plasmid. These plasmids have similar gene structures and organization suggesting that they have evolved from a common ancestor (Harayama *et al.*, 1987).

Plant phenolics are important transcriptional signals to soil bacteria (Peters and Verma, 1990). Studies have indicated that plant phenolics induce catabolic genes in rhizospheric microorganisms as well as genes that regulate plant-microbe interactions (McCutcheon and Schnoor, 2003; Yen and Serdar, 1988; Schell, 1990; Mahaffey *et al.*, 1988; Chen and Aitken, 1999; Barnsley, 1975; Kamath *et al.*, 2004). Rentz *et al.* (2004) stated that *Pseudomonas putida* utilized root exudates as a carbon and energy source, which is consistent with other studies that report root exudates provide nutrients that enhance the abundance of rhizospheric microorganisms. In some cases, genetic stimulation is necessary for microbes with the capability to degrade organic pollutants to degrade that pollutant over other more easily degradable carbon sources.

1.8.1 Salicylate induces PAH degradation

The degradation of PAHs by aerobic bacteria has received some attention due to the success in inducing genes responsible for encoding catabolic enzymes. All genes involved in the early steps of PAH degradation are inducible (McCutcheon and Schnoor,

2003; Yen and Serdar, 1988; Schell, 1990; Yi and Crowley, 2007). As mentioned before, salicylate, an intermediate in the naphthalene dioxygenase pathway, has been shown to induce the *nah* genes, which are responsible for encoding the catabolic enzymes that perform naphthalene degradation (Yen and Serdar, 1988; Schell, 1990). In addition to naphthalene degradation, salicylate also induces the degradation of benz(a)anthracene by *Sphingomonas yanoikuyae* (McCutcheon and Schnoor, 2003) and as well as the degradation of other high molecular weight PAHs by *Pseudomonas saccharophila* P15 (Chen and Aitken, 1999). Salicylate is not only an intermediate in the naphthalene dioxygenase pathway, but is also a root lysate released by willow and poplar trees. Plants that release this inducer have shown to stimulate petroleum-degrading microorganisms and the degradation of PAHs. Yi and Crowley (2007) tested the effects of different plant-released rhizospheric compounds and found that of the 21 sugars, carboxylic acids, amino acids and aromatic compounds tested, only salicylic acid, 4-methylsalicylic acid, and acetylsalicyclic acid induced the *nah* operon in HK44 cells. In this way, particular plant species could select for and maintain the growth of microorganisms capable of degrading recalcitrant organic pollutants in the rhizosphere.

1.8.2 Salicylate contributes to cometabolism

A variety of environmental conditions and plant released compounds may assist in the biodegradation of recalcitrant compounds in the rhizosphere. Successful stimulation of high-molecular-weight PAH degradation has also been noted after the addition of salicylate (Chen and Aitken, 1999). *Pseudomonas saccharophila* P15 uses the salicylate pathway for phenanthrene metabolism (Chen and Aitken, 1999) and after growing on phenanthrene, the species can cometabolize a wide range of PAH substrates that cannot be used for growth (Aitken *et al.*, 1998). This strain expressed both the catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities after being exposed to salicylate, suggesting that salicylate induced the entire phenanthrene degradation pathway (Chen and Aitken, 1999). That research suggests that a link between the degradation pathways of low and high-molecular weight PAHs may exist, especially in synthesis of similar

degradative enzymes and activity. The naphthalene degradative enzyme, naphthalene dioxygenase, has also been shown to be induced in specific *Pseudomonas* species grown on salicylate (Barnsley, 1975).

A variety of degradative enzymes have been shown to be responsible for the breakdown of naphthalene to non-aromatic products (Davies and Evans, 1964) and are expressed by *Pseudomonas* NCIB9816 and *Pseudomonas putida* (strains PPG7, ATCC 17484, PpG7, NCIB 9816 and ATCCI7483) when grown on salicylate (Barnsley, 1975; Barnsley, 1976). The presence of salicylate has been shown to induce naphthalene oxygenase, 1-2-dihydroxynaphthalene oxygenase, salicylaldehyde dehydrogenase, and salicylate hydroxylase (Barnsley, 1975). Rentz *et al.* (2004) observed that salicylate-induced cultures of *Sphingomonas yanoikuyae* increased the degradation and mineralization of benzo[a]pyrene over cultures exposed to other root products.

1.9 Studies of petroleum rhizoremediation

Research has shown that vegetated soils not only foster a healthy and abundant microbial community but also influence the rhizosphere community to degrade petroleum products. In experiments conducted by Reilley *et al.* (1996), pyrene and anthracene concentrations were significantly lower in PAH contaminated soil planted with fescue, alfalfa, sungrass and switchgrass when compared to unplanted soil after 24 weeks. Significant pyrene degradation has also been demonstrated in vegetated petroleum contaminated soil in only eight weeks compared to unvegetated soil (Liste and Alexander, 1999). Higher removal of TPH was also demonstrated in the rhizosphere soil of grasses, legumes (Banks *et al.*, 2003a) and sorghum (*Sorghum bicolor L.*) (Banks *et al.*, 2003b) when compared to unvegetated soil. Other research has demonstrated that the rhizosphere soil significantly removed 90% of phenanthrene and 61% of pyrene when compared to the non-planted treatments (Ling and Gao, 2004). Parrish *et al.* (2004) has shown that the presence of tall fescue, annual rye grass and yellow sweet clover were all successful at removing PAHs in composted soil. Furthermore, Kim *et al.* (2004) and

Reilley *et al.*(1996) have both stated that tall fescue and switchgrass removed 30-40% more pyrene and anthracene than non-planted treatments. Dominguez-Rosado and Pichtel (2004) measured the degradation of used motor oil (1.5% w/w) and discovered that after 150 days, oil was no longer detected in the clover treatment and 67% of the oil was removed in the sunflower/mustard treatment, but after fertilizer was added, the oil was completely removed. A hybrid willow species (*Salix alba* x *matsuda*) has also shown significant naphthalene degradation (Yi and Crowley, 2007).

Research conducted by Siciliano *et al.* (2003) suggested that the enhancement of catabolic activity may be the result of an increase in microbial activity due to the release of plant lysates and root exudates. Siciliano *et al.* found that tall fescue (*Festuca arundinacea*) increased the prevalence of naphthalene mineralization in the rhizosphere soil compared to that in bulk soil. Ho *et al.* (2007) conducted research to determine if tall fescue promotes PAH degradation using a salicylate-similar compound to induce degradative gene expression. It is suggested that the treatments containing the root compounds stimulated the rhizosphere degradation of naphthalene over the control without roots. Miya and Firestone (2000) observed enhanced phenanthrene biodegradation in the presence of oat root exudates and root debris, and found that these plant organics facilitated larger microbial degrading populations, especially after much of the phenanthrene had been utilized. This research suggests that these root products could have offset low bioavailability and enhanced phenanthrene removal from the soil. Kamath *et al.* (2004), simulated root turnover by evaluating the potential of root derived extracts on the induction and repression genes that encode naphthalene biodegradation. This study showed that naphthalene degradation was faster in reactors amended with naphthalene plus root extracts than in controls amended with naphthalene alone, especially for reactors with root extracts from mulberry, osage orange, switch grass and willow (Kamath *et al.*, 2004). Furthermore, a higher abundance of a bioluminescence marker of PAH catabolic enzymes was observed in reactors with root extracts and naphthalene that suggests the potential for enhanced cooxidation of other PAHs in the

rhizosphere. Due to the structural similarity of naturally occurring plant compounds and anthropogenic pollutants, it is reasonable to assume that the former can induce the appropriate microbial degradative pathways (Leigh *et al.*, 2002). It is also important to note that other microbial and fungal taxa may also influence PAH degradation through different pathways other than naphthalene dioxygenase.

Unfortunately, not all rhizoremediation studies have been successful, and it is sometimes challenging to determine the cause. Unfavorable soil conditions such as oxygen, temperature, and moisture could affect the viability of the rhizosphere community. Challenges associated with the bioavailability of contaminants and the nutrient variability in selected soils are also factors that could negatively impact the success of rhizoremediation (Graham *et al.*, 1995; Bossert and Bartha, 1984; Cooney, 1984).

In some cases, the competition for nutrients has shown to decrease biodegradation rates. Among the most common components of root turnover, sugars such as glucose and organic acids such as acetate and succinate have been shown to inhibit the degradation of aromatic compounds such as benzene (Kosaric, 2001), catechol (Kosaric, 2001), and toluene (Uyttebroek *et al.*, 2006). When carbon is abundant in the environment, *Pseudomonas* species growing in the presence of toluene and succinate have shown to repress the *xyl*S gene (Kosaric, 2001). On the other hand, when grown in succinate-limiting conditions, the same genes in *Pseudomonas* species were easily inducible. Other research conducted on an *Acinetobacter* sp. strain closely related to some species of *Pseudomonas,* found that the biodegradation of the aromatic p-hydroxybenzoic (a phenolic derivative of benzoic acid) was deferred until acetate and succinate had been consumed (Kosaric, 2001). It is possible that primary plant metabolites would be preferentially degraded over the contaminant resulting in decreased biodegradation.

The ability of plants to stimulate PAH degradation is highly variable depending upon the plant species. The presence of easily degradable plant components of root turnover has shown to inhibit specific degradative genes (*nah*G) and may overshadow any positive effects of potential inducers that may be present at lower concentrations (Yi and Crowley, 2007). However, the presence of a large and diverse rhizospheric microbial community could aid in the reduction of these inhibitory carbon substrates and thus allow the exposure of plant components that induce PAH degradation.

1.10 Evaluating the rhizoremediation potential of *Salix alaxensis*

The genus *Salix* is a large, taxonomically complex genus, with about 350 to 500 different species worldwide (Argus, 1999). Of the 40 willow species known to occur in Alaska, 29 species are found growing in the interior of Alaska (Collet, 2004). The majority of willow trees exist as shrubs and can grow up to 20 meters in height (Verwijst, 2001; Collet, 2004). Willow trees are known for their rapid growth, flood tolerance and ease of vegetative propagation. In a recent experiment by Spriggs *et al.* (2005), the biomass of willow roots exceeded the biomass of green ash and hybrid poplar. This extensive rooting in willow trees has been shown to correlate with higher microbial populations and lower PAH concentrations (Spriggs *et al.*, 2005). In places where contaminated soil or waste is concentrated, willows can be used to contain the contaminants at the site by means of increased evaportranspiration, acting as a solar pumping system (Verwijst, 2001).

Plants synthesize a wide variety of phenolic compounds in both root and shoot tissues during normal growth and development (Peters and Verma, 1990). Willow tissues characteristically contain a diverse assortment of phenolic compounds such as salicylates, cinnamic acid derivatives, flavonoids, and condensed tannins (Yi and Crowley, 2007). However, the occurrence of salicin is a dominant characteristic of willows (*Salix* sp.) (Collet, 2004; Egloff, 1982). The phenylpropanoid biosynthetic pathway produces phenolic compounds that produce plant pigments (Ebel and Hahlbrock, 1982), provide

structure in cell walls (Hahlbrock and Griseback, 1979), protect against ultraviolet light, and provide a defense against pathogens (Dixon *et al.*, 1983).

The leaves, bark and roots of most willow species contain salicin (Julkunen-Titto, 1989), a phenolic glycoside that acts as a defense against herbivory (Collet, 2004; Tahvanainen *et al.*, 1985). Plant phenolics are released in response to wounding (Lawton and Lamb, 1987), as their bitter taste is said to deter predators. Willow trees also release phenolic compounds into the rhizosphere as cells lyse during fall root turnover. The quantity of phenolics released by plants has been shown to increase with age (Hedge and Fletcher, 1996) and environmental stresses associated with water, light, oxygen, temperature and nutrient availability (Kraffczyk *et al.*, 1984; Dakora and Phillips, 2002; Gaffney *et al.*, 1993). When salicin is consumed by the rhizosphere community it is hydrolyzed and oxidized, producing salicylic acid (Lambers *et al.*, 2008).

1.11 Willows in rhizoremediation

Applications of willow systems focusing on phytoremediation are becoming more and more popular (Aronsson and Perttu, 2001). The release of salicylic acid via root turnover and the resulting induction of degradative genes makes rhizoremediation with *Salix* species an attractive potential phytoremediation solution to petroleum contamination.

A laboratory based experiment investigated the advantage of using willow tress (*Salix babylonica*) to phytoremediate groundwater contaminated with gasoline-ethanol mixtures. Results showed that the willow cuttings were able to reduce ethanol and benzene concentrations by more than 90% in less than a week (Corseuil and Moreno, 2001). The degradation of polyaromatic hydrocarbons (PAH) in an aged creosote-contaminated soil in the presence of *Salix viminalis* was investigated in a greenhouse experiment. Phenanthrene and pyrene were degraded 100% and 80%, respectively, in the presence of plants but only 68% and 63% without plants. Viable counts and active

biomass were highly correlated in all treatments and demonstrated that *S. viminalis* greatly increased petroleum degrading microbial populations (Zalesny *et al.*, 2005). Spriggs *et al*. 2005, demonstrated in greenhouse conditions, that willows enhanced the degradation of PAHs in soil. Again, higher microbial populations were measured in the willow treatment and were shown to correlate with extensive rooting. This relationship was significant because it provides supporting evidence that the presence of deep-rooting vegetation (i.e. willow trees) can stimulate PAH-degrading microbial populations and thus contribute to the degradation of PAHs. A field trial was designed by Vervaeke *et al*. (2003) to assess the growth of willow on the dissipation of mineral oil and PAHs in dredged sediment. After 1.5 years, a significant decrease of 57% in the mineral oil concentration in the planted treatment was observed.

1.12 Thesis objectives and hypothesis

The goal of this study was to evaluate the potential of *Salix alaxensis* to rhizoremediate diesel contaminated soil. Specifically, the project objectives included the evaluation of the effects of salicylic acid and simulated root turnover (crushed willow roots) on the abundance of diesel degrading microorganisms; as wells as the growth of willow (with and without fertilizer) on the abundance of diesel degrading microorganisms and the disappearance of DROs from soil. It was hypothesized that *Salix alaxensis* will promote rhizoremediation of diesel contaminated soil.

Chapter 2
Rhizoremediation of Diesel Contaminated Soil using *Salix alaxensis*[1]

Abstract

An outdoor pot study and a microcosm study were conducted to evaluate the potential for *Salix alaxensis* (felt leaf willow) to rhizoremediate diesel-contaminated soil. The pot study was conducted for 96 days during an Alaskan interior summer with *S. alaxensis* grown in soil contaminated with diesel fuel oil #2. The concentration of diesel range organics (DRO) and the most probable number (MPN) of diesel degrading microorganisms in the rhizosphere were measured initially and compared to final values. A microcosm study was also performed with crushed willow roots to simulate root turnover, in which the abundance of diesel degrading microorganisms was also determined. It was hypothesized that treatments containing willow and fertilizer would foster the greatest abundance of diesel degrading microorganisms and thus would provide the largest decrease of diesel range organics. In the pot study, growth of *S. alaxensis* resulted in the largest decrease of DROs, although treatments amended with fertilizer contributed to a significant increase in MPN of diesel degrading microorganisms. The microcosm study indicated that the addition of crushed willow roots to contaminated soil produced a similar abundance of diesel degrading microorganisms as the addition of salicylic acid. The findings suggest that *S. alaxensis* can be a useful plant for rhizoremediation of diesel-contaminated soil.

[1]McFarlin, K.M., Schiewer, S., and Leigh, M.B. 2009. Rhizoremediation of Diesel Contaminated Soil Using *Salix alaxensis*. Prepared for submission in the International Journal of Phytoremediation.

2.1 Introduction

Diesel spills are a widespread problem due to leaking storage tanks, land disposal of petroleum waste and accidental or intentional spills (Bossert and Bartha, 1984). The use of fuel oil is very common in Alaska as a main heating source. In 2007, the residential and industrial consumption of fuel oil #2 in Alaska averaged approximately 30,000,000 gallons each. (DOE, 2007). Diesel fuels are used in internal combustion engines and are different from fuel oils used in lamps and heaters. Diesel fuel #2 is similar in chemical composition to diesel fuel oil #2, with the exception of additives (ATSDR, 1995). Diesel fuel oil predominantly contains a mixture of carbon and hydrocarbons that typically include between 8 and 28 carbon atoms per molecule (ADEC, 2002; Trapp *et al.*, 2001).

Phytoremediation has the potential to treat petroleum hydrocarbon contaminated soil in areas accessible by roots at lower costs than conventional remediation strategies. Phytoremediation has been estimated to cost approximately $162 per m^3 of petroleum-contaminated soil compared to approximately $810 per m^3 for excavation and incineration (Rock and Sayre, 1998). For hydrophobic petroleum components such as diesel range organics (DRO), the main phytoremediation technology contributing to degradation of contaminated soil is rhizoremediation (Yi and Crowley, 2007; Alexander, 1994; Burken and Schnoor, 1996; Siciliano *et al.*, 2003).

In the rhizosphere, the presence of roots is known to increase microbial biomass and alter microbial community structure relative to bulk soil. As roots grow and die back due to fluctuating water tables, soil moisture and seasonal fine root turnover; organic carbon, nutrients, and secondary metabolites are released into the surrounding soil (Yi and Crowley, 2007; Kapulnik, 1996; Leigh *et al.*, 2002). The input of organic compounds into the rhizosphere has been shown to increase the abundance and metabolic activities of a diverse rhizospheric community (Schnoor *et al.*, 1995; Siciliano and Germida, 1998;

Barber and Lynch, 1977; Bowen and Rovira, 1999; Lynch and Whipps, 1990; Kamath *et al.*, 2004). Because plant species differ substantially in their chemical composition, particularly secondary compounds, different plants can foster different rhizosphere communities. Therefore, some plants can be more effective than others at facilitating microbial biodegradation rates of certain contaminants.

Many plant species have been shown to enhance the biodegradation of diesel range organics (Aprill and Sims, 1990; Binet *et al.*, 2000; Liste and Alexander, 1999; Miya and Firestone, 2000; Miya and Firestone, 2001). A few examples include black willow (*Salix nigra* marshall) (Spriggs, *et al.*, 2005), winter rye (*Secale cereal* L.) (Muratova *et al.*, 2008), tall fescue (*Festuca arundinacea* Schreber) (Banks *et al.*, 1999; Siciliano *et al.*, 2003), alfalfa *(Medicago sativa* L.) (Reilley *et al.*, 1996; Nichols *et al.*, 1997), green ash (*Fraxinus pennsylvanica* Marshall) (Spriggs *et al.*, 2005), and hybrid poplar (*Populus deltoids* x *P. nigra* DN 34) (Spriggs *et al.*, 2005). Since most DROs are not water soluble, research has shown that they are not taken up by plants in substantial quantities (Yi and Crowley, 2007; Cofield *et al.*, 2007; Reilley *et al.*, 1996; Schnoor *et al.*, 1995). Increased biodegradation rates of diesel range organics are thus attributed to microbial degradation rather than plant uptake and metabolism (Banks *et al.*, 1999; Alexander, 1994; Siciliano *et al.*, 2003; Binet *et al.*, 2000; Reilley *et al.*, 1996). Bacterial numbers in DRO-contaminated soil have been shown to be 70-200 times greater in the presence of plants than in their absence (Schwab and Banks, 1994; Reynolds *et al.*, 1999).

Many petroleum degrading bacterial species have been identified in the environment, such as bacteria in the genera *Pseudomonas, Mycobacterium, Flavobacterium, Arthrobacter, Burkholderia,* and *Sphingomonas* (Watanabe, 2001; Wackett and Hershberger, 2001; Walter, 1991; Ye *et al.*, 1996; Mueller *et al.*, 1990; Kosheleva, *et al.*, 2000). Of the organisms that have been shown to degrade hydrocarbons, pseudomonads are the most studied. All genes involved in the early steps of PAH degradation by pseudomonads are inducible (Yen and Serdar, 1988; Schell, 1990). Salicylate, an

intermediate in the naphthalene dioxygenase pathway, has been shown to induce the *nah* genes, which are responsible for encoding the catabolic enzymes that perform naphthalene degradation (Yen and Serdar, 1988; Schell, 1990; Barnsley, 1975; Schell, 1985; Kamath *et al.*, 2004).

PAH biodegradation is often attributed to cooxidation, which depends on the presence of lower molecular weight aromatic compounds to trigger enzyme induction (Mueller *et al.*, 1990; Weissenfels *et al.*, 1991; Ye *et al.*, 1996; Dean-Ross *et al.*, 2002). The naphthalene dioxygenase pathway is widely spread among naphthalene-degrading soil bacteria and its non-preferential substrate specificity allows cooxidation of numerous aromatic hydrocarbons such as phenanthrene, anthracene, biphenyl, toluene and fluorene (Resnick *et al.*, 1996; Kosheleva *et al.*, 2000). In some cases, the degradation of high molecular weight PAHs has been attributed to cometabolism by cells growing on low molecular weight PAHs and plant secondary compounds such as salicylate (Chen and Aitken, 1999; Kosheleva *et al.*, 2000; Sutherland *et al.*, 1995).

Because salicylate is important to PAH degradation by potentially serving as inducer of gene expression and/or a substrate promoting cooxidation, plants producing salicylate have the potential to rhizoremediate PAHs. Willows, which are known for their production of salicylate, are very abundant, diverse and among the most rapidly growing woody plants in Alaska. An outdoor pot study and laboratory microcosm experiment with crushed willow roots were conducted with *Salix alaxensis* (felt leaf willow) to measure the rhizospheric effects on the abundance of diesel degrading microorganisms and the biodegradation of diesel fuel #2. Because *Salix* species are known to contain salicylic acid throughout their roots, bark and leaves, we hypothesized that the rhizosphere of *S. alaxensis* would foster significantly higher numbers of diesel degrading microorganisms and a greater extent of diesel biodegradation in contaminated soil.

2.2 Methods

2.2.1 Plant material

Willow (*Salix alaxensis*) clippings were obtained from a single stand adjacent to the Fairbanks International Airport (64°79'84"N, 147°89'89"W) and were rooted vegetatively. Stem cuttings (approximately 20 cm) were rooted by planting in saturated sand in 100% humidity tents. Cuttings were saturated three times a week with a water-fertilizer solution (52 $mg \cdot L^{-1}$ Ca, 3.4 $mg \cdot L^{-1}$ Mg, 145 $mg \cdot L^{-1}$ total N, 76 $mg \cdot L^{-1}$ P and 125 $mg \cdot L^{-1}$ K) to promote root growth. After two weeks, clippings had sufficient root growth to sustain the newly formed leaves and were subsequently acclimated to outside conditions prior to planting into the diesel contaminated soil. At the time of planting, the clippings were carefully removed from the sand, rinsed, and transplanted into pots containing diesel contaminated soil.

2.2.2 Soil preparation and characterization

The experimental soil was obtained from the Great Northwest Inc. (Fairbanks, AK) and consisted of a local blend of 60% silt and 40% peat. The absence of petroleum contamination in the original soil samples was verified by GC-MS analysis. Measurements of initial carbon and nitrogen were made using a Carlo-Erba C/N analyzer; nitrogen (0.167% dry weight), carbon (2.87% dry weight). Initial values of percent moisture (9.52%) were measured gravimetrically. Initial percentage of organic matter (6.5%) was measured following the ASTM D 2974 dry ashing method. The soil was spiked with diesel fuel oil #2 (University of Alaska Fairbanks incinerator) to a concentration of 12.96 ± 3.03 ppm by spraying and homogenizing in small batches using a cement mixer. Spiked soil was stored in 55 gallon (208 L) drums for approximately one month before the start of the experiment.

2.2.3 Experimental design

The experiment commenced on June 24th 2008 and included the potting of all treatments and the planting of willow trees. One-liter plastic pots were filled with diesel contaminated soil. Three grab samples (approximately 50 grams each) were taken as the soil was placed in each pot: one when the soil filled 1/3 of the pot, another when the soil filled 2/3 of the pots' volume, and the final sample when the soil was approximately 2.5 cm from the top of the pot. The three grab samples from each pot were then combined, homogenized and sampled for later DRO concentration and most probable number (MPN) analyses of diesel degrading microorganisms.

Pots were randomly assigned to one of the four treatments: control (unplanted, unfertilized), willow only, fertilizer only, and willow plus fertilizer. Pots were randomly placed in an outdoor grid with pots set in plastic bins (2-3 pots per bin) in order to ensure that drainage from fertilized treatments was separated from non-fertilized treatments. All bins were placed in a wooden frame lined with plastic to contain any contaminated runoff in the event of heavy rain. Shade cloth was placed over the surface of the soil to help maintain natural low-temperature soil conditions. All pots were watered on the first day and watered as needed for the remainder of the experiment. All runoff was collected and stored for hazardous waste disposal. Fertilized treatments were fertilized with 500 mL (100 $mg \cdot L^{-1}$ Ca, 6.8 $mg \cdot L^{-1}$ Mg, 290 $mg \cdot L^{-1}$ total N, 153 $mg \cdot L^{-1}$ P and 250 $mg \cdot L^{-1}$ K) two times per week for the total duration of the experiment. The experiment was run for approximately three months until September 28th 2008 when all pots were destructively harvested and sampled for DRO concentration, MPN analysis and plant biomass.

At the time of harvest, plants were separated into roots, stems and leaves for biomass determinations. The stem was cut at the soil surface, any attached leaves were removed, and large roots (>1.0 mm) were carefully separated from the soil, and then the remaining soil was homogenized and sampled for DRO concentration and MPN. DRO samples were stored at 4°C for 5 to 9 months and MPN samples were stored at 4°C for no longer

than 2 weeks prior to analysis. The remaining soil was dried and sieved with a #200 (0.075 mm) sieve to remove fine roots. Roots (large and fine) and shoots were washed. All plant biomass collected was dried for 48 hours at 60°C and weighed to determine dry weight. Approximately 10 g of soil was collected from each pot for percent moisture determination, which was measured gravimetrically by comparing fresh weight of soil to dry weights of soil following oven-drying at 60°C for 48 hours.

2.2.4 DRO concentration analysis

Soil diesel concentrations were determined by GC/MS analysis. The extraction procedure was adapted from Schwab *et al.* (1999). Samples were thawed for 2 hours at room temperature before three replicates of 1 ±0.03 grams of contaminated soil were added to clean 15 mL glass centrifuge tubes. Using a glass syringe, 250 μL of 893 $mg \cdot L^{-1}$ naphthalene-d8 in methylene chloride was added to each sample as a surrogate and allowed to adsorb to the soil particles for 30 minutes. Ten milliliters of dichloromethane was added to each soil sample and. The centrifuge tubes were then sealed with a Teflon-lined lid and shaken horizontally on a rotary platform shaker for 30 minutes (120 r.p.m.). Tubes were then centrifuged at 45,362 g for ten minutes and the extract solution decanted. Ten more milliliters of fresh dichloromethane was then added to each tube and the process was repeated for a total of three times, producing 30 mL of extract. All extracts were stored at 4°C until DRO analysis in GC vials with Teflon lined septa.

Total DRO concentration was determined by GC/MS and is defined as the sum of alkanes and aromatics from C_{10} to C_{25}. One milliliter of each extract was transferred to a GC vial and spiked with 5 μL of 2,000 ppm 5α-androstane as an internal standard. The internal standard was also added to each calibration standard. Diesel fuel oil #2 was used to prepare stock calibration standards in methylene chloride. Four diesel standards (100 $mg \cdot L^{-1}$, 300 $mg \cdot L^{-1}$, 600 $mg \cdot L^{-1}$, and 1000 $mg \cdot L^{-1}$) were run with each sample series. GC/MS analyses were performed using an Aglient 6890N GC with an Aglient 5973 mass selective detector (MS) and a column of 30m x 320 um x 0.25 um. The GC was

programmed to run at an initial temperature of 35°C, held for 1 minute, then ramped up by 12°C per min to 320°C and held for 15 minutes. The total run time was 40.75 minutes. The injection port and the MS detector were both maintained at 280°C using a 1- μL pulsed splitless injection. Peak areas were manually integrated using data output from Agilent MSD Chemstation E02.00.

2.2.5 Soil microbial enumeration

The abundance of diesel degrading microorganisms was determined by a 96-well plate most probable number (MPN) method adapted from Haines *et al.* (1996). Microbes were removed from the soil particles by adding ten milliliters of 1% w/v sodium pyrophosphate (Fisher Scientific, Fairlawn) to one gram of soil and 3-4 grams of glass beads and then shaking horizontally on a rotary platform shaker for one hour (Leigh *et al.* 2006). Three replicate 1 gram soil samples were taken from each pot. The soil slurry was allowed to settle in upright tubes for 30 minutes before inoculating MPN plates. Each 96-well plate contained 180 μL of sterile Bushnell-Haas (BD, Sparks) medium per well. A 20 μL sample of microbial suspension was pipetted into the first column on the 96-well plate. Each well was thoroughly mixed before placing 20 μL into the adjacent well, resulting in ten-fold serial dilutions of the sample across the plate. Each plate included three replicate series from each soil suspension, a positive control (microbial suspension, diesel fuel, and Bushnell-Hass) and a negative control (diesel fuel and Bushnell-Hass). After inoculation, 20 μL of filtered (0.22 μm) diesel fuel oil #2 was added to each well, except the negative control. The plates were sealed with parafilm and placed in plastic bags to reduce evaporative loss. All plates were incubated in the dark at room temperature for 14 days. After the 14 day incubation, 50 μL of $3 g \cdot L^{-1}$ p-Iodonitrotetrazolium (MP Biomedicals, Salon) was filtered (0.22 μm) and then added to each well. This indicator dye deposits a red precipitate in the presence of active respiring microorganisms. After 48 hours the wells were scored, with a red or pink color producing a positive result. The MPN values were calculated from published MPN tables (Man, 1983).

2.2.6 Microcosm study

A laboratory microcosm study was also conducted in parallel with the pot study to examine the effects of crushed willow roots, salicylate and fertilizer on the abundance of diesel-degrading microbes in diesel contaminated soil. The method for this experiment was adapted from (Yi and Crowley, 2007) and included 4 relevant treatments with 5 replicates each: control, fertilized (0.40 mL of 104 ppm Ca, 6.8 ppm Mg, 290 ppm N,153 ppm P and 250 ppm K)), salicylic acid (20 mg), and crushed *Salix alaxensis* roots (20 mg); all containing the same diesel-spiked soil as the pot study. The most probable number of diesel degrading bacteria was measured in all treatments after a dark, room temperature, 90 day incubation.

2.2.7 Statistical analysis

GCMS and the MPN data were found to be normally distributed with the Kolmogorov- Smirnov (K-S test). Statistical differences among treatments were evaluated using the analysis of variance (ANOVA) with SPSS 16.0 (IBM, Chicago) and least significant differences were calculated ($p<0.05$).

2.3 Results

2.3.1 Plant biomass

The addition of fertilizer to pots containing *S. alaxensis* significantly increased the root and stem biomass (Table 1). A portion of the difference in leaf biomass is due to the timing of harvest, since plants were undergoing senescence. The majority of leaves on the unfertilized plants had already fallen, while the fertilized plants were at a much earlier stage of senescence, exhibiting small amounts ($\leq 30\%$) of color change at the time of harvest. Aside from the biomass differences accountable to early leaf senescence in the unfertilized plants, the increased root and stem biomass of the fertilized willow treatment indicates that fertilizer contributed to an increase of plant biomass in this contaminated soil.

2.3.2 Most probable number results

In all treatments, including the control (untreated diesel contaminated soil) the abundance of diesel degrading bacteria significantly increased over the growing season (Figure 1).

Fertilization, both with and without willow plants, resulted in significantly higher (3.4-fold) numbers of diesel degrading microorganisms in comparison to the unamended, unplanted control. The highest mean quantity of diesel degrading microorganisms was observed in the fertilized willow treatment, and this value was significantly similar to the fertilized and willow treatments. Although the mean MPN was approximately 5-fold higher, the combination of willow and fertilization did not result in a statistically significant increase in numbers of diesel degrading microorganisms over fertilization. In the absence of fertilizer, willow plants were not associated with significantly higher MPNs of diesel-degraders over the unamended control at the 95% confidence interval ($p<0.05$) although they were different at a 90% confidence interval, $p<0.10$.

In the microcosm study, fertilization also resulted in significantly higher numbers of diesel-degraders in comparison to unamended control (Figure 2). However, the highest numbers of diesel degrading bacteria were found in the statistically similar ($p<0.05$) treaments of crushed willow roots and salicylic acid. These treatments were also found to be statistically different from the fertilized and control treatments.

2.3.3 Removal of diesel range organics

The growth of *S. alaxensis* in diesel contaminated soil significantly decreased the concentration of DROs beyond the addition of fertilizer (Table 2). The concentration of DROs in the fertilized treatment did not differ significantly from the control nor did the fertilized willow treatment produce a significant difference from the fertilized treatment or the control. The willow and fertilized willow treatments were significantly different at a 90% confidence interval, but not at a 95% ($p<0.05$).

2.4 Discussion

The growth of *S. alaxensis* in diesel contaminated soil significantly increased the removal of DRO (Table 2). *S. alaxensis* may have genetically induced the diesel degrading rhizospheric community by releasing salicylic acid through cell lyses. The fertilized willow treatment did not produce a significant reduction in DRO and it is believed that the presence of fertilizer delayed fall root-turnover. Research has shown that nutrient-rich conditions produce significantly lower amounts of salicin than minimal nutrient conditions (Julkunen-Tiitto *et al.*, 1993). The lack of abscission in the willow trees amended with fertilizer is an indicator that they seasonally lagged behind the willow treatments. If abscission is postponed in fertilized willow treatments, it is probable that root turnover is also delayed. Postponement of fall turnover in the presence fertilizer may have an effect on root lyses and the subsequent release of salicylic acid into the rhizosphere.

The addition of fertilizer to pots containing *S. alaxensis* significantly increased the root and shoot biomass (Table 1). This is aligned with previous research that suggests that fertilizer contributes to an increase of plant biomass. With a higher biomass of roots, more salicylic acid has the potential to become released into the soil. The presence of fertilizer and plant secondary compounds have been shown to stimulate microbial growth and the degradation of petroleum hydrocarbons (Siciliano *et al.*, 2003; Bragg *et al.*, 1994; Walworth *et al.*, 1997; White *et al.*, 2003; Yen and Serdar, 1988; Schell, 1990; McCutcheon and Schnoor, 2003; Yi and Crowley, 2007). In comparison, this study shows that the unfertilized willow had significantly lower biomass, but the greatest DRO loss. Prior to harvest, the unfertilized willow had undergone leaf senescence, which is generally temporally associated with fine root turnover; whereas the fertilized plants were just beginning to undergo leaf color change.

Fertilizer significantly increased the abundance of diesel degrading microorganisms in diesel contaminated soil (Figure 1, Figure 2). Fertilizer provides limiting nutrients and

an enriched environment favorable for microbial growth. The *S. alaxensis* treatment was not significantly different than the unamended control, with respect to MPN of diesel degraders, but produced the greatest reduction of DRO (Figure 1, Table 2). Given that >99% of environmental microorganisms cannot be cultured in the laboratory, it is likely that the MPN method did not detect many of the diesel-degraders present. These non-culturable diesel degrading microorganisms may have contributed to the biodegradation of diesel fuel oil #2 in response to the input of salicylic acid (via root turnover) rather than fertilizer.

In the microcosms, the addition of crushed willow roots to diesel contaminated soil was found to have the same effect as salicylic acid; in which they both increased the abundance of diesel degrading microorganisms beyond the addition of fertilizer and the unamended control (Figure 2). In the pot study, the fertilized treatments contained the greatest abundance of diesel degrading microorganisms (Figure 1). The effect of fertilizer in the pot study may have been magnified by increasing plant biomass, including fine root biomass, which increases the amount of soil impacted by the rhizosphere of living and dead roots.

The planted treatments provided the greatest reduction in DRO and it is possible for DRO to sorb onto roots. In this study, sorption is not believed to be a cause of DRO disappearance as the fertilized treatment had significantly more root biomass, but a change in DRO concentration similar to the control. More roots did not result in increased DRO loss.

2.5 Conclusion

This research demonstrates that rhizoremediation with *Salix alaxensis* can be effective in reducing terrestrial fuel oil #2 contamination and may be a cost-effective strategy suited for cold-regions where remoteness, cost and extreme temperature differences provide limitations. The presence of *S. alaxensis* has been shown to

significantly increase the degradation of diesel range organics. In order to enhance rhizoremediation of hydrophobic and recalcitrant DROs it is important to understand the major role of root-associated microorganisms and their interactions with specific plant compounds.

As shown in prior research, fertilizer increases plant biomass, which in turn fosters a diverse microbial community in the rhizosphere. The application of fertilizer is beneficial in that it enhances the abundance of diesel degrading microorganisms, but application requires special attention. This research suggests that the chosen application rate and dose may have contributed to a delay in root turnover and in turn, the release of salicylic acid into the rhizosphere. The process of root-turnover is especially important in rhizostimulating the degradation of petroleum hydrocarbons and any factors that affect this process require additional consideration.

Phytoremediation strategies that include both plant released carbon compounds and inorganic fertilizer are likely to have the highest potential for rhizodegradation. This study demonstrates that the addition of fertilizer significantly contributes to the abundance of diesel degrading microorganisms, but not the degradation of diesel range organics. The greatest degradation of diesel fuel occurred in the willow treatment. The diesel degrading microorganisms that were cultured and estimated in the lab may not have been the rhizospheric community members that performed the degradation of diesel range organics. It is possible that other members of the rhizospheric community such as fungi are contributing to the degradation of diesel range organics. Stable Isotope Probing is currently underway to identify the rhizospheric microorganisms involved in DRO degradation.

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2.6 Figures

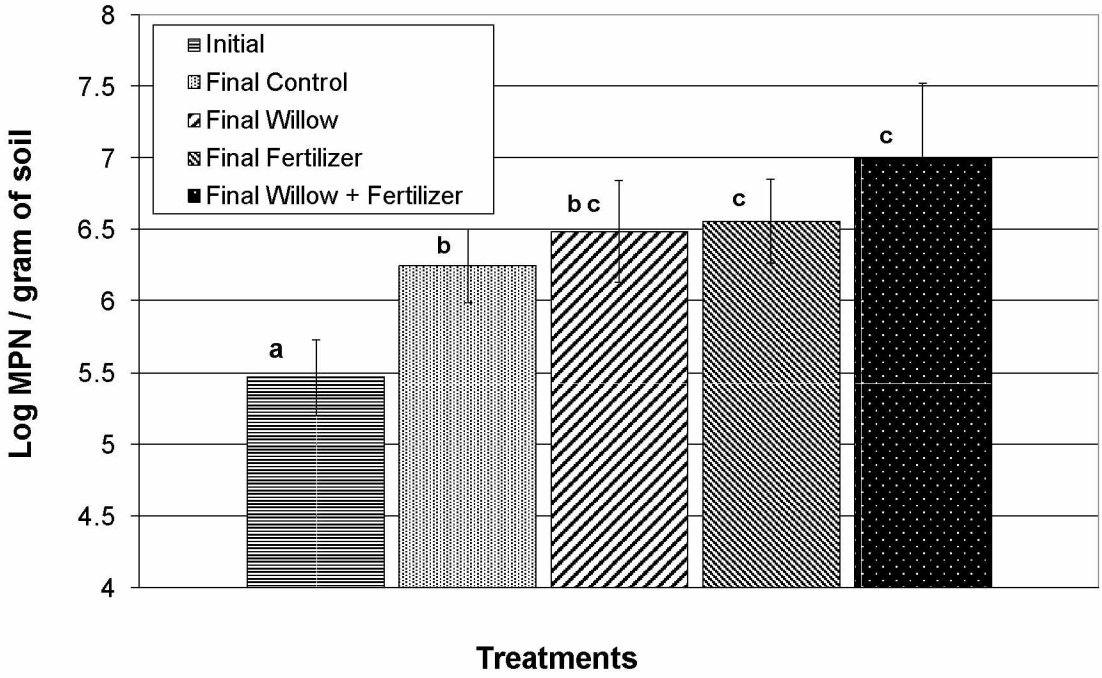

Figure 1. Pot Study MPN. Most probable number of diesel degrading bacteria in soils following the 96-day pot study in comparison to initial soil. Measurements from each pot were based on 3-replicate 1-g soil samples. The number of pots measured (n) for each treatment were: initial (45), control (10), willow (10), fertilized (16) and fertilized willow (15). Different letters correspond to treatments that are significantly different ($p<0.05$).

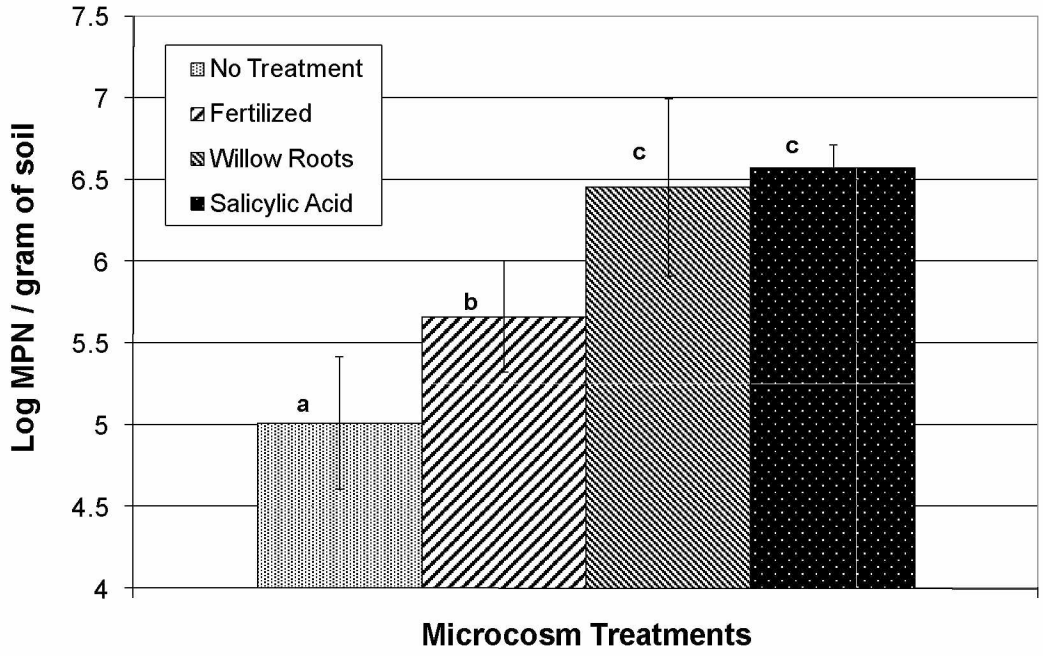

Figure 2. **Microcosm Study MPN**. Most probable number of diesel degrading bacteria in microcosms of diesel contaminated soil after 21 days incubation. Three 1-gram samples were analyzed from each replicate microcosm in each treatment, with N=6 microcosms for treatments and N=3 for the control. The different letters correspond to treatments that are significantly different ($p<0.05$).

2.7 Tables

Table 1. Plant Biomass. The mean dry weight (grams) of the roots, shoots, and leaves from the willow treatment (N=15) and the fertilized willow treatment (N=16). Corresponding letters refer to significantly similar pairs, $p<0.05$. Fine roots in the fertilized willow treatment were significantly greater at a 90% confidence interval, $p<0.10$. Total biomass includes stems and roots.

Treatment	Leaves	Stems	Fine Roots	Large Roots	Total Biomass
Willow	0.38 ± 0.41^{a}	14.29 ± 2.42^{c}	1.23 ± 0.59^{e}	3.13 ± 1.61^{f}	18.65 ± 4.18^{h}
Willow + Fertilizer	2.63 ± 1.78^{b}	18.71 ± 5.45^{d}	1.51 ± 0.67^{e}	4.58 ± 2.88^{g}	24.80 ± 7.47^{i}

Table 2. DRO Concentration. Concentration of DROs in soil samples before and after the 12 week pot study determined by GC-MS. Three 1-gram soil samples were taken from each pot. A minimum of four analytical standards were analyzed with every run. Different letters correspond to treatments that are significantly different, $p<0.05$.

Treatment	Initial Concentration mg/kg	Final Concentration mg/kg	Change in Concentration mg/kg	Percent Reduction in DRO Concentration
Control (n=9)	12.42 ± 2.99	9.43 ± 2.79	2.30 ± 4.66^{a}	20%
Fertilizer (n=9)	13.51 ± 3.00	10.83 ± 2.67	2.35 ± 1.49^{a}	20%
Willow (n=15)	13.71 ± 2.68	8.94 ± 1.13	4.51 ± 2.12^{b}	35%
Willow + Fertilizer (n=15)	12.20 ± 3.08	8.92 ± 1.65	$3.01 \pm 2.03^{a,b}$	23%

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Conclusions

Rhizoremediation with *S. alaxensis* is a potential cost-effective remediation solution suited for cold-regions where remoteness, cost and extreme temperature differences provide limitations. In order to enhance the rhizoremediation of hydrophobic and recalcitrant DROs, it is important to understand the major role of root-associated microorganisms and their interactions with specific plant compounds. Enhancing major pollutant degrading species in the rhizosphere directly contributes to the increased degradation of pollutants. To optimize the rhizoremediation potential of DROs, one must choose plants wisely and provide the optimal environmental conditions for microbial and terrestrial growth.

Plants that deposit salicylic acid into the rhizosphere have been shown to increase polyaromatic hydrocarbon (PAH) degradation. Successful rhizoremediation begins with a contaminant tolerant species that can overcome limits such as bioavailability, insufficient microbial biomass, and limited genetic induction or metabolic pathways. *S. alaxensis* is believed to release salicylic acid into the rhizosphere and induce the degradation of PAHs. In understanding the plant-microbe relationship and the mechanisms of PAH degradation, rhizoremediation can further succeed as a cleanup technology for DRO contaminated sites.

This research, as prior research, has shown that fertilizer contributes to an increase in plant biomass. At the final sampling, the fertilized treatments contained a significantly larger amount of roots, shoots, and leaves when compared to the non-fertilized treatments. The more concentrated rhizosphere in the fertilized treatment is believed to foster a healthier abundance of rhizospheric microorganisms, as a significantly larger population of diesel degrading microorganisms was measured in the fertilized treatments. This research found that fertilizer contributed to an increase in plant biomass and an increase in the abundance of diesel degrading microorganisms.

This research also suggests that the concentration of fertilizer may have contributed to a delay in fall root-turnover. The lack of salicylic acid would affect the genetic induction of microorganisms capable of degrading diesel range organics. The fertilized willow treatment had significantly less diesel range organic degradation when compared to the willow treatment at a 90% confidence interval. The presence of fertilizer is important to foster an abundant community of diesel degraders, but its application may also delay the rhizospheric input of important plant secondary compounds that induce petroleum hydrocarbon degradation. More research is necessary to develop a beneficial range of concentrations and application rates of fertilizer.

Crushed willow roots in microcosms containing diesel contaminated soil significantly increased the abundance of diesel degrading microorganisms compared to the addition of fertilizer or the control. The addition of pure salicylic acid produced a statistically similar increase in diesel degrading microorganisms as the addition of crushed willow roots. This suggests that crushed willow roots and salicylic acid, both foster a community of diesel degrading microorganisms. This effect is similar to what would be expected during fall root-turnover.

It was hypothesized that the fertilized willow treatment would have the greatest abundance of diesel degrading microorganisms and thus the largest degradation of diesel range organics. The addition of fertilizer was shown to significantly contribute to the abundance of diesel degrading microorganisms, but not the degradation of diesel range organics. The greatest degradation of diesel fuel occurred in the willow treatment. The diesel degrading microorganisms that were cultured and estimated with the MPN technique, may not have been the rhizospheric community members that performed the degradation of diesel range organics. The willow treatment, which showed the highest degradation, did not have significantly more diesel degrading microorganisms than the control. It is suggested that other members of the rhizospheric community such as fungi are contributing to the degradation of diesel range organics.

This research concludes that the addition of *S. alaxensis* to diesel contaminated soil has a greater impact on the biodegradation of DROs than the input of fertilizer. It is recommended to test these findings in a field study.

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