# The use of plant growth promoting rhizobacteria to enhance phytoremediation of petroleum hydrocarbon-impacted soils

by

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# AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## Abstract

Phytoremediation is a cost-effective and environmentally conscious technology that utilizes plants to remediate a variety of contaminants from the soil. Petroleum hydrocarbons (PHC), as a result of their widespread use are a common environmental problem in soils, and one where phytoremediation would be well suited as a solution. Phytoremediation of PHC proceeds mainly through microbial degradation in plant root zone (rhizosphere) and plant degradation of PHC. The high concentrations of PHC at many sites are an impediment to phytoremediation. The toxicity of PHC hinders plant growth and prohibits remediation. One way to overcome this decrease in plant growth is by employing plant growth promoting rhizobacteria (PGPR). PGPR are naturally present soil bacteria that influence plant growth through direct and indirect methods, and can ultimately act to reduce plant stress. If PHC toxicity could be overcome with the use of PGPR, phytoremediation may become a viable option for remediation of PHC contaminated sites.

This study was divided into a field component and a laboratory component, both of which focused on different aspects of phytoremediation of PHC enhanced with PGPR. Previous studies have shown that PGPR-enhanced phytoremediation was successful in the greenhouse. As a result of this, long-term field studies of this system were initiated. Effectiveness was evaluated by assessing plant performance and remediation of PHC in the field. The long-term impact of PGPR on the resident microbial community was evaluated, to ensure there was no detriment to microbial diversity. Following success in the field, lab studies were performed to evaluate the effect of PGPR and PHC by examining both physiological and molecular changes in plants exposed to PHC where PGPR effects were demonstrated.

When PGPR were used in field experiments to facilitate phytoremediation, PHC toxicity was alleviated, and this was evident through improved germination, increased plant growth, and improved photosynthetic performance of selected grass plant species. These findings were corroborated at two field sites, one with high and one with low levels of weathered PHC. Addition of PGPR consistently improved remediation at each site.

When remediation of PHC was followed over a period of three years, it was found that despite increased plant growth and increased remediation with addition of PGPR, remediation slowed in the second and third year of growth; this was particularly evident at the site with low levels of PHC. Further investigation revealed that the decrease in remediation may be attributed to increases in biogenic material over the growing season that may not be adequately removed when standard regulatory protocols are followed during PHC analysis in soil. The possibility of degradation being masked by biogenic material was supported by the fact that specific, recalcitrant components of the PHC, such as polycyclic aromatic hydrocarbons (PAHs), continue to degrade over time. Microbial community changes during phytoremediation of PHC soils with PGPR were investigated. It was found that addition of PGPR resulted in higher microbial numbers in the soil but did not produce significant changes in the indigenous microbial populations.

Following field study, phytoremediation of PHC contaminated soil was further studied in the laboratory. The effects of PHC on gene expression of *Secale cereale* were evaluated using differential display PCR. Six differentially expressed genes were found: poly(A)-binding protein, phytoene desaturase, cytochrome P450, plasma membrane $H^+$ATPase, and knotted-like homeobox transcriptional activator. Their expression was confirmed using quantitative PCR (qPCR). In addition, the expression of four reference genes was evaluated and most stable reference genes in *Secale cereale* were recommended. Phenolic and flavonoid content in *Secale cereale* and *Festuca arundinacea* was evaluated and it was found that PGPR and PHC affected phenolic production in shoots of these plants, albeit differently in the two plant species.

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Thank you Zack for teaching me that work isn't everything.

# Dedication

To my grandparents Kazia i Edziu,

To Zack

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# List of Abbreviations

| | |
|---|---|
| $^{O}C$ | degrees Celsius |
| µL | microlitre |
| µg | microgram |
| ACT | actin |
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| ACT | actin |
| AL | actinic light used in PAM fluorometry |
| AWCD | average well color development |
| BOC | biogenic organic compounds |
| BTEX | benzene, toluene, ethylbenzene and xylenes |
| CAS | chrome S azurol |
| CCME | Canadian Council of Ministers of the Environment |
| CHS | chalcone synthase |
| CFU | colony forming units |
| Ct | threshold cycle |
| d | days |
| $ddH_2O$ | double distilled water |
| ddPCR | differential display polymerase chain reaction |
| DRO | diesel range organics |
| EF1A | elongation factor 1-alpha |
| EPA | Environmental Protection Agency |
| FHA1 | transcriptional factor containing forkhead-associated domain |
| FR | Far-red light used in PAM fluorometry |
| FID | flame ionization detector |
| $F_v/F_m$ | energy transfer efficiency in dark adapted plant tissues |
| g | grams |
| GC | gas chromatography |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |

| | |
|---|---|
| HPLC | high performance liquid chromatography |
| IAA | indole-3-acetic acid |
| KNOX | knotted-like homeobox gene |
| L | litre |
| M | molar, g/mol |
| mg | milligram/miligrams |
| min | minute/minutes |
| mM | milimolar |
| MS | mass spectrometry |
| NWT | Northwest Territories |
| OD | optical density |
| PABP | poly(A)-binding protein |
| PAH | polycyclic aromatic hydrocarbon |
| PAM | pulse amplitude modulated fluorometry |
| PAR | photosynthetically active radiation (400 – 700 nm) |
| PCA | principal component analysis |
| PCR | polymerase chain reaction |
| PDS | phytoene desaturase |
| PEPS | PGPR enhanced phytoremediation system |
| PGPR | plant growth promoting rhizobacteria |
| PHC | petroleum hydrocarbons |
| PMA | plasma membrane $H^+$ATPase |
| ppm | parts per million |
| PSII | photosystem II |
| P450 | cytochrome P450 |
| qN | non-photochemical quenching |
| qP | photochemical quenching |
| qPCR | quantitative polymerase chain reaction |
| ROS | reactive oxygen species |

| | |
|---|---|
| rpm | revolutions per minute |
| rRNA | ribosomal ribonucleic acid |
| RT | room temperature |
| s | seconds |
| SD | standard deviation |
| SE | standard error |
| UCM | unresolved complex mixture |
| UVA | ultraviolet-A (320 – 400 nm) |
| UVB | ultraviolet-B (290 – 320 nm) |
| UW3 | PGPR isolate, *Pseudomonas* sp. |
| UW4 | PGPR isolate, *Pseudomonas putida* |
| T/B/A | timothy/brome/alfalfa |
| TSB | tryptic soy broth |
| TSEM | total solvent extractable material |
| TUB | tubulin |
| Tm | melting temperature |

# Chapter 1

# Introduction to Phytoremediation of Petroleum Hydrocarbons (PHC)

## 1.1 Overview

The discovery that contaminants disappear in the vicinity of plant roots has lead to the development of a field of research, and usage of the technology called phytoremediation (Cunningham et al., 1995; Cunningham and Ow, 1996). It has been successfully applied to a broad spectrum of contaminants in a variety of settings, both terrestrial and aquatic. Steady efforts have been undertaken to optimize phytoremediation for organic contaminants such as petroleum hydrocarbons (PHC), polycyclic aromatic hydrocarbons (PAHs), polychlorinated phenols (PCBs), trichloroethylene (TCE) (Salt et al., 1998) and metals (Alkorta and Garbisu, 2001).

The objective in phytoremediation of PHC is to progressively lower contaminant concentrations *in situ* through uptake and degradation by plants and by microbial degradation of PHC in the rhizosphere, the area immediately surrounding the root zone (Wenzel, 2009). In the remediation of PHC, plants fulfill a dual role. They predominantly act by providing a suitable habitat, in the form of the rhizosphere for a contaminant-degrading bacterial population to thrive in (rhizoremediation). Plants may also degrade or sequester contaminants themselves (phytotransformation, phytodegradation). The final goal is the complete disappearance of PHC, or to reach generic targets set by the regulatory bodies such as the Canadian Council of Ministers of the Environment (CCME). This introduction will review current literature on phytoremediation of PHC, PHC composition and weathering, mechanisms of remediation of PHC, and amendments tested in greenhouse and field trials. The focus of this introduction will be primarily on grasses. The discussion of trees used for remediation of PHC will be omitted.

## 1.2 Petroleum hydrocarbons

Crude oil and its products exist as complex mixtures, consisting mostly of hydrocarbons and lesser amounts of non-hydrocarbon compounds with heteroatoms such as nitrogen, sulfur, or oxygen species (Farrel-Jones, 2003; Wang et al., 2006). The organic components of this mixture are further divided into aliphatic hydrocarbons (non-aromatic compounds) and aromatics such as benzene and benzene-like compounds (Farrel-Jones, 2003). Petroleum components are classified in bulk groups of saturates (hydrocarbon compounds with no double bonds, can be both straight and branched chain, e.g. decane, Figure 1.1), olefins (alkenes, hydrocarbon compounds with some double bonds, e.g. 1,13-tetradecadiene, Figure 1.1), aromatics (cyclic planar compounds that resemble benzene, Figure 1.1), resins (small polar compounds, e.g. dibenzothiophene, Figure 1.1) and asphaltenes (large, undissolved polar compounds) (Wang et al., 2006; Howard et al., 2005). The average composition of crude oil prior to processing is ~ 57% saturated hydrocarbons, ~ 29% aromatic hydrocarbons and ~ 14% polar compounds (Tissot and Welte, 1984). Of the many thousands of petroleum derived compounds only a few hundred have been fully identified and characterized (Howard et al., 2005), and even fewer individual compounds are regularly monitored in the environment.

A simple way to think of the complex petroleum mixture is to divide the compounds based on the number of carbon atoms they contain. This type of classification has been widely adopted by regulatory agencies. In Canada, the Canadian Council of Ministers of the Environment (CCME) has made the following divisions for PHC compounds where C is the number of carbon atoms in the molecule. Fraction 1: C6-C10, Fraction 2: C>10 to C16, Fraction 3: C>16 to C34, Fraction 4: C34+. In the United States the divisions made by the Environmental Protection Agency (EPA) is Gasoline Range Organics (GRO) corresponding to small chain alkanes (C6-C10) and Diesel Range Organics (DRO) that includes longer chain alkanes (C10-C40) as well as PAHs (Kamath et al., 2004). Both organizations have

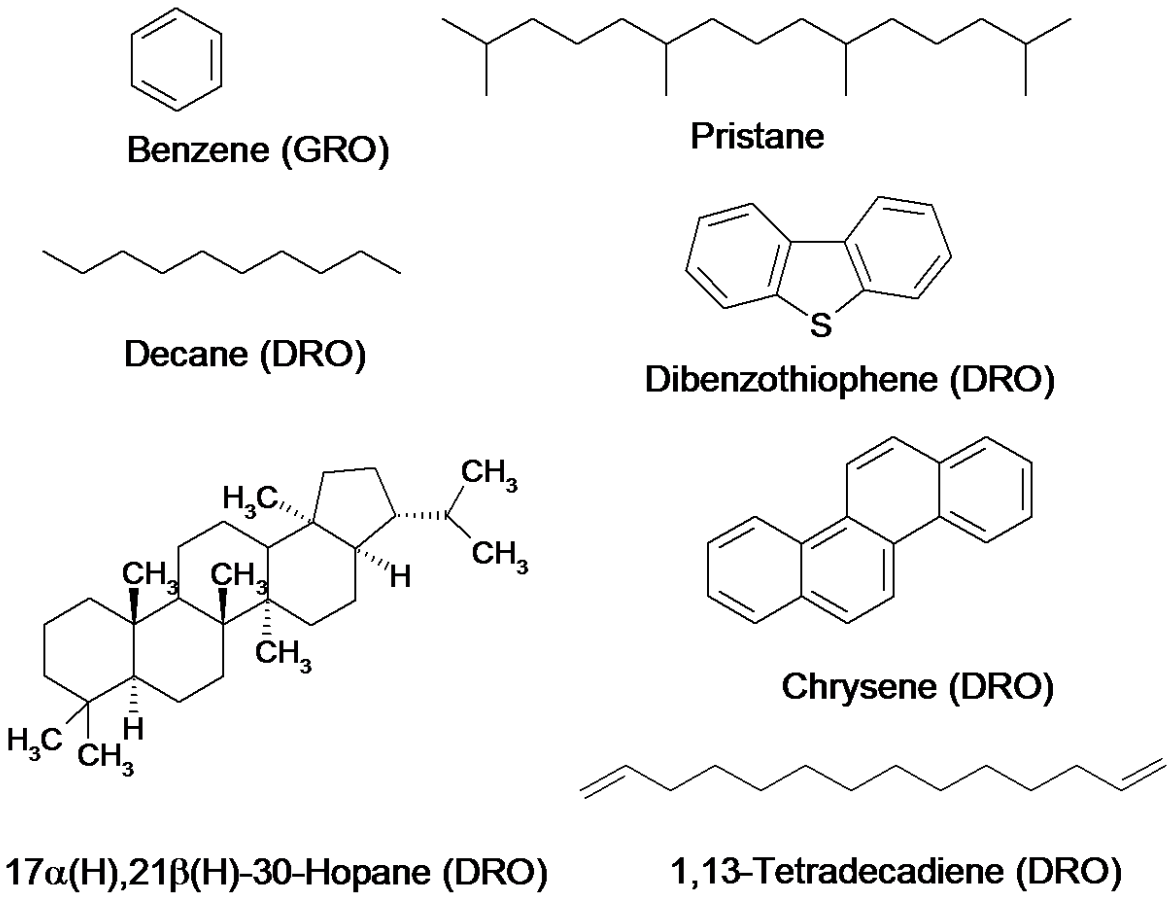


**Figure 1.1 Groups of compounds found in petroleum and its products.**

Saturates are composed of carbon (C) and hydrogen (H) only and contain no double C-C bond (e.g. decane). Saturates include straight chain, branched chain (also called paraffins) and cycloalkanes (also called naphthenes, e.g. hopane). Olefins (alkenes) are partially unsaturated hydrocarbons (e.g. 1,13-tetradecadiene) with one or multiple double C-C bonds. Aromatic hydrocarbons are benzene-like cyclic, planar compounds, with single (e.g. benzene) or multiple rings (e.g. the PAH chrysene). Polar compounds carry negative or positive charges, typically from nitrogen, oxygen or sulfur atoms (e.g. dibenzothiophene, (Hegazi and Andersson, 2007)). Small polar compounds are called resins. Asphaltenes are large heteroatom-containing compounds, undissolved and present as colloids (complex structures, not shown). GRO, gasoline range organics (C6-C10) and DRO (C10-C40) diesel range organics, represent categorical conventions used in American literature.

implemented allowable levels of petroleum hydrocarbons in soil and they are dependent upon the end use of the contaminated land (CCME, 2001a).

### 1.2.1 Contamination with petroleum and petroleum products

Many oil and gas industry contamination issues exist due to a lack of regulations in the past. For example, historically it was common practice to use earthen pits to store and burn fluids produced at upstream processing sites such as wells (Rutherford et al., 2005). In addition to oil products, these pits would often contain brine used to extract oil or other chemicals used in drilling or processing operations (Rutherford et al., 2005). These sites now need to be addressed through various remediation methods, often involving digging up of the contaminated soil and movement off site. As 'dig and dump' strategies (dig up soil and dispose in a landfill) are cost prohibitive, especially for sites where toxicity is low and does not require immediate intervention; there is a need for economically viable remediation methods for these types of sites.

Another source of petroleum contamination comes directly from the refining process. Crude oil requires refining through distillation to produce gasoline, diesel fuel, jet fuel or heating oil, with producing the unwanted remnants of processing, petroleum sludge. Petroleum sludge production represents a substantial portion of the refining process and requires ongoing disposal, often performed by continuously spreading the sludge on refinery land. While land farming was considered as an "environmentally attractive alternative" in 1982 (Arora et al., 1982), petroleum land farms have since become highly regulated to minimize migration of contaminants through leaching or volatilization (Maila and Cloete, 2004). As a result, in Canada land disposal of petroleum sludge is becoming increasing difficult due to changes in land disposal regulations (Ministry of the Environment, 2009) and this will produce many decommissioned petroleum-impacted sites. At these sites, petroleum sludge was continuously spread throughout the year, with ongoing tilling and addition of nutrients to increase microbial growth in the soil. As a result of tilling and increased growth of microbial petroleum degraders, most of the light petroleum compounds are removed and heavier petroleum products remain (Hutchinson et al., 2001; Maila and Cloete, 2004).

The widespread use of petroleum and petroleum products increases the likelihood of accidental contamination during transport, pipeline spills or contamination during the refining processes. In Canada, it is estimated that over 60% of all contaminated sites are contaminated with PHC (CCME, 2001a). In the United States, the US petroleum industry spent 0.8 billion dollars on remediation in 2001 (Collins, 2007). This problem is not confined to North America and in many countries worldwide, such as the United Kingdom, Russia, Portugal and India, PHC dominate polluted soils and require accessible remediation technologies (Collins, 2007; Lyubun and Tychinin, 2007; Nabais et al., 2007; Prasad, 2007).

### 1.2.2 PHC analysis

The variable composition of PHC mixtures in the environment poses a difficulty in choosing appropriate methodology to quantify these mixtures. PHC content is defined by the amount of extractable material, and the exact amount of contaminants extracted can widely vary based on the methods (TPHCWG, 1998). The process of PHC analysis includes sampling and/or sample preservation, extraction with solvents, sample cleanup and detection. Selections made at each of these steps will alter the PHC values and thus must be taken into consideration when discussing the final result. Choice of solvent will impact the type and amount of PHC extracted, for example if methylene chloride is the extracting agent, aromatic and aliphatic hydrocarbons cannot be separated and if volatile PHC must be preserved, methanol is used (TPHCWG, 1998). Common extraction methods include sonication (to loosen particles adhering to soil), Soxlet (heating and passing of solvent continuously through the soil sample for an extended period of time) and shaking or vortexing. Shaking and vortexing may be less efficient, while Soxlet requires large volumes of solvent, further requiring sample concentration following extraction. Clean up of samples may be necessary, as many methods cannot differentiate between biologically- and petroleum-derived hydrocarbons, which may lead to an overestimation of the concentration of PHC. A commonly used clean up method is the silica gel cleanup, which results in binding of polar (likely biologically-derived) material, with the PHC remaining in the solvent. Notably, no single method is able to extract the entire PHC content and various aspects of methodology used must be taken into account when evaluating PHC data (TPHCWG, 1998).

A very straightforward PHC quantification method is the gravimetric method where any PHC components that may be extracted and weighted will be measured; the PHC extracted will somewhat vary with the type of solvent that is used for gravimetric extraction. This method allows for fast, simple and cost effective monitoring of PHC, however it does not provide any information about the specific components present in the PHC mixture. Without a previous silica cleanup, the measurements may include PHC hydrocarbons and polar plant derived compounds (TPHCWG, 1998).

A more sensitive method for PHC detection than gravimetric analysis is gas chromatography (GC). GC separates the mixture based on the boiling point, polarity and affinity to the chromatographic column (TPHCWG, 1998). GC methods provide more information about the identity of PHC. Compounds may be quantified with a GC with flame ionization detector (GC-FID), where any compound that elutes from the chromatographic column and burns will be detected. This detection is non-specific and individual components can co-elute and identification and quantitation of individual analytes is almost impossible, although, the relative distribution of lighter and heavier PHC components can be investigated from the chromatogram (Wang and Fingas, 1997). Increased resolution is possible if GC is coupled with mass spectrometry (GC-MS). GC-MS is frequently used to identify specific components of the PHC mixture; mass spectrometer detects ionized fragments of molecules. While a FID detects multiple molecules with the same retention time, these compounds are not likely to have the same ionization pattern in the mass spectrometer and thus allows for a firmer identification of compounds than with GC-FID. MS can also be run in selected ion monitoring (SIM) mode where only compounds of interest are identified, greatly increasing the sensitivity of this method (Wang and Fingas, 1997) Polar, nonpetroleum hydrocarbon components are highly reactive and do not reach the detector, thus, interferences in GC methods from those compounds are less likely (TPHCWG, 1998; Howard et al., 2005) However this also means that any polar PHC components containing sulfur, oxygen or nitrogen will not be detected and further, some non-petroleum biogenic compounds are still detected if they are non-polar (TPHCWG, 1998). GC analyses work best for compounds with 6-50 carbon atoms. Often, these methods are employed in tandem, for example heavy

hydrocarbons can be detected by employing a follow up method such as the gravimetric method where samples are extracted, dried then weighted to estimate PHC content. The current CCME methodology states that if the GC-FID chromatogram does not return to zero (baseline) when it reaches C50, a gravimetric method must be used to measure total extractable PHC. Further PHC analysis methods such as infrared spectroscopy and immunoassay method exist, however GC based and gravimetric methods predominate PHC detection in the phytoremediation trials literature. The Total Petroleum Hydrocarbon Criteria Working Group wrote an excellent review of other methods for PHC analysis (TPHCWG, 1998).

### 1.2.3 Weathering of petroleum compounds

When a complex mixture of petroleum products is released into soil, it begins a continuous process called weathering. This is dependent on factors such as soil type, climate and the composition of PHC (Wang et al., 2006). Weathering reactions such as volatilization, leaching, natural dispersion, chemical photooxidation and microbial degradation (biodegradation) continue to evolve the chemical composition of the product and as such, no specific petroleum product could ever result in an identical spill, unless it took place in identical environmental conditions (Wang et al., 2006). The rate of biodegradation is largely governed by the composition of PHC; while *n*-alkanes (straight chain alkanes, e.g. decane, Figure 1.1) are some of the fastest to degrade, compounds such as PAH (e.g. chrysene, Figure 1.1) demonstrate variable degradation.

The common order of biodegradation organized by decreasing degradation rates is saturates > low molecular weight aromatics > high molecular weight aromatics/polar compounds (Leahy and Colwell, 1990). Many high molecular weight compounds, such as multiple ring cycloalkanes (naphthenes, e.g. hopane, Figure 1.1) show resistance to degradation and are often used as persistent biomarkers (Howard et al., 2005). Others, such as pristine (Figure 1.1) and phytane, initially thought of as almost entirely resistant to degradation have been shown to be degraded (Howard et al., 2005). Low molecular weight compounds of PHC, such as the BTEX (BTEX is benzene, toluene, ethylbenzene and xylene

combined together), migrate through the soil quickly compared to larger compounds and are subject to both aerobic and anaerobic microbial degradation. Thus BTEX disappears quickly from the soil (Leahy and Colwell, 1990; Jindrova et al., 2002; Kamath et al., 2004). Larger components of the PHC mixture such as the toxic, mutagenic and carcinogenic polycyclic aromatic hydrocarbons (PAHs) tend to be less mobile, bind to soil organic matter and are not as easily degraded in part due to this limited bioavailability (Kuiper et al., 2004; Lampi et al., 2007; Siciliano and Germida, 1998b; Kamath et al., 2004). The potential for biodegradation of PAHs deteriorates further with increasing size of the molecule (Howard et al., 2005).

In general, lightly weathered oils have a low abundance of *n*-alkanes and complete loss of BTEX and $C_3$-benzene, while severely weathered oils have complete loss of n-alkanes, branched and cycle-alkanes. These oils show low resolution in GC-MS and GC-FID chromatograms evidenced by the presence of a large unresolved complex mixture (UCM), as opposed to small UCM and more defined peaks for less weathered oils. In highly weathered oils, PAHs and their alkylated homologues (e.g. PAHs with two additional methyl groups or an ethyl group) are substantially degraded. The rate of degradation decreases with increasing number of carbons in alkyl groups. This results in a characteristic degradation of members of each PAH family (Wang et al., 2006). Once the easily biodegradable compounds disappear, what remains is the UCM, which appears as an unresolved hump in the GC chromatograms.

Molecules resistant to biodegradation consist of highly branched and cyclic saturated, aromatic naphthenoaromatic and polar compounds (Sutton et al., 2005). The UCM may account for as much as half to all of the total mass of weathered oil; it will not however include the polar and asphaltene compounds, which do not volatilize during chromatographic analysis and would not be detected; these compounds would be extracted in gravimetric PHC analyses however (Prince and Walters, 2007). The large diversity of compounds that comprise PHC mixtures results in very unpredictable behavior of those mixtures in soil environments. It is these recalcitrant compounds that often remain in the soil and lead to exceedances of allowable PHC levels in soil, and thus need to be consumed by plant-assisted bioremediation.

### 1.2.4 Bioavailability of PHC

Biodegradation of organic contaminants, including PHC, may be limited by sorption to soil particles, incorporation of soil organic material, and by entrapment of PHC within soil micropores; processes collectively referred to as aging (Alexander, 1995). This sequestration of PHC may make it unavailable for microbial or plant degradation. Aging is different from processes of weathering of contaminants in that weathering implies removal of the contaminant, whereas aging merely implies sequestering. Organic compounds, such as PAHs, have been shown to have biodegradation kinetics with a reoccurring pattern. Initially the contaminant disappears from soil at prompt rates followed by a noticeable decline (reviewed in Alexander, 1995). Thus, several lines of evidence including toxicity assessments, extractability and diminishing availability to microorganisms suggest that sequestered contaminants may not be toxic, as a result of the movement of contaminants to inaccessible sites within soil particle pores (many of those < 1.0 μm in size, (Alexander, 1995)). However, current methodology, often employing harsh solvent extractions used to measure PHC concentrations in soil are capable of extracting PHC that would not otherwise be accessible by biological organisms such as bacteria, animals, or plants. Thus, if regulatory decisions and site assessments are solely based on those, the overestimation of risk and poor choice of remediation, or site management strategy, may occur (Alexander, 1995).

There currently are no generally accepted methods of estimating bioavailability of PHC. An approach to address the bioavailability issue that is currently used is PHC toxicity testing (risk assessment) that can be performed on a site-specific basis. A suggestion by Collins (2007) is to carry out risk assessment when PHC levels are below 1% (following phytoremediation) to estimate if PHC at a particular site are no longer toxic and thus the site is deemed to meet site-specific criteria. This approach may be used when the exhaustive chemical extractions reveal values that exceed regulatory thresholds where little toxicity was observed. The often missing historical site information resulting from frequent changes in site ownership leaves gaps in the origins and age of contaminating material, making the predictions about bioavailability of PHC even less reliable. Obtaining data on remediation of

weathered PHC contaminated sites will provide insight into usable methods to deal with PHC contaminated sites, including if remediation of weathered PHC is possible.

### 1.3 Biological remediation strategies for PHC

Many remediation approaches that employ *ex situ*, and highly engineered physical and chemical methods exist (reviewed in Reddy et al., 1999 and Khan et al., 2004). Although those can be performed rather quickly, in most cases the soil is disrupted and chemical treatment requires movement off site, and is not economically viable. This, combined with the knowledge that contamination is low and not immediately dangerous to human health, makes it appealing to explore cost effective, albeit more time consuming, biological remediation strategies. Because the potential to degrade PHC with biological methods is high, these methods have been investigated for a number of years and are discussed below.

Landfarming is a remediation strategy where naturally present microbes biodegrade available PHC components, e.g. n-alkanes. With aeration from tilling and application of fertilizer, the microbes that are naturally present in the soil (petroleum degraders have been shown to be enhanced with PHC contamination (Muratova et al., 2003a)) are provided with the right conditions to degrade PHC. Problems arise when the easily bioavailable PHC has been degraded, and the recalcitrant, less bioavailable compounds remain. In this case, landfarming does not suffice and will not bring PHC below criteria.

Bioaugumentation or enhanced bioremediation is a process where contaminant-degrading microbes are added to the contaminated soil. Although a plethora of microbes with the ability to degrade specific xenobiotics exist, either as wild types or as genetically modified strains, the shortfall of this remediation approach lies in the inability to support the growth of these microbes at the remediation site (Kuiper et al., 2004). Reasons cited for this problem are the low concentrations of the xenobiotics or their low bioavailability and the inability of the inoculants to reach the contaminant. Thus, there is a lack of substrate for growth of the microbes and they do not reach sufficient titers to affect remediation. Added microbes may also be eliminated by natural predators such as the protozoa or microbes may prefer a substrate other than PHC (Kuiper et al., 2004). A more complex bioremediation approach

may involve engineered irrigation/fertilization systems that reach lower soil depths, or an *ex situ* remediation approach where excavated soil is bioremediated off site. However, both of these approaches add significant costs in comparison to the straightforward *in situ* bioremediation discussed above. Often, both landfarming and bioremediation suffer from lack of sufficient microbial biomass production. However, if some of these obstacles can be overcome with suitable conditions to increase total number of microbes and the activation of necessary metabolic pathways, even recalcitrant organics can be biodegraded **(**Fulthrope et al., 1996; Olson et al., 2003).

Phytoremediation can be considered as a biological nutrient delivery system that utilizes the root network to transport nutrients, water and air into the contaminated soil. With the production of plant biomass, a greater area of the contaminated site can be infiltrated with roots, and the microbes, air and nutrients associated with plant root growth than when typical aeration, landfarming and microbes alone are used. Plants, through their roots systems, take up and transpire large amounts of water, also taking up some of the contaminants in the process (Schnoor, 2002). Contaminants can be removed through a variety of mechanisms. This may occur in the rhizosphere, such as microbe-assisted degradation (rhizodegradation), or phytosequestration (also referred to as immobilization) (Kamath et al., 2004). Mechanisms requiring uptake into the plant include phytoextraction/phytoaccumulation in the plants, phytodegradation of contaminants by the plant, and phytovolatilization or release of contaminant to the atmosphere (Khan et al., 2004). Reportedly plants can provide as much as 100 million miles of roots per acre, illustrating the considerable potential to reach soil contaminants with proper plant growth (Boyajian and Carreira, 1997). Phytoremediation can offer significant cost savings comparing to other methods. Whereas phytoremediation may cost $60,000 to $100,000 USD per one acre of soil to 50 cm depth, a similar quantity of soil could cost as much as $400,000 to $1,700, 000 USD to excavate and landfill. In comparison, construction of the biological *ex situ* treatments discussed above would cost between $125,000 to $2 000,000 USD (Khan et al., 2004). The lower cost makes phytoremediation a suitable method for many contaminated sites, where resource limitations would prevent remediation activities from ensuing.

## 1.4 Mechanisms of PHC phytoremediation

### 1.4.1 Phytodegradation by plants

Plants play a dual role in the phytoremediation of PHC, acting directly and indirectly. They act indirectly by stimulating the growth of microorganisms inhabiting their root zone, the rhizosphere. The microorganisms in the rhizosphere consume the organic contaminants that are available to them. Plants also act directly by taking up contaminants, leading to their sequestration, degradation or transformation (Salt et al., 1998; Alkorta and Garbisu, 2001; Kamath et al., 2004).

Direct uptake of chemicals into plants necessary for plant degradation of contaminants depends on several factors: the uptake efficiency, transpiration rate, and concentration of chemicals in the soil. Uptake efficiency will rely on physico-chemical properties, chemical speciation and plant type (Schnoor, 1998). Transpiration varies with plant type, surface area of the leaf, nutrient and moisture levels in the soil, temperature, wind and relative humidity (Burken and Schnoor, 1996). Plants can take up organic chemicals and transport them through the xylem to the shoots and leaves (Schnoor, 2002). Contaminants enter the roots together with water, similar to nutrients. Contaminants can be transported within the plant in two ways. One route, along the apoplast, or the free intercellular space, passes the Casparian strip barrier. Second route passes through the symplast, through cells and the plasmodesmata that connect the cells (Figure 1.2). Molecular mass of the substance, temperature, and hydrophilicity of the substance are some of the factors that dictate the rate and route of entry of contaminants (Kvesitadze et al., 2006).

Direct phytodegradation of PHC occurs when contaminants are broken down following uptake into the plant (Kamath et al., 2004). Plant uptake of PHC occurs when PHC

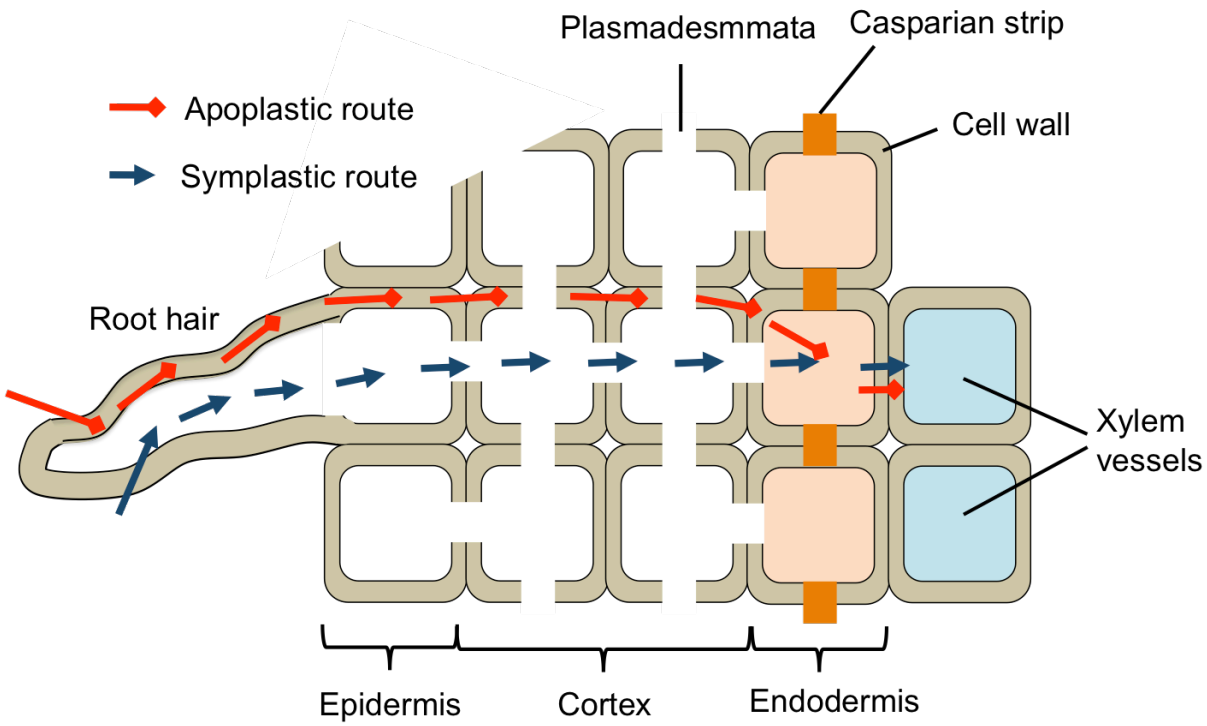


**Figure 1.2 Chemical transfer through root cells.**

Contaminants can be transported within the plant through the apoplast (diamond head arrow) or through the symplast (triangle head arrow). Apoplast is the free intercellular space. The route through the symplast, proceeds through cells and the plasmodesmata that connect the cells. The apoplast route passes the Casparian strip barrier. When Casparian strip is reached, chemicals must use the symplastic route. Adapted from Kvesitadze et al., (2006).

compounds are in the acceptable range of solubility, or hydrophobicity and size. For these compounds, the octanol water partition coefficient (log $K_{OW}$) is within the range of between 1-3. Highly hydrophilic chemicals (log $K_{OW}$ less than 1) are not sorbed to roots and are not actively transported through plant membranes (Schnoor, 2002). Hydrophobic chemicals (log $K_{OW}$ larger than 3.5) remain strongly bound to the root surface (solid surfaces) and cannot be readily translocated into the plant for transformation, because of their low concentration in the water phase and low bioavailability (Schnoor, 2002). Although, the ability of a contaminant to cross the lipid bilayer of the plasma membrane increases as the log $K_{OW}$ increases (Boese et al., 1999; Sung et al., 2001). Some hydrophobic compounds such as the PAH phenanthrene, pyrene (Gao and Zhu, 2004; Xu et al., 2009) and anthracene (Bałdyga et al., 2005) have been detected in plant roots and shoots. Shoot accumulation of PAHs is thought to occur through a foliar route however (Gao and Zhu, 2004; Xu et al., 2009) and it is thought that translocation of PAHs from roots to above ground portions of the plant would be minimal with compounds that possess four or more rings (reviewed in Schnoor, 2002). Other PAHs have also been predicted to be taken up by plants (e.g. chrysene (Sung et al., 2001)). Although their log $K_{OW}$ (chrysene, log $K_{OW}$ of 5.50) may fall out of the favorable range for water solubility, their lipophilic properties likely increase their uptake into root tissues (Sung et al., 2001).

When examining the complex PHC mixtures typically encountered in contaminated soils, the extent of plant uptake of the particular fractions can be inferred based on size and the log $K_{OW}$ values of compounds typically found in particular fraction. Compounds in F1 (C6-10) with log $K_{OW}$ 3-6 are expected to be taken up by the plants based on the previously discussed criteria and their small size. Fraction 2 (C>10-C16) with log $K_{OW}$ between 2.5 and 8 might be subject to direct plant uptake to some extent. F3 (C>16-C34) has a much higher expected log $K_{OW}$ (5-11) and based on this criteria will not be directly taken up by the plants, whereas F4 (C>16-C34) would have approximate log $K_{OW}$ values of above 11.5 and would likely remain bound to roots and soil particles (TPHCWG, 1998). In a study by Watts et al. exploring uptake of PAHs in salt marsh plants grown in contaminated sediments, it was found that heavier, complex compounds with higher log $K_{OW}$ were indeed less mobile (Watts

et al., 2005). A study of PHC uptake by poplar trees performed with twelve organic compounds commonly found at hazardous waste sites, resulted in predictive relationships for the translocation and partitioning to plant tissues. Translocation and partitioning estimation based on log $K_{OW}$ were most successful (Burken and Schnoor, 1998). These findings suggest that the majority of compounds in PHC mixtures will not be available for plant uptake and plant performed degradation as they are outside the range of absorption as defined by the log $K_{OW}$ values and will most likely be subject to microbial degradation (Aprill and Sims, 1990). Further, some studies with diesel-range compounds showed accumulation of PHC compounds at 10 g $kg^{-1}$ dry plant tissue (Kvesitadze et al., 2006).

Although the knowledge of degradation pathways in plants is much more limited than knowledge of bacterial degradation, many studies to date have provided evidence of plant organic contaminant degradation. The detoxification process has been most widely studied with herbicides (Schröder, 2007) and it is thought that detoxification pathways in plants somewhat resemble that of mammalian liver with the first two steps of detoxification and breakdown, and the final step as storage within the plant (Sandermann, 1994). This final step involves transport and storage into the vacuole and finally cell wall binding or excretion (Schröder, 2007). Phytodegradation has been demonstrated for some organic compounds such as trichloroethene (Collin et al., 2002) however this is not the case for many PHC components. Majority of PHC constituents have not yet been identified (only few hundred compounds have been identified (Howard et al., 2005)) and cannot be quantified in the soil, thus are not typically monitored in plants.

### 1.4.2 Rhizodegradation (Rhizoremediation)

Plants play a pivotal role in the phytoremediation of PHC by stimulating the growth of microorganisms inhabiting their root zone, the rhizosphere. Phytoremediation relies heavily on plant and endogenous microbial growth in the rhizosphere to degrade contaminants (Liste and Alexander, 2000; Hutchinson et al., 2001). The microbial composition of the rhizosphere differs greatly from the rootless, bulk soil in terms of both biotic and abiotic characteristics (Olson et al., 2003). From vegetation growth, plant roots greatly improve soil quality and

deposit large amounts of organic material that can be utilized by bacteria and fungi. In fact, microbial counts in the rhizosphere soils can be 1 to 2 orders of magnitude higher than in non-rhizosphere soils (Curl and Truelove, 1986; Atlas and Bartha, 1993); this phenomenon is referred to as the rhizosphere effect (Lynch, 1990). Plants exude large portions of their photosynthetically fixed carbon as sugars, amino acids, aromatics, organic acids, volatile compounds, vitamins and phenols. Plants also secrete carbohydrates and enzymes, deposit lysates such as cell walls, sloughed whole cells and gasses such as ethylene and $CO_2$ (Curl and Truelove, 1986; Whipps, 1989; Buyer et al., 2002). This plethora of compounds shapes the diverse microbial community that is supported by plant root systems. The plant-supported rhizosphere microbial community is actively involved in the decomposition and cycling of organic carbon in the environment (Whipps, 1989) and more importantly the degradation of contaminants as part of their normal metabolic processes (rhizoremediation or rhizodegradation) (Cunningham et al., 1995; Hutchinson et al., 2003).

As vegetation increases the total number of root-associated bacteria and fungi through exudation of compounds into the rhizosphere, the diverse microbial community shaped through this deposition may be capable of degradation of contaminants in the rhizosphere. Many bacteria naturally present in the soil environment have evolved the capability to biodegrade contaminants because they resemble naturally occurring organic compounds such as root exudates or allelopathic chemicals (Olson et al., 2003). For example, PAHs like phenanthrene and anthracene somewhat chemically resembles the root exudate morin and the allelopathic chemical physcion and would thus be expected to be degraded (Siciliano and Germida, 1998b). Bacteria that degrade aromatic compounds such as the PAHs are ubiquitous in the environment (Olson et al., 2003). Although the increased microbial numbers in the soil do not insure that degraders will also be present, the release of plant phenolics, that may resemble contaminants, may stimulate microbes with the ability to degrade organic contaminants (Siciliano and Germida, 1998b); albeit knowledge of this influence is still limited. Phenolic compounds exuded in the plant rhizosphere have been shown to promote the growth of PAH degraders (Donnely et al., 1994; Fletcher and Hedge, 1995; Siciliano and Germida, 1998b) and to induce PAH degrading genes in bacteria

(Reardon et al., 2001). Furthermore, particular vegetation may increase bioavailability of recalcitrant organic contaminants in the rhizosphere through production and release of organic acids and biosurfactants (Olson et al., 2003). In addition, bacteria, such as the commonly present soil bacteria *Pseudomonas*, may produce biosurfactants (e.g. rhamnolipids), which can further aid in the increase of bioavailability of recalcitrant organic contaminants. The biosurfactant-producing bacteria may be selected for in the rhizosphere (Reardon et al., 2001; Olson et al., 2003). Most importantly, for contaminants present at very low concentrations in the soil, where the contaminants would not be sufficient to spur the growth of degraders, when plants are present these contaminants can be co-metabolized with other carbon sources in the rhizosphere (Olson et al., 2003). This stimulation of contaminant degraders with rhizosphere compounds may bear similarity to priming with a low molecular weight PAH, which then stimulates degradation of high molecular weight PAHs (Olson et al., 2003). Taken together, the rhizosphere sustains a community where plants and microbes may synergistically degrade organic contaminants.

### 1.4.3 Effects of petroleum on plants

PHC phytotoxicity studies indicate that PHC effects on germination start at 5000 mg $kg^{-1}$ for sensitive plant species, with most species affected at 25 000 mg $kg^{-1}$. However, there are reports of plants being able to tolerate as much as 50 000 mg $kg^{-1}$ (reviewed in (Collins, 2007)). These numbers were obtained with unweathered PHC however, and tolerance is expected to increase in soils where weathering has occurred (see section 1.2.4). Plant biomass decreases with increasing petroleum concentration and further, plants may increase root biomass allocation with increasing PHC concentration, releasing increased amounts of carbon from roots (Nie et al., 2010). PHC negatively affect plant performance with respect to plant size, biomass and chlorophyll content (Nie et al., 2011b). Photosynthesis may be impacted with PHC, and PAHs have been shown to negatively impact photosynthesis and significantly changed the appearance of the plant cell nucleus (Huang et al., 1997; Marwood et al., 2001; Kvesitadze et al., 2006). PAHs were demonstrated to be phytotoxic, especially with environmentally relevant UV exposure (Huang et al., 1994). Further, low molecular-mass alkanes may swell the chloroplast and cause morphological and structural changes to

the membrane, with implications to photosynthetic apparatus (Kvesitadze et al., 2006). Thus, plants are expected to reveal a variety of toxicity symptoms in response to complex PHC mixtures although the effective concentration to toxicity are high, indicating PHC is not very toxic to plants.

### 1.4.4 PHC and plant effects on soil microorganisms

High PHC concentrations have been shown to significantly affect microbial populations and high contaminant concentrations often result in lower numbers of microorganisms relative to control soils, in some cases by as much as 75% lower (Banks et al., 2003a; Muratova et al., 2003a). However, soils contaminated with bitumen have been shown to have increased number of hydrocarbon degrading microorganisms (Muratova et al., 2003a). The increase in hydrocarbon degraders may be due to selection for these types of organisms in impacted soils. Specifically, the bacterial PAH catabolic genes and BTEX degradation genes have been shown to be located on plasmids, which sometimes carry genes for chemotaxis towards the contaminant (reviewed in (Kuiper et al., 2004) and (Leahy and Colwell, 1990)). Some of the plasmids have been shown to be self-transmissable suggesting their presence as a probable cause of the adaptation of the indigenous microbes towards PAH or other pollutants as (Kuiper et al., 2004).

There have been opposing views whether the relationship between contaminant degraders and plants is non-specific (i.e. the nutrients released from roots indiscriminately stimulate microbial growth) (Siciliano and Germida, 1998b). However specificity has been suggested in numerous cases. Plants will increase degradation of specific contaminants by releasing polyphenolic compounds that stimulate microbial enzymes (Siciliano and Germida, 1998b). Plants may also promote growth of degrading microbes through specific exudation of polyphenolic compounds (Fletcher, 2001). There has been evidence that alfalfa plants have a stronger PAH degrading community, in part perhaps due to what compounds are exuded into the rhizosphere (Muratova et al., 2003b; Phillips et al., 2006), although this may not be specifically increased by PHC. Altogether, there is strong evidence that microbial communities adapt to the presence of PHC and often PHC degradation is increased in

previously contaminated soils (reviewed by Leahy and Colwell, 1990). When plants are present, these previously present communities may proliferate.

## 1.5 Requirements for phytoremediation of PHC

Phytoremediation of PHC is highly appealing due to its ease of implementation and cost efficiency. Some of the disadvantages experienced with transfer of this biotechnology to the field are longer remediation times, its climate dependence and variable results (Kamath et al., 2004). The variety of mechanisms through which phytoremediation can take place, allow for its use to remove a wide range of contaminants that vary widely in chemical and physical properties. Regardless of the type of contaminant that is being removed a number of criteria must be met for phytoremediation to succeed. Firstly, contaminants must be within the rooting depth of plants, which falls between 2 to 10 feet for grasses, annuals and shrubs and as far as 20 feet for certain tree species (EPA, 2000). However, contaminants deeper in the soil can be brought to the surface. Plant growth must be supported at the contaminated sites; therefore environmental conditions such as rainfall and temperature must be evaluated. Finally, contaminants must be treatable with phytoremediation processes, thus issues such as bioavailability must also be considered.

To aid in determining the applicability of phytoremediation for a test site, Collins provided a number of essential criteria for successful remediation (2007). These included acceptable conditions for adequate plant growth, namely sufficiently long growth season and precipitation. Further, present toxic fractions, such as BTEX, would require amendments, such as *in situ* volatilization to remove the lighter PHC fractions (for BTEX characteristics see section 1.2.2). Collins suggests PHC levels not to exceed 3% PHC (Collins, 2007) with the understanding that weathering my result in loss of toxicity, thus increasing this threshold. This suggested number is not based on empirical evidence, however, and would result in fewer sites phytoremediated if this guideline was followed. Although the time available for remediation of a site may be constrained by the length of growing season, the success of field applications with short growing seasons should be further evaluated. The limited depth of the root system, and plant growth resulting from short growth seasons can be overcome by

addition of appropriate growth promoters (Glick, 2003). Presence of BTEX may not justify considerable cost increases for additional remedial treatments if this toxicity can be overcome through plant growth promotion (Glick, 2003; Huang et al., 2005). Taken together, many parameters routinely disregarded in phytoremediation applications should be thoroughly evaluated, as this is a promising technology, which may aid in restoration in many PHC contaminated sites.

### 1.6 Plants used in phytoremediation of PHC

For phytoremediation to be effective, it is desirable that the vegetation is fast growing and robust, easy to sow and maintain, and that plants are able to transform the contaminants to compounds that are less toxic or non-toxic (Schnoor, 2002). Plant species that are less sensitive to the contaminants are desirable because they are capable of surviving in contaminated soil, and generating large amounts of biomass, despite the toxic effects of the contaminants. The many plant species tested for phytoremediation applications to data can be fall into the main categories of grasses (tall fescue, annual ryegrass) and other herbaceous plants (Indian mustard, sunflower), legumes (clover, alfalfa), trees (hybrid poplars, cottonwoods, willows), aquatic plants (parrot feather, water lettuce, duckweed) and less so shrubs and vines (Huysen et al., 2004; Newman and Reynolds, 2004; Ran et al., 2004; Boonyapookana et al., 2005; Gujarathi et al., 2005; Kirk et al., 2005; Parrish et al., 2005; Hall et al., 2011). Trees are often used when ground water contamination is a concern and when contaminants are deeper than the reach of grass roots (Novak et al., 2000) (Parrish et al., 2005) and have been used with petroleum contamination. Aquatic plants have been extensively studied with halogenated compound (Jacobson et al., 2003) but their application in phytoremediation of PHC would be limited.

Grasses are often preferred because their fibrous root systems allows for establishment of a large microbial population as they may contain more sites (larger surface area) for nutrient exudation and ferry microorganisms through soil (Aprill and Sims, 1990; Kirk et al., 2005; Collins, 2007; Kuiper et al., 2004; Schröder, 2007). Grasses provide a vast amount of fine roots, which are effective at binding, transforming, and degrading hydrophobic contaminants

such as PAHs, PHC and BTEX. Grasses are often less sensitive to contaminants than other plant species and their quick growth provides surface cover to prevent transfer of contaminants with dust (Schnoor, 2002; Collins, 2007). There is not a general agreement on a most effective PHC degrading grass species that are also hydrocarbon tolerant, however there is a growing body of literature examining many plant species. A tool developed at University of Saskatchewan, the Phytopet© database summarizes hydrocarbon tolerance, degradation capability and any studies performed with a given plant species. The database allows researchers to submit their findings making it a valuable resource (www.phytopet.usask.ca). Many phytoremediation applications use various grasses combined with legumes. The research on the efficacy of mixtures versus single species is ongoing. Phillips et al. (Phillips et al., 2006) examined single species versus mixtures and showed that mixtures had a more significant impact on the rhizospheric community, including most culturable PAH degraders than other treatments, but it was not the most successful when PHC degradation was examined. Grasses such as *Festuca arundinacea, Lolium perenne, Cynodon dactylon* have been shown to be viable for revegetating oil and brine spills (Colgan et al., 2002). Kirkpatrick and colleagues (2006) suggested using a sequence of cool-warm-cool-season grasses and legumes to ensure that at all times during the growing season actively growing roots are present in contaminated soil, thus degradation would be increased (Kirkpatrick et al., 2006). Although this approach has not yet been tested in the field, some findings indicate that actively growing plants in vegetative as opposed to reproductive growth stages, may have higher degradative potential, due to increased number of microbes with PHC degrading genes (Nie et al., 2011b).

Alfalfa is one member of the Fabaceae family, commonly known as the bean family. The superior performance of some legumes, especially alfalfa can be attributed to their creation of soil macropore spaces, which increases oxygenation of the soil and thus degradation by aerobic microbes (reviewed in Hall et al., 2001). Alfalfa, has previously been used for phytoremediation of PHC (Kirk et al., 2002; Muratova et al., 2003b). The Research Technologies Development Forum (RTDF) recommends a combination of cool and warm season grasses and a legume for phytoremediation applications. The legumes provide

nitrogen, which can be beneficial for microbial action (Collins, 2007) and also grow in N poor soils where other plants may not be able to thrive (Adam and Duncan, 2003). Adding legumes in a phytoremediation seed mixture may act to improve the N economy of the contaminated soil if senescent plants are tilled into the soil (Hall et al., 2011). In one study, alfalfa showed more degradative potential of rhizosphere microflora than reed plants (Muratova et al., 2003b). However, Phillips et al. (2006) examined alfalfa alone and in the recommended RTDF mix. It was found that although alfalfa and alfalfa containing mixtures had the most significant impact on the rhizospheric community, including more culturable PAH degraders than other treatments without alfalfa, it was not the most successful when PHC degradation was examined (Phillips et al., 2006). Contrasting findings were observed where diesel fuel remediation was examined and addition of legume resulted in faster disappearance than in non-legume treatments (Palmroth et al., 2002). Thus, legumes show great potential in phytoremediation applications but their effectiveness is still inconclusive.

Thus, plant selection for treatment of PHC contamination in soil should be based on many considerations including: root morphology, climate suitability, hydrocarbon tolerance, microbial community sustained by chosen plants, ease of growth and exudate quality.

## 1.7 Phytoremediation amendments

The increased interest in phytoremediation led to a number of approaches where addition of amendments would increase degradation, mostly through biomass increase and thus expedite remediation.

### 1.7.1 Fertilizer

Fertilizer applications are often critical in phytoremediation and insight into optimal approaches in fertilizer treatment at PHC contaminated sites could contribute to success in the field. Although carbon is most often the limiting nutrient in the environment, in PHC contaminated soils carbon may be abundant to resident microorganisms while other nutrients may become relatively limited as they are consumed. Thus, soils contaminated with organic contaminants are often limited in nutrients, and both plant growth and microbial degradation

will be restricted (Steffensen and Alexander, 1995; Hutchinson et al., 2003; Rentz et al., 2003). Naturally, there was interest in applying and modifying fertilizer rates in hopes of increasing plant growth and remediation. From bioremediation studies, it has been reported that microbial degradation decreased when nitrogen (N) and phosphorous (P) were added. This suggests the importance of slow growing oligotrophs, capable of withstanding low nutrient levels, acting in an important biodegrader role. The addition of nutrients may promote the growth of faster growing non-degrading microbes, which may outcompete oligotrophs, causing this decrease in biodegradation (Morgan and Watkinson, 1992; Steffensen and Alexander, 1995; Johnson and Scow, 1999). Atagana et al. found that a lower N supplement was more effective at enhancing microbial growth than higher N supplementation (Atagana et al., 2003).

Hutchinson et al., (2001) found that best PHC degradation in vegetated treatments occurred where application of inorganic N fertilizer was in excess of what is needed to maintain plant growth (Hutchinson et al., 2001). Other studies also indicated a more pronounced decrease in PHC concentrations in fertilized vegetated plots than unfertilized vegetated plots ((Kirkpatrick et al., 2006; Palmroth et al., 2006). The idea behind overfertilizing is to decrease the competition for nutrients between microorganisms in the soil and plants. Another viewpoint is that plants and microorganisms may also be in competition for inorganic nutrients (Siciliano and Germida, 1998b) or organic nitrogen (Kaye and Hart, 1997). In a study by Hutchinson et al. (2001), microbial growth in the rhizosphere was not affected by fertilizer concentration and only plant growth was increased with increasing fertilizer concentrations. Further studies in this direction suggest that fertilizing with inorganic N may lead to lower plant growth and deterioration of soil quality if the N application rates are too high (Kirkpatrick et al., 2006), or result in undesirable osmotic effect on PHC biodegradation from fertilizer salts partitioning into the pore water (reviewed in (Walecka-Hutchinson and Walworth, 2007)). A resolution to this may be to only add nutrients when they are lacking, instead of overfertilizing to increase microbial growth. Supporting of this approach is the fact that often plants that are fertilized and watered well do

not develop extensive root systems (Schröder, 2007). These studies emphasize the importance of optimizing fertilizer applications for phytoremediation applications.

In recent years, several studies have examined the use of organic versus inorganic fertilizer in phytoremediation application providing insights on this subject. Nie et al. (2011a) found that petroleum negatively impacted nitrogen mineralization rates but dissolved organic nitrogen was positively affected (Nie et al., 2011b). Another study by the same group (Nie et al., 2011) indicated that plants can adjust their use of fertilizer, possibly lowering the competition with microbes for inorganic fertilizer, by using organic fertilizer, possibly through increased arbuscular mycorrhiza fungi colonization (Killham, 1994; Nie et al., 2011b). The use of a combination of organic and inorganic fertilizer would be in agreement with agricultural applications where combined applications of inorganic and organic fertilizer are superior to single form of inorganic N (Nie et al., 2011a). This knowledge may lead to the use of combination of inorganic and organic fertilizers for phytoremediation.

### 1.8 Plant growth promoting rhizobacteria

Soil has numerous microbial inhabitants including fungi, protozoa, and algae, with the most pronounced being bacteria (Curl and Truelove, 1986). The bacterial consortium occupying the rhizosphere is enormous with some representative genera *Agrobacterium*, *Achromobacter, Asorhizobium, Bacillus*, *Burkholderia, Cytophaga* and *Pseudomonas*, (Campbell and Greaves, 1990; Glick et al., 1999). Some of these can be beneficial to plant growth, directly or indirectly (by preventing damage to plants or providing plants with a synthesized compound or facilitating nutrient uptake) (Glick et al., 1999). Bacteria that provide benefits to plants may be symbiotic bacteria, which are closely associated with plants (for example *Rhizobia*), and also bacteria that are free-living in the soil. A particular group of free-living bacteria found in the rhizosphere that are beneficial to plants are referred to as plant growth-promoting rhizobacteria (PGPR) (Glick et al., 1998). They can belong to any of the genera listed above, excluding symbiotic bacteria. PGPR have the ability to reduce plant stress level directly by providing the plant with a compound that is made by the

bacterium or facilitating uptake of nutrients. Importantly, PGPR can decrease ethylene levels, a plant hormone thought to be a mediator of stress response (Glick et al., 1998).

Many PGPR strains can synthesize a phytohormone, indoleacetic acid (IAA) that acts to enhance various stages of plant growth (Glick et al., 1998). IAA is taken up by the plant, where it can stimulate plant cell proliferation and cell elongation. It can also stimulate the activity of the enzyme 1-aminocycloproane-1-carboxylic acid (ACC) synthase, which is responsible for conversion of S-adenosylmethionine (SAM) to ACC, a precursor for the plant stress hormone – ethylene (Glick et al., 1998). The increase in ACC synthase activity leads to a rise in the concentration of ACC, and a higher availability of ACC to the bacteria through plant exudation of this molecule. Bacterial strains that possess ACC deaminase can use ACC as a source of nitrogen, effectively lowering the outside concentration of ACC (Glick, 2003). To maintain the concentration gradient of internal and external ACC and to compensate for bacterial utilization of this compound, the plant must increase ACC exudation. When the internal ACC concentration is lowered, the downstream effect is decreased production of ethylene and lowering of the stress responses induced by this molecule in the plant. When added as a seed inoculant, the two *Pseudomonas* strains used throughout the study, UW3 and UW4 promote plant growth leading to a larger, healthier, richer rhizosphere, thus increasing the potential for remediation. The supplementation of crops or soils with PGPR was first reported in the 1950s (reviewed in Zehnder et al., 2001). PGPR were first used to improve crop fertility by increasing the amount of nitrogen available to the plant. PGPR are now used as biological control agents for the suppression of soil born pathogens (Zehnder et al., 2001), plant growth promotion in field, and remediation (Huang et al., 2005; Gurska et al., 2009).

One of the challenges faced in phytoremediation of PHC is phytotoxicity, resulting from toxic PHC components such as PAHs (Baek et al., 2004; Alkio et al., 2005; Palmroth et al., 2006). Remediation is dependent on plant growth, and is thus impeded if plants cannot grow due to high levels of toxicants. Similarly, populations of bacteria, including PGPR, may be weakened by prolonged exposure to organic contaminants (Huang et al., 2004b). A way to alleviate these difficulties is by adding PGPR during seed application to aid plant growth.

PGPR used can be isolated from native soils, so that upon re-inoculation into the soil the soil ecosystem structure may be preserved. PGPR can relieve environmental stress brought on by organic pollutants and can promote plant growth, especially root elongation, which is crucial for successful phytoremediation.

### 1.9 Plant and microbe communication

During plant-microbe interactions, there are a number of possible ways of communication between the participants. For PGPR to exert a beneficial effect on plants and for plants to stimulate indigenous bacterial growth there is a need for effective signaling to spur the initial colonization and to maintain intra-species contact. Plant-microbe associations can be nonspecific, as is the case with root exudates from normal plant processes stimulating the expansion of microbial consortia (Figure 1.3). When root exudates are efficiently utilized, the microbes consuming then have an opportunity to thrive in the rhizosphere. Curiously, plants and bacteria can also form specific associations, where the plant provides bacteria with a specific carbon source that induces the bacteria to reduce the phytotoxicity of the contaminated soil (Fletcher and Hedge, 1995).

Studied plants naturally produce over 8000 different phenolic compounds. The abundance and diversity of these compounds make phenolic compounds ideal candidates to mediate plant and soil microorganism interactions. It has long been established that flavonoids, common 15-carbon plant secondary metabolites, are exuded in the rhizosphere. Flavonoids impact microbes by either inducing microbial gene expression, or through their antimicrobial properties and finally, simply as nutrients (reviewed in Pillai and Swarup, 2002). Flavonoids, following synthesis, are glycosylated and stored in vacuoles; it has been hypothesized that these will eventually be released to the rhizosphere during root senescence (Shaw et al., 2006). Thus, there is great potential for flavonoids to exude influence on the microbial community in the soil.

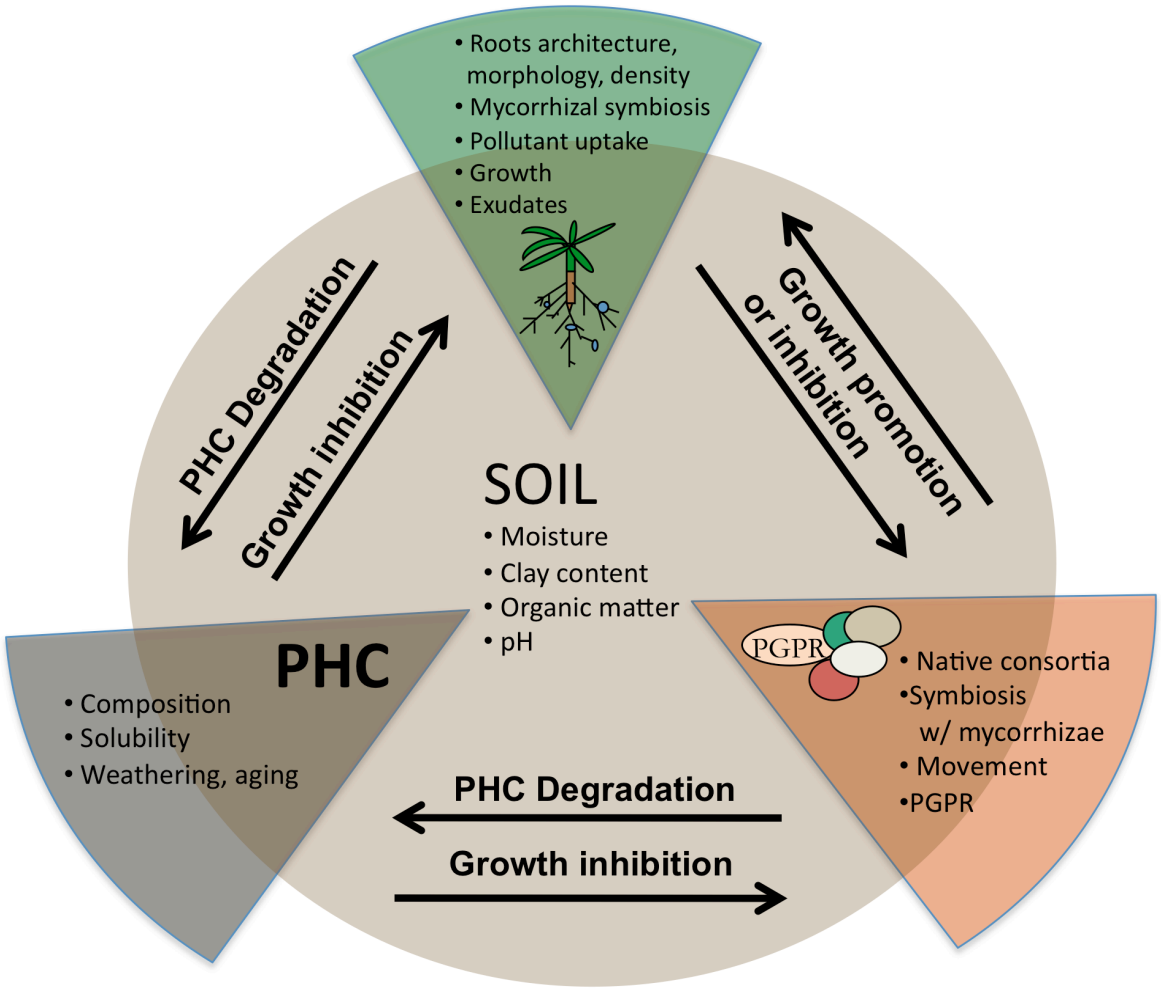


**Figure 1.3 Overview of processes and factors in phytoremediation of petroleum hydrocarbons.**

## 1.10 Field applications of phytoremediation of PHC

Phytoremediation research has grown extensively over the past decade, with many PHC remediation bench scale studies with flare pit soil (Rutherford et al., 2005) army test sites contaminated with diesel fuel and heavy oil over 20 years (Siciliano et al., 2003), soil spiked with petroleum sludge (Hutchinson et al., 2001), diesel spiked soils (Palmroth et al., 2002), and with PGPR enhanced phytoremediation of PHC (Huang et al., 2005). What is still thought to be lacking are sufficient successful field trials with high starting contaminant concentration that will help to accept this remediation technology as a viable method for use by industry and regulatory bodies.

Some studies that have been performed to date include work by Nedunuri et al. (2000) that showed significant remediation at sites contaminated with crude oil. This study was performed with a recent spill that would likely result in high availability of contaminants for plant degradation. At a series of sites, Schwab and Banks (1999) investigated disappearance of PHC contaminated with crude oil, diesel fuel and petroleum refinery wastes with PHC levels from 1,700 to 16, 000 mg $kg^{-1}$ PHC. Specifically, at a crude oil contaminated site, after 21 months of growth, annual rye-soy bean rotation plot and a St. Augustine grass-cowpea rotation plot was significantly better at PHC removal than sorghum-sudan grass or unvegetated control plots (Schwab and Banks, 1999). At a refinery waste site, vegetated plots appeared to increase PHC remediation more so than unvegetated plots, although the experiment length was not sufficient to analyze these results statistically (Schwab and Banks, 1999). Andreotti et al. (2001) followed remediation of PHC contaminated soils over three growing seasons and found that the rate of degradation became significant in the second growing season; it was also observed that corn, sorghum and triticale were better than clover and alfalfa. Euliss and colleagues (2008) found that in a 12-month field study no significant differences were found between vegetated and unvegetated treatments when PHC and PAH concentrations were examined with starting PHC concentrations of ~20-80 g $kg^{-1}$. Although a greenhouse trial with the site soil indicated significant remediation with some plant species (Euliss et al., 2008). This indicates a large discrepancy between greenhouse and field performance. Palmroth et al. (2006) found that after four growing seasons, with starting PHC

concentrations of ~ 11 000 mg $kg^{-1}$ and metal contamination resulting from bus maintenance activities, significant 60-65% remediation was observed, however remediation was not significant in the first 12 months of study (Palmroth et al., 2006). Others found 6-40% of PHC removed in a 5-month study (Porta et al., 1999) and 65% in a one-year study (Hutchinson et al., 2001), although in both studies these numbers were within 10% of landfarming and unvegetated controls respectively. Yet another study did not see increased removal of PHC with vegetation (Angehrn et al., 1999). Taken together, these studies indicate there are many variable results in the field with some prevailing trends and that although significant remediation was achieved, greenhouse results may not be easily extrapolated to the field. Further, phytoremediation proceeds at a slower pace in the field compared to the greenhouse, thus any amendments to expedite the process may make this remediation option more effective. Transfer to field and extensive field testing is critical in evaluating phytoremediation as a viable remediation option for PHC contaminated sites. There is a need for further investigation of PHC phytoremediation in the field where contamination is weathered, which would be more representative of sites that may qualify for phytoremediation.

Huang et al. investigated the application of PGPR in combination with landfarming practices to accelerate remediation of PHC (Huang et al., 2005) and PAHs (Huang et al., 2004b) in contaminated soils. The initial greenhouse testing indicated that combination of microbe additions and landfarming practices greatly increased degradation rates. If successfully transferred into the field, this phytoremediation enhancement would likely increase remediation achieved in each year of plant growth and expedite the clean up of PHC contaminated sites. Thus, phytoremediation applications with the use of PGPR in the field must be investigated further, including comparison of remediation kinetics between standard phytoremediation applications. It would be desirable to expedite phytoremediation so that significant remediation could be observed as early as year one of field applications and this could be achieved with PGPR enhanced phytoremediation, based on the success of the greenhouse trials.

## 1.11 Rationale and Objectives

Phytoremediation of PHC is a complex process where many of the variables, both known and unknown, often make it difficult to draw clear conclusions. First and foremost however there is a need for demonstration of this biotechnology in the field, where if successful, this will become the driving force for optimization of variables in phytoremediation. Current greenhouse success with applications of PGPR must be transferred into the field, where optimal growing conditions that are observed in the greenhouse are less likely to be encountered. Also, PHC spiked soil used in many greenhouse trials cannot be extrapolated to the field, where the majority of the contamination will be highly weathered. PGPR may present a good alternative to chemical fertilizers without the drawbacks of low use efficiency of N, and decrease in P solubility (Adesemoye et al., 2009). Overfertilizing may lead to outcompeting of PHC degraders by other microbes reducing the efficiency of the process, or, with continued fertilization, leaching of nitrates to ground water (Adesemoye et al., 2009). These potential problems would not happen with PGPR. The ability of PGPR to increase PHC degradation must therefore be investigated in the field extensively.

The current knowledge of xenobiotic metabolism in plants stems mostly from crops and a few ornamental plants; many reaction mechanisms were derived from herbicide metabolism. Understanding the molecular mechanisms of detoxification may help increase the efficiency of phytoremediation and this in turn may widen its acceptability as a remediation method. Further, PGPR have shown promise as biofertilizers and in degradation of PHC in greenhouse trials, as some PGPR increase uptake of nutrients such as nitrogen (N), phosphorous (P) and through siderophore synthesis, iron (Glick et al., 1998). It is unknown however, how PGPR may assist in phytoremediation of PHC. It would be of great benefit to investigate the PGPR interaction with plants under PHC stress. The effect of PGPR on the other microorganisms in the PHC contaminated soils must also be investigated. This will increase our understanding of why this system performs better than standard phytoremediation applications and allow for further improvement to field applications.

The numbers obtained with unweathered PHC indicate that phytotoxicity begins at 5 000 mg $kg^{-1}$ for some plant species, with most species affected at 25 000 mg $kg^{-1}$ with some

plants being able to withstand as much as 50 000 mg $kg^{-1}$ (Collins, 2007). Phytotoxic effects such as decrease in plant biomass, plant size, chlorophyll content and photosynthetic performance would give a good indication of plant performance on PHC contaminated soils. Specifically, photosynthesis may be impacted with PHC, as PAHs have been shown to negatively impact photosynthesis (Marwood et al., 2001; Kvesitadze et al., 2006). These parameters should be investigated at sites where high levels of high molecular weight compounds accumulate, especially at weathered sites where, despite high levels of PAHs, their decreased bioavailability may make these mixtures less toxic. Plants are expected to reveal a variety of toxicity symptoms in response to complex PHC mixtures. Although plant performance in phytoremediation is critical, microbial community is thought to be predominantly responsible for degradation of PHC, thus their performances in a system where PGPR inoculation is used should be investigated. Most importantly, the impact of PGPR on microbial community structure is of interest, and the changes it imposes on the community structure and population and their permanence will be integral in decision making by regulators.

PHC measurements suffer from inherent variability that comes with the methodology used. The appropriate techniques must be employed to establish if phytoremediation is indeed occurring. For example, in a study by Muratova and colleagues (2003a), paraffinic bitumen was measured with the IR spectroscopy, which determined alkanes or/and alky-containing hydrocarbons. These data suggested that plants had no significant effect on remediation of PHC. A subsequent study by the same group revealed that plants distinctly enhanced the biodegradation of persistent polycyclic aromatic hydrocarbons (Muratova et al., 2003a). Reliable PHC analyses will help establish phytoremediation as a viable remediation alternative. Thus, a closer look at the dynamic interaction of a number of factors in field applications of phytoremediation of PHC with and without PGPR will provide meaningful insight into optimizing this remediation strategy in the field and expedite its application to PHC contamination in Canada and worldwide.

Based on the above, the objectives of this thesis revolved around investigating the use of PGPR to enhance phytoremediation in PHC contaminated soils. The study was divided into a

field component and a laboratory component. In the field, the objective was to perform phytoremediation trials at sites with both low and high levels of PHC contamination and evaluate plant and microbial performance temporally during those trials. In the laboratory, soils from remote Northwest Territories were used to isolated native PGPR species and these were characterized. This was performed to expand application of PGPR enhanced phytoremediation to sites where phytoremediation may not otherwise succeed due to factors such as short growing season. Further, soils from field trials were used to investigate physiological and genetic changes in a model plant species, *Secale cereale*, with and without the presence of PGPR. The interactions between plants and microbes with PHC stress were investigated by investigating changes in phenolics and flavonoid levels with and without PHC and PGPR. The overarching goal was to gain more in depth understanding of the complex interactions in the phytoremediation triad of PHC, plant and bacteria.

# Chapter 2

# Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste

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## 2.1 Overview

Phytoremediation of petroleum hydrocarbons (PHC) has the potential to be a sustainable waste management technology if it can be proven to be effective in the field. Over the past decade, a plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation system (PEPS) was developed that, following extensive laboratory testing, was shown to be effective at remediating PHC from soils. This system consists of physical soil manipulation and plant growth following seed inoculation with PGPR. PGPR elicit biomass increases, particularly in roots, by minimizing plant stress in highly contaminated soils. Extensive development of the root system enhances degradation of contaminants by the plants and supports an active rhizosphere that effectively promotes PHC degradation by a broad microbial consortium. Following promising greenhouse trials, field tests of PEPS were performed over a period of three years at a Southern Ontario site (~130 g $kg^{-1}$ PHC) used for land farming of refinery hydrocarbon waste for many years. The low molecular weight fractions (the Canadian Council of Ministers of the Environment [CCME] fractions 1 and 2) were removed through land farming and bioremediation; the high molecular weight, recalcitrant fractions (CCME fractions 3 and 4) remained at high levels in the soil. Using PEPS, we substantially remediated fractions 3 and 4, and lowered PHC from 130 g $kg^{-1}$ to ~ 50 g $kg^{-1}$ over a three year period. The amount of plant growth and extent of oil remediation were consistently enhanced by PGPR.

## 2.2 Introduction

The large number of petroleum products released into the environment characteristically consist of aliphatic, aromatic, heterocyclic and asphaltene/tar hydrocarbons ranging in size from C6 to C>50. These compounds, collectively labeled petroleum hydrocarbons (PHC), have been classified into various fractions, where fraction 1 (F1) contains C6 – C10; fraction 2 (F2), C10 – C16; fraction 3 (F3), C16 – C34; fraction 4 (F4), C34 – C50+ and higher fractions contain compounds C>50 (CCME, 2001a). F1 and F2 are volatile or semi-volatile, whereas F3 and F4 are very hydrophobic and recalcitrant to breakdown. Compounds from F3 are often highly toxic and are regulated due to their mutagenicity and carcinogenicity (CCME, 2001a; Farrel-Jones, 2003).

Toxicity of PHC has been a motivating force in finding sustainable biological methods of remediation for these compounds. Among the many existing biological remediation approaches, strategies such as land farming or *in situ* bioremediation are of great interest. However, these methods are often not capable of sustaining dense growth of indigenous microorganisms nor those added to promote degradation, due to nutrient restrictions and toxicity of contaminants (Leahy and Colwell, 1990; Strand et al., 1993; Alexander, 1999; Boopathy, 2000). Phytoremediation provides a viable option for efficient and cost-effective remediation of contaminated soils because adding plants to the remedial system addresses the biomass production limitations of other biological methods (Cunningham and Ow, 1996; Salt et al., 1998; Singh and Jain, 2003). Plants exude soluble nutrients that can be utilized as an energy source by microorganisms, and the plant roots provide a substratum to increase microbial growth in the rhizosphere (Curl and Truelove, 1986; Shann and Boyle, 1994; Cunningham and Ow, 1996; Alkorta and Garbisu, 2001; Burken, 2003; Hutchinson et al., 2003). Many plant species, grasses in particular, have extensive root systems that can generate substantial biomass into large volumes of soil (White et al., 2006), thus promoting an active rhizosphere, which can consume soil contaminants. Indirectly, the presence of the plants enhances degradation through changes in the soil profile induced by root growth, such as creating channels for water/air penetration, increased surface area for microbial growth and changes in pH in response to nutrient status (Hutchinson et al., 2003).

Although phytoremediation has potential as a viable remediation strategy for persistent organics, several limitations hinder its widespread application in the field (Cunningham and Ow, 1996; Salt et al., 1998; Glick, 2003). Contaminants can affect plant photosynthesis, respiration and metabolism resulting in low plant biomass and, subsequently, low remediation (Medina et al., 2004). To increase plant biomass in contaminated soils, plant growth promoting rhizobacteria (PGPR) can be used to mitigate plant stress responses, and enhance degradation of contaminants (Siciliano and Germida, 1998b; Huang et al., 2004b; Huang et al., 2005). Many PGPR have the ability to consume 1-amino-cyclopropane-1-carboxylic acid (ACC), a precursor to the plant stress hormone ethylene. This degradation is directly dependent on the bacterial activity of ACC deaminase (Glick et al., 1998). Lowered ethylene biosynthesis in plants can facilitate growth under stress conditions in highly contaminated soils (Glick, 2003). These PGPR act by alleviating contaminant induced stress, thus allowing adequate biomass accumulation for acceptable rates of remediation (Glick, 2003; Huang et al., 2004b; Huang et al., 2005). In greenhouse studies, PGPR have proven effective for improving phytoremediation of petroleum and other contaminants (Huang et al., 2004b; Huang et al., 2005; Muratova et al., 2005). Although some field scale phytoremediation studies have emerged (Kamath et al., 2004), the benefits of using PGPR in PHC remediation have not been fully validated in the field (Reed and Glick, 2004; Zhuang et al., 2007).

A PGPR enhanced phytoremediation system (PEPS) was developed specifically for the removal of toxic, persistent and recalcitrant contaminants from soil (Huang et al., 2004a, 2004b; Huang et al., 2005). In PEPS, phytoremediation is performed with the addition of PGPR to increase plant biomass in contaminated soils via alleviation of plant stress by ACC deaminase activity. Based on the success of the greenhouse experiments, where PEPS outperformed microorganisms alone and plants alone (Huang et al., 2004b; Huang et al., 2005), the objective of the research reported here was to test the efficacy of PEPS in the field at an oil refinery land farm site in Sarnia, Ontario, Canada. This was carried out between April 2004 and October 2006. This particular site was chosen due to high contaminant levels, which presented a tremendous remediation challenge for PEPS; a literature search revealed that no attempts to phytoremediate concentration as high as 130

g $kg^{-1}$ PHC have been reported. Parameters such as plant growth and photosynthesis were assessed as indicators of plant health and remediation. Most remediation technologies are site specific, and successful application depends on careful experimental design and adjustments based on changes in soil and chemical properties as the remediation process progresses (Khan et al., 2004). Indeed, we accelerated the transition of PEPS from laboratory to field trials by applying lessons learned in the lab and field immediately in the subsequent years of field trials reported here. Most importantly, plant growth (annual ryegrass, tall fescue, barley and fall rye) in the field was enhanced by PGPR, remediation was commensurate with plant growth, and remediation appeared to follow first order kinetics.

## 2.3 Materials and Methods

### 2.3.1 Site description

Field tests were performed for three consecutive years from May 2004 at an oil refinery land farm in Sarnia, Ontario, Canada. At this site, petroleum sludge was regularly spread on the soil horizon and tilled over the last 20 years, between the months of May and October. Two rectangular plots of 130 by 40 meters each, i.e. site 1 and site 2, were divided into experimental plots with paired block designs (Figure A1, Appendix A). Both sites were somewhat homogenous in PHC concentrations. Constant land farming prevented establishment of any vegetation and plant growth had not been attempted on this site prior to the study. At the onset of the trial, PHC levels were approximately 130 g $kg^{-1}$, ranging from 99 to 148.5 g $kg^{-1}$.

### 2.3.2 Soil remediation using PEPS

The remediation strategy consisted of physical manipulation of the soil performed through tilling, sunlight exposure (aeration/photooxidation), and plant growth with PGPR. Plants with PGPR treated seeds are hereafter referred to as PGPR plants or as PGPR treated plants. Plants were selected based on their suitability to the Southern Ontario climate, and their tolerance to hydrocarbon stress (Huang et al., 2004a; Huang et al., 2004b; Huang et al., 2005). In 2004, site 1 was planted with annual ryegrass (*Lolium multiflorum*) with and without PGPR treatment. Subsequently, site 1 was chosen to

evaluate remediation kinetics. Temporal analysis was performed with PGPR treatment only, and there was no direct comparison between ± PGPR treatments for 2005 and 2006 for site 1. In 2005, annual ryegrass and tall fescue (*Festuca arundinacea,* var. Inferno) were planted on site 1 and fall rye (*Secale cereale*) was used to overseed in the fall. Tall fescue was added because it is a perennial plant species and fall rye was added to introduce plant growth in the spring (fall rye germinates early in the spring, prior to planting with annual ryegrass and tall fescue). In the spring of 2006, barley (*Hordeum vulgare*) was added to the annual ryegrass/tall fescue mixture. Barley was added because it grows quickly in the spring, and matures early in the season, making way for the slower growing annual ryegrass and tall fescue. The following plants, and plant mixtures, were used in this study at site 2: year two (2005) - annual ryegrass, annual ryegrass and tall fescue mixture, barley and fall rye mixture; year three (2006) - annual ryegrass and tall fescue mixture, and annual ryegrass, tall fescue and barley mixture. Seeds were bought from Ontario Seed Co., Waterloo, Ontario, Canada (annual ryegrass, tall fescue, fall rye), and from Cribit Seeds, West Montrose, Ontario, Canada (barley). Every year, ± PGPR treatments were tested on site 2. Seed planting density was approximately 300 seeds $m^{-2}$. Plants were allowed to grow for the entire growing season (~150 d) and were irrigated as needed. The control area consisted of soil that was not vegetated and received tilling treatments concurrently with planted sites, at the beginning of every growing season. Treatment with herbicides was not necessary to keep this area free of vegetation.

### 2.3.3 Seed treatment with PGPR

Two PGPR *Pseudomonas* strains, UW3 and UW4 (Glick et al., 1995) were introduced to the rhizosphere by applying them to seeds. UW4 has previously been identified as *Pseudomonas putida* (Hontzeas, 2004), and UW3 has been identified as *Pseudomonas* sp. UW3 has been characterized using biochemical tests, 16S rDNA sequence and other biochemical and microbiological tests and found its closest species matches to be those of non-pathogenic bacteria from genus *Pseudomonas*. Both strains are susceptible to tetracycline and kanamycin, and will not grow at 37$^{o}$C. For seed treatment, bacterial strains were grown in tryptic soy broth (30 g $L^{-1}$, Fisher Scientific, Ottawa, Ontario, Canada) at RT for 24 hours, until they reached an absorbance of 2-3 at 600 nm. A Hege

11 Liquid Seed Treater (Wintersteiger, Saskatoon, Saskatchewan, Canada) was used to apply PGPR to the seed. The bacterial culture was centrifigued and the pellet was resuspended in deionized water. A methyl-cellulose polymer (Sigma-Aldrich, Oakville, Ontario, Canada) coating was used to facilitate PGPR adhesion to the seeds. Seeds treated with PGPR were also treated with colorant (Color Coat Blue, Becker Underwood, Saskatoon, Saskatchewan, Canada) to distinguish treated and untreated seeds. The colorant did not affect plant growth or PGPR efficacy (data not shown).

### 2.3.4 Soil sampling and chemical analysis

Soil samples (250 g) from planted and unplanted areas were collected at least three times per year, using an Edelman auger (Eijelkamp Agrisearch Equipment, Giesbeek, the Netherlands). Single grab soil samples were taken systematically, in a grid pattern in each plot to ensure complete coverage of the site. A sampling grid was established with a point taken every 10 meters in all directions on the site. The sample was taken randomly within a 4 $m^2$ area around a given grid point to a depth of 30 cm, in areas with representative amounts of plant growth. Duplicate samples were collected regularly and analyzed to ensure accuracy of sampling and analyses, and to control for sampling anomalies. Samples were placed in glass jars, and stored at 4$^oC$ until further analysis. Levels of PHC were determined gravimetrically. The use of ultrasonic solvent extraction was chosen for this particular site because contamination consisted mostly of the heavier petroleum fractions, F3 and F4, therefore the loss of volatile hydrocarbons was not a major concern. Briefly, air-dried soil samples (2 g) were extracted three times by ultra-sonication for 50 min into a total of 20 mL of 1:1 hexane/acetone mixture (EPA, 1998). To ensure extraction efficiency (completeness) the soil was periodically extracted for a fourth time and those extracts always contained less than 5% of the PHC in the soil. As well, in-house gravimetric data was compared to those from certified analytical laboratories (see below); the extraction efficiency of these analytical laboratories was generally >95% and our data were on par with this data (Figure A2, Appendix A). Extracts were dried by completely evaporating the solvent under a stream of nitrogen gas. The amount of petroleum sludge was determined by weighing the dried extracts. Replicate variation was <10%. The PHC content and CCME Fractions 1-4

measurements in soil samples were analyzed independently by two certified laboratories: ALS Environmental (Waterloo, Ontario, Canada) and Maxxam Analytics Inc. (Mississauga, Ontario, Canada). These analyses were performed according to standard protocols of the CCME (CCME, 2001b). CCME Fractions 1-4 were determined using gas chromatography with flame ionization detector and a 100% poly(dimethylsiloxane) column, following a hexane:acetone extraction (CCME, 2001b). For QA/QC requirements, to ensure accuracy and efficiency of in-house PHC extraction, samples with known PHC concentration were analyzed along with new field samples on an ongoing basis. Concentration for the known samples had to be within 10% of the established value for the analytical run to be considered acceptable. To further assess the accuracy of analysis, at least 15% of all soil samples were sent to the independent laboratories for analysis; split samples were sent to compare the gravimetric analysis PHC results of our laboratory to that from an analytical laboratory. Only if the data from our laboratory and the analytical laboratory were consistent was the data used. An example of this evaluation is in Figure A2 (Appendix A) where the comparison yielded a straight line with a slope close to one and a y-intercept close to zero ($r^2 = 0.85$). Further, our gravimetric data collected can be correlated to the F3 and F4 data obtained from the analytical laboratories (data not shown).

### 2.3.5 Plant biomass, plant length and ground cover measurements

Plant samples were collected at least three times per year. Plant growth was measured in one of two ways: root and shoot length, or root and shoot biomass. Plant biomass was measured by isolating a 50 cm by 50 cm square of soil, 30 cm in depth, with as little disturbance of roots as possible. Soil samples were further divided into three sub-samples. Plants, including roots, were isolated from the soil and washed with water to remove all soil particles adhering to the roots. Roots and shoots were separated and blotted dry to obtain fresh weight. To determine dry weight, plant samples were dried for 2 days at 40$^{o}$C in an oven and re-weighed. All measurements were normalized to those of untreated plants and averaged to obtain annual plant performance in terms of root and shoot biomass (fresh and dry weight). Ground cover measurements were performed in

triplicate by recording percent plant ground cover in a 1 $m^2$ quadrant, with a minimum three measurements taken each time.

### 2.3.6 Plant photosynthetic activity

Effects of hydrocarbon stress were assessed by measurements of the photosynthetic activity of plants measured by chlorophyll-*a* (Chl *a*) fluorescence induction using a Pulse Amplitude Modulated (PAM) fluorometer (PAM-101, Walz, Effeltrich, Germany). The protocol as described in Marwood et al. (2001) was used. Values obtained from PAM fluorescence were: Yield ($F_m$'-$F_t$/$F_m$'), qP and qN. The parameter $F_m$' is defined the chlorophyll fluorescence signal at its intermediate maximum and $F_t$ as the steady state signal. Yield is the effective quantum yield of photosystem II (PSII) under steady state conditions. qP and qN are coefficients of photochemical and non-photochemical (heat) fluorescence quenching, respectively (Maxwell and Johnson, 2000). Sampling was performed at least three times per year from site 2. PAM fluorometry measurements were performed in 2005 and 2006, on plants with and without PGPR treatment.

### 2.3.7 Statistical analysis

To determine significance, ANOVA was used along with Tukey-Kramer post-test. Degradation rates were calculated by assuming first-order kinetics. Degradation curves were fitted using non-linear regression by Systat Software (Systat Software, Point Richmond, California, USA) to the first-order kinetic equation given below:

$$C = C_0 \exp(-kt) \qquad \textbf{(Equation 2.1)}$$

C is the PHC concentration (g $kg^{-1}$), t is time (months), $C_0$ is the initial PHC concentration and k is the kinetic rate constant ($month^{-1}$).

## 2.4 Results

### 2.4.1 Overview of experimental results

In year one (2004), the complexity of the experimental design was kept to a minimum. The main goal was to determine whether plant growth was feasible on soil contaminated with PHC averaging 130 g $kg^{-1}$ of soil, whether PGPR treatment improved growth, and if remediation could be measured. Annual ryegrass was planted on site 1. To determine the protective effect of PGPR on the plants, treatments were plants with and without PGPR. In 2004, the weather conditions were optimal, and excellent growth of PGPR treated annual ryegrass was achieved, with more than a 40% increase in shoot length and 2 to 3 fold increase in ground cover compared to untreated plants (Table 2.1). Dispersal of PGPR throughout the site, due to tilling, could not be controlled or determined in the spring of year two, therefore to avoid experimental artifacts only PGPR treated plants were planted on site 1 in years two and three. Concurrently, starting in year two (2005), a series of small plots in a paired block design were set up on site 2, to examine plant growth and remediation with PGPR treated and untreated plants. Biomass accumulation and photosynthetic performance were also determined for plants with and without PGPR on site 2 in years two and three.

**Table 2.1 Plant growth parameters.**

A. Plant shoots measurements (mm) of annual ryegrass in year one at site 1 (n=10, ± SD). B. Ground cover measurements (%) in year one and two at the experimental sites 1 and 2 (n = 3, ± SD). Ground cover measurements were performed by estimating percent vegetation cover in a 1 $m^2$ area. AR - Annual ryegrass, TF – tall fescue, B – barley, FR – fall rye, N/A - not applicable. * indicates significant differences between control and treated samples with $P < 0.01$.

| Date | Site | Plant type | - PGPR | + PGPR |
|---|---|---|---|---|
| **A** | | | **Shoot length** | **(mm)** |
| 07/2004 | 1 | AR | 91.8 ± 22 | 128.7 ± 35* |
| 08/2004 | 1 | AR | 191.0 ± 28 | 274.9 ± 32* |
| **B** | | | **Ground cover** | **(%)** |
| 07/2004 | 1 | AR | 23 ± 8 | 74 ± 9* |
| 08/2004 | 1 | AR | 38 ± 8 | 85 ± 4* |
| 08/2005 | 1 | AR/TF | N/A | 76 ± 9 |
| 08/2006 | 1 | AR/TF/B | N/A | 87 ± 6 |
| 06/2005 | 2 | B/FR | 45 ± 5 | 70 ± 6* |
| 06/2005 | 2 | AR/TF | ~5 | ~5 |
| 07/2005 | 2 | B/FR | 60 ± 4 | 83 ± 8* |
| 07/2005 | 2 | AR/TF | 9 ± 6 | 14 ± 6 |
| 08/2005 | 2 | B/FR | 5 ± 1 | 45 ± 7* |
| 08/2005 | 2 | AR/TF | ~5 | ~5 |

### 2.4.2 The effect of PGPR on plant biomass, plant length and ground cover

During the course of the study, petroleum contamination was at its highest (~130 g $kg^{-1}$) in the first year of planting at site 1 (Figure 2.1). Plants without PGPR grew poorly; plant size and ground cover were much greater for PGPR treated plants (Table 2.1). In 2005, similar effects were observed when site 2 was planted for the first time using the same experimental protocols (Table 2.1). Soil contamination decreased in each successive year, while plant growth improved (Figure 2.1 and Table 2.1). Each year, plants with PGPR inoculation germinated and grew well, and exhibited fewer signs of phytotoxicity, such as stunted growth, lower seed yield, and extent of chlorosis, than untreated plants. Plant growth measurements showed a consistent improvement in shoot length (Table 2.1) and plant biomass (Figure A3, Appendix A) when PGPR were used. Greatly increased ground coverage was also evident in plots with PGPR treated plants, relative to plots with untreated plants (Figure 2.1 and Table 2.1). Note that, in 2005 on site 2, the annual ryegrass and tall fescue mixture did not grow well (< 5% ground cover) due to early season drought, and this was the only case where PGPR improved plant growth was not observed (Table 2.1).

### 2.4.3 The effect of PGPR on plant photosynthesis

Small, yet significant, differences in photosynthetic parameters were apparent when different plant species were examined using PAM fluorometry. An increase in qN, the non-photosynthetic quenching parameter, has been used as an indicator of plant stress. In 2005, qN was lower in both mixtures of plant species (tall fescue and annual ryegrass, fall rye and barley) treated with PGPR than in their untreated counterparts (Figure 2.2). No significant changes in qP were detected in either 2005 nor 2006. In 2005, PGPR treated mixture of fall rye and barley plants had higher $F_v/F_m$ and Yield value than untreated plants. However, PGPR treated tall fescue and annual ryegrass mixture showed lower $F_v/F_m$ and Yield values than untreated plants. In 2006, PAM fluorometry did not indicate major differences between plants with and without PGPR, aside from a small rise in barley treated with PGPR; qN appeared to increase with PGPR treatment. Thus, PAM fluorometry measurements of photosynthesis indicate that both annual ryegrass and tall fescue, and barley and fall rye mixtures were performing better with PGPR treatment,

according to qN parameter. Further, annual ryegrass and tall fescue treated with PGPR were better able to thrive in a contaminated environment than untreated plants according to $F_v/F_m$ and Yield parameters.

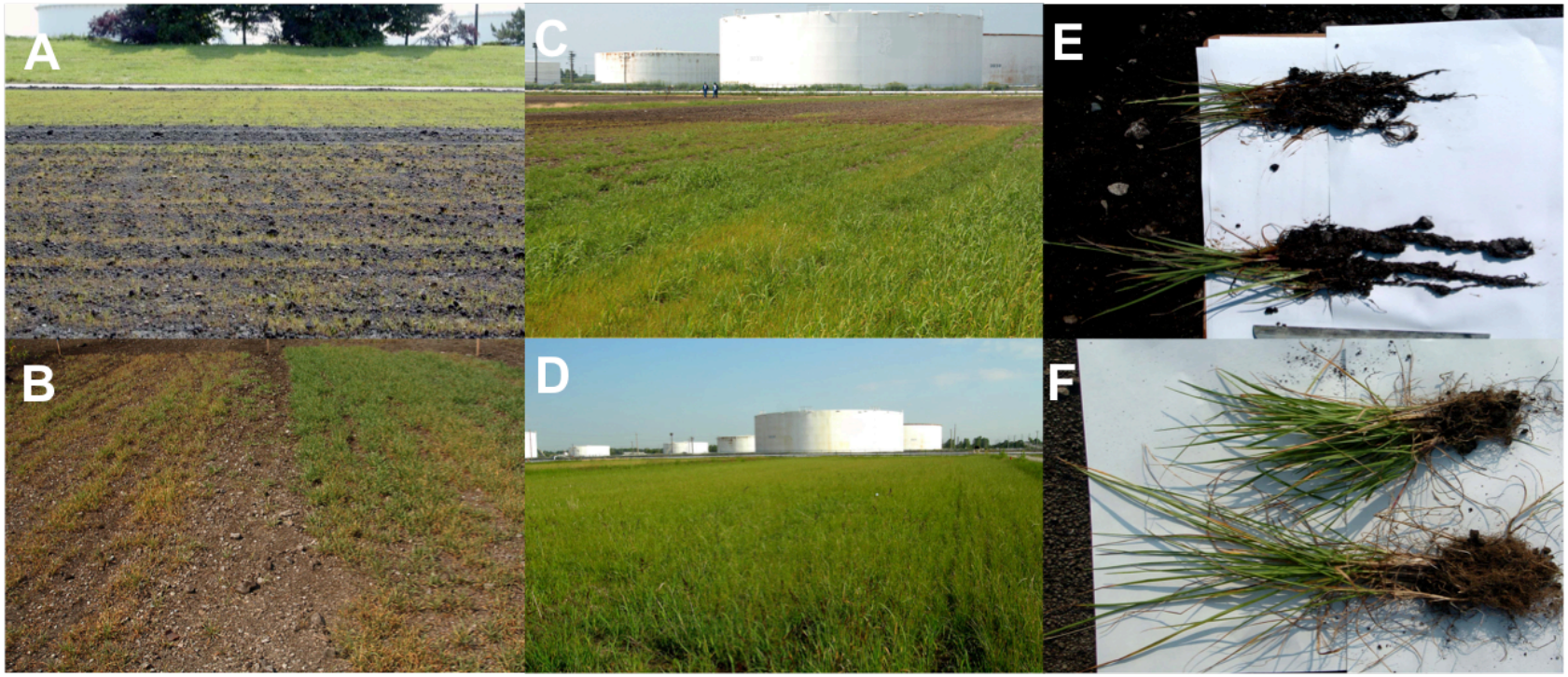


**Figure 2.1 Plant growth with PGPR in PHC contaminated soils.**

(A) PGPR enhanced growth in contaminated soils at site 1 in 2004 growing season using annual ryegrass, 60 days after planting. (B) Site 2 in 2005 using barley and fall rye, 35 days after planting. (C) Site 1 in 2005 using tall fescue and annual ryegrass, 35 days after planting. (D) Site 1 in 2006 planted with barley, tall fescue and annual ryegrass, 110 days after planting. (E) Annual ryegrass from site 1 in 2004, 60 days after planting, and (F) Annual ryegrass from site 1 in 2004, 120 days after planting. In panel E and F, top plants are – PGPR, bottom plants are + PGPR plants.

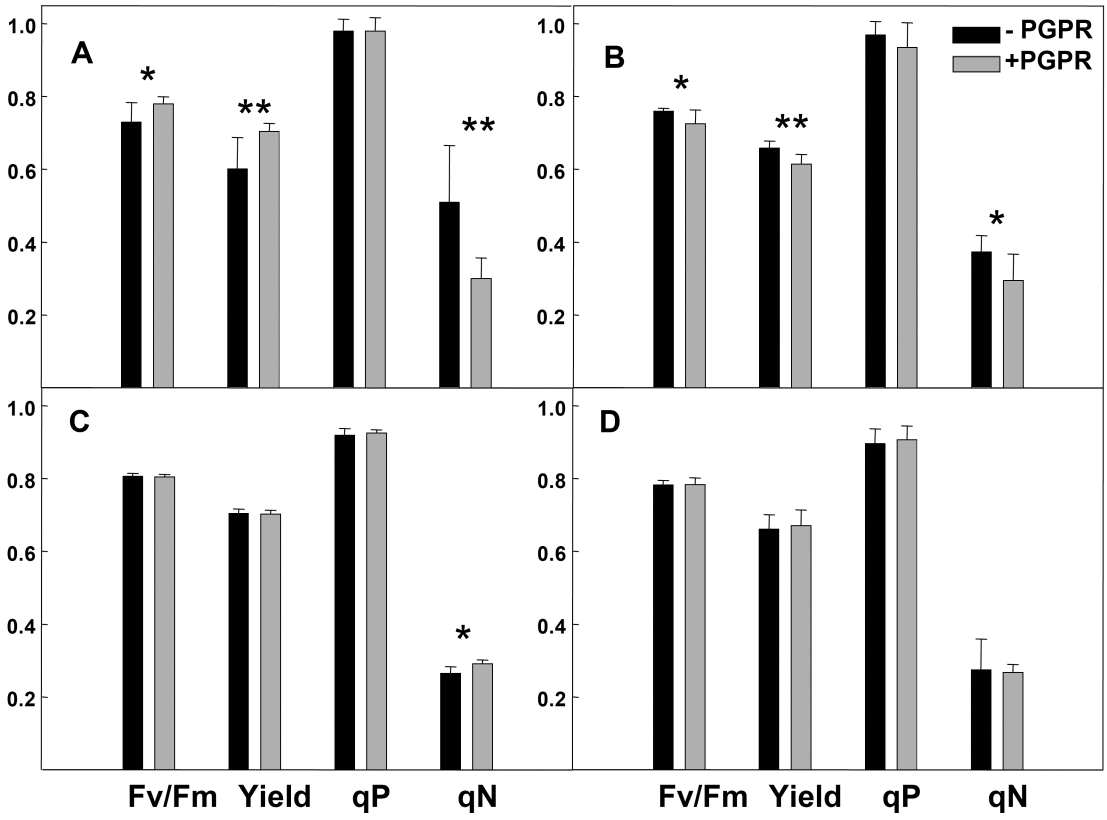


**Figure 2.2 PAM parameters in plants grown on the PHC landfarm.**

PAM parameters obtained at site 2 from barley and fall rye in 2005 (**A**), annual ryegrass/tall fescue in 2005 (**B**), barley in 2006 (**C**), and annual ryegrass/tall fescue in 2006 (**D**). Measurements are the mean (± SD) from two sampling events (n = 6) where similar trends were observed. The x-axis is the same for panel C and D as it is for panels A and B. All values are a ratio and therefore a unit-less number. Asterisks indicate significant differences between medians of PGPR and untreated samples, *$P < 0.05$ and ** $P < 0.01$, determined with Welch t-test.

### 2.4.4 Phytoremediation of PHC

In year one (2004), annual ryegrass was grown on site 1 with and without PGPR. Plants with PGPR grew very well and approximately 50% PHC remediation was observed (Figure 2.3). PEPS was able to lower the concentration of specific CCME fractions, also on the order of 50% (Figure 2.3). Without PGPR treatment, plant growth was poor, only about 20% remediation was observed and the change in PHC levels relative to the time zero ($t_0$) control was not significant (Figure 2.3). In May 2005, PGPR treated annual ryegrass and tall fescue were planted together on site 1. Plants from the previous year were tilled into the soil prior to sowing. The average PHC concentration at the onset of the 2005 field season was 106.3 g $kg^{-1}$, greater than the final concentration in 2004 of 65.2 g $kg^{-1}$. In year two, significant remediation was once again achieved (Figure 2.3), total hydrocarbon concentration decreased to 69.6 g $kg^{-1}$ PHC. Most importantly, F3 and F4 were remediated further (Figure 2.3). In year three (2006), following tilling, site 1 was planted with PGPR treated annual ryegrass, tall fescue and barley. Approximately 15% remediation of total hydrocarbons was achieved (Figure 2.3), with ~ 20-25% remediation of both F3 and F4 (Figure 2.3).

Remediation kinetics for the three year field trial are presented in Figure 2.4. Overall, 65% remediation was achieved using plants with PGPR, down to 46.4 g $kg^{-1}$, while the unplanted control had a total drop in PHC concentration of only 22%, down to 87.9 g $kg^{-1}$ (note different starting concentration for control and planted areas). While a total drop in PHC concentration in PGPR treated plants was significant for all but one time point, none of the PHC decreases in unplanted control were significantly different from the zero time point. Data for plants with PGPR and for the unplanted control were fitted to a first order kinetics model. The first order kinetics model was a good fit for the PGPR treatment ($r^2$ of 0.916) and for the unplanted control ($r^2$ of 0.974) data. Remediation kinetics remained first order throughout the three years of field trials and the rate constant k for remediation with PGPR treated plants was 0.027 $month^{-1}$ versus a k of 0.009 $month^{-1}$ without plants.

Remediation was also monitored at site 2 using a series of small plots that were planted for two consecutive years. Remediation data collected (presented in Supporting Information section) showed comparable magnitude of remediation to site 1 and were

approximately 10 to 30% each year (Table A2, Appendix A). Four times out of five, the PGPR treated plants had greater levels of remediation than the untreated plants. To create a robust dataset for statistical analysis of the effect of PGPR, the data from all plots was pooled into + PGPR and – PGPR (regardless of plant type), thus combining all plant species from both 2005 and 2006. In that case, we were able to show that + PGPR plants in small plots had a statistically significant level of remediation of 18% ($p = 0.05$), whereas with the – PGPR plants remediation was not statistically significant (15% remediation).

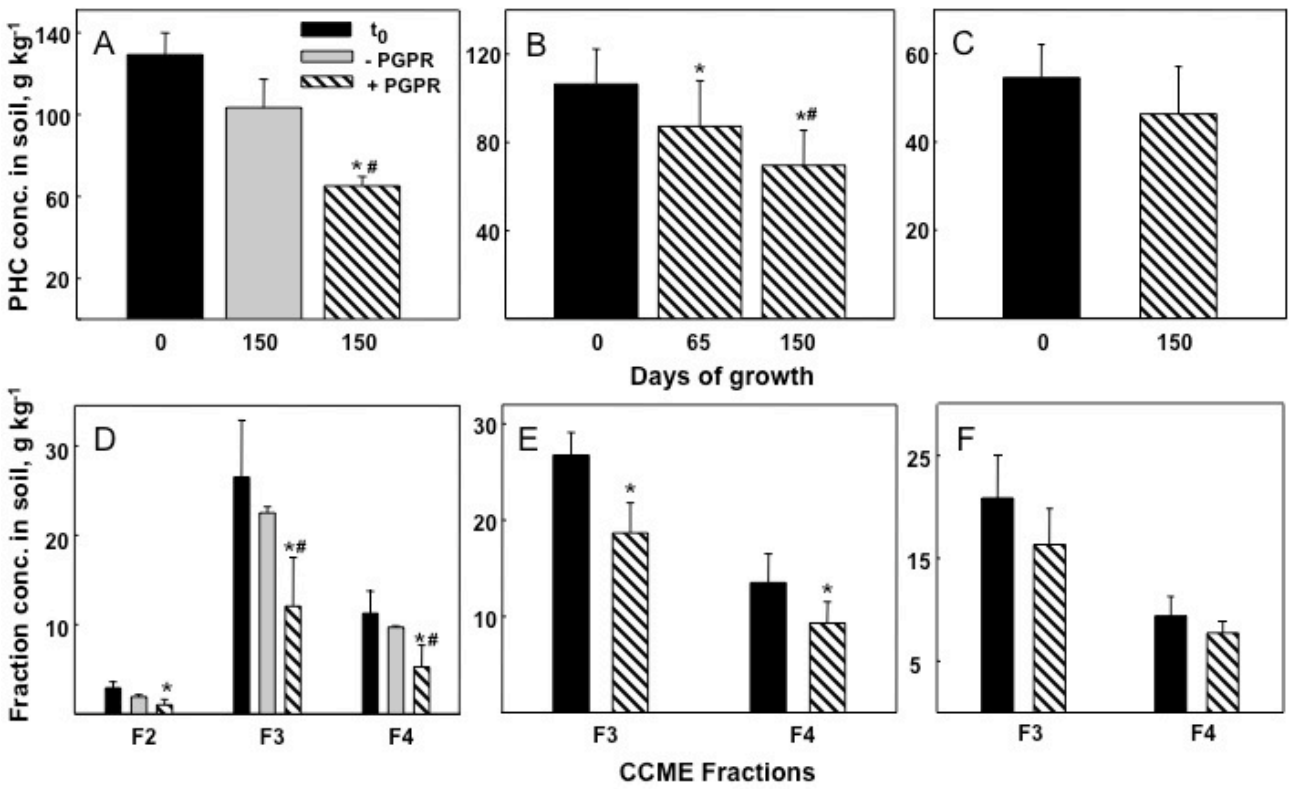


**Figure 2.3 PHC concentrations at the landfarm.**

Decrease in PHC (A-C) and F2, F3, F4 (D) and F3, F4 (E, F) concentrations. PHC concentrations in 2004, using annual ryegrass (AR) after a growing period of 150 days (n≥5) (A), in 2005 using AR and tall fescue (TF) mixture after a growing period of 65 and 150 days (n≥5) (B), and in 2006 using AR, TF and barley mixture after a growing period of 150 days (n≥5) (C). CCME fractions F2, F3 and F4 in 2004, at 150 days of growth using AR (n≥3) are shown (D). F3 and F4 in year 2005 at 150 d of growth are shown using AR and TF mixture (n≥3) (E) and from 2006 using AR, TF and barley mixture (n≥3) (F). Asterisks indicate values significantly different from t0 control ($P < 0.05$), # indicate values significantly different from 150 days growth – PGPR ($P < 0.1$). Unplanted controls PHC concentration decreased 22%, down to 8.79 ± 0.59 g kg$^{-1}$. Two replicates were analyzed. Error bars indicate ±SD.

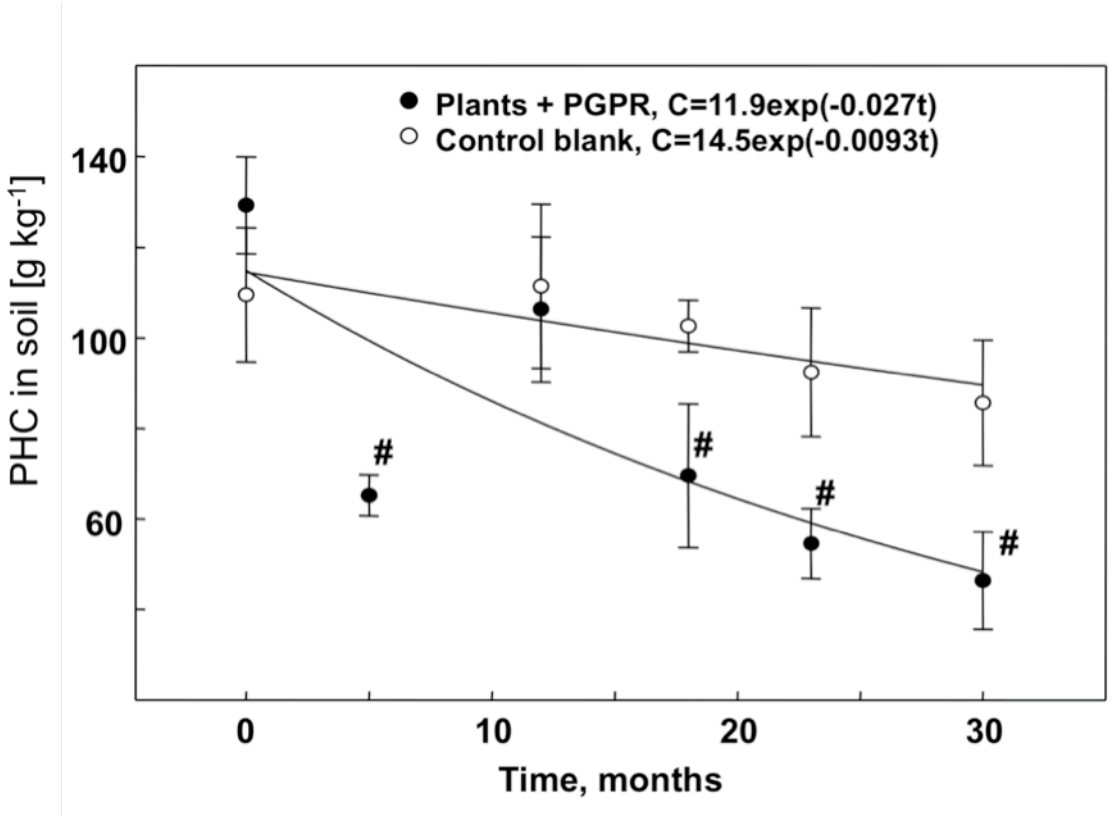


**Figure 2.4 PHC remediation kinetics.**

Remediation kinetics of PEPS and unplanted control. PHC removed from the soil were determined at various time points throughout the 30 month remediation project. Note: blank and planted samples were collected from different parts of the field and thus had slightly different starting concentrations. Error bars indicate ± SD. # indicates values significantly different from the respective PHC concentrations for PGPR planted and unplanted treatments from time zero.

## 2.5 Discussion

Successful remediation of PHC in the field has been hindered by low biomass accumulation, particularly when petroleum hydrocarbon levels are high. PGPR have previously been used to improve plant growth in agriculture (Cook, 2007) and in greenhouse phytoremediation applications (Huang et al., 2004b; Huang et al., 2005). However, few full scale studies have been performed to evaluate PEPS in the field (Reed and Glick, 2004). Our study showed that PEPS can be effective, even in highly contaminated soils. Here, it was shown that PEPS was more successful relative to natural attenuation or plant growth alone.

The ability of PEPS to overcome the limitations of other biological remediation strategies stemmed from its capacity to generate high levels of biomass. PGPR plants are better able to withstand the stress of growing in the contaminated soils than plants without PGPR. This was observed as both the 40% increase in shoot length in year one (Table 2.1) and the 40% increase in shoot fresh weight in PGPR plants in subsequent years (Figure A3, Appendix A). Treatment of seeds with PGPR enabled the plants to initiate and sustain more vigorous growth. This was also evident in the ground cover data which was much greater with PGPR treated plants compared to untreated plants (Table 2.1) Addition of PGPR also stimulated root growth, albeit to a smaller degree than shoot growth, putatively resulting in a greater rhizosphere that is likely responsible for remediation of recalcitrant F3 and F4 (Alkorta and Garbisu, 2001; Beauchamp and Dzantor, 2002). The increase in size of the roots and shoots also would effectively lower the ratio of phytotoxic contaminants to the amount of plant tissue, lowering stress on the plants.

Often, photosynthetic activity can be affected in plants under persistent hydrocarbon stress, such as that coming from polycyclic aromatic hydrocarbons (PAHs), photomodified PAHs and creosote (Huang et al., 1997; Marwood et al., 2000; Marwood et al., 2001). For this reason, photosynthesis was used as an endpoint. Despite poor growth resulting from the high contaminant load in experimental soils, the plants appeared healthy and expectedly there was not a large degree of photosynthetic stress. Plants without PGPR were able to carry out photosynthesis efficiently with only minor signs of environmental injury. Nonetheless, in 2005, the small negative effects of petroleum contamination that were observed were

ameliorated in the presence of PGPR. This was evident as higher $F_v/F_m$ and Yield for treated barley and fall rye plants. These data indicate less damage to PSII (Marwood et al., 2001). However, in some cases, treated plants had slightly lower Yield and $F_v/F_m$ values. In 2005, PGPR ryegrass and tall fescue as well as fall rye and barley had lower non-photosynthetic quenching (qN), indicating less environmental injury. Taken together, treated and untreated plants showed relatively good values for the photosynthetic parameters, confirming our visual observations that the plant tissue produced was healthy.

In 2006, the negative effects of petroleum contamination on growth were not as pronounced, and consistent with this, photosynthesis was even less impacted. The PAM measurements for tall fescue and annual ryegrass in 2006, showed no significant differences between plants with and without PGPR. This could be due to diminished stress as result of lowered phytotoxicity due to remediation in the previous year. Overall, the photosynthetic data suggest that although PGPR may improve photosynthesis, the changes were small and as a result did not contribute to the growth improvement due to PGPR treatment. Thus, improved plant performance is likely due to other factors, such as lowering stress ethylene levels. Through the use of ACC deaminase, PGPR consume the precursor to ethylene, ACC, resulting in improved plant growth, despite the high levels of contaminants present in the soil. In fact, visual observations of the plants did not reveal stress effects, which is consistent with only minor impacts on photosynthesis. This indicates there may be little stress on the plants, so there was no physiological reason for growth to be impeded. Thus, when PGPR is employed, the putative ethylene block on root growth is alleviated allowing more vigorous plant growth without negative impacts on the plants.

It is clear from our findings that favorable conditions for petroleum degradation were created at a highly contaminated site using proper plant selection, land farming techniques and application of PGPR. Phytoremediation was successfully applied to remediate PHC contaminated soils using annual ryegrass, tall fescue, barley and fall rye. We have shown that PEPS continues to remediate soils, with first order kinetics when employed in successive field seasons. During this 30 month trial PHC concentration in the soil declined from 129.3 g $kg^{-1}$ to 46.4 g $kg^{-1}$.

In 2004, the weather was excellent for plant growth (moderate temperatures and sufficient precipitation) in the Sarnia, Ontario region. That year, we observed the most pronounced effects of PEPS, both in terms of plant growth and PHC remediation (Figure 2.1, Figure 2.3). The increased growth of PGPR plants was reflected in remediation, with approximately 50% decrease in PHC using PGPR treated plants. Remarkably, both recalcitrant F3 and F4 were remediated. Without PGPR, plants grew poorly and the 20% remediation observed was not significantly different from the control. Without plants no drop in PHC was observed that year. The final PHC concentration at the end of year one was 65.2 g $kg^{-1}$. In 2005, the PHC concentration at the beginning of the 2005 field season rose to 106.3 g $kg^{-1}$. This was likely due to tilling performed prior to planting. We propose that because root density decreases with increasing soil depth, tilling would bring contaminants from the zone of lower root density, which had experienced less remediation, to the surface. The final concentration of hydrocarbons in the soil at the end of year two was found to be 69.6 g $kg^{-1}$. Thus, 35% remediation was realized in year two. The unplanted control showed a small 8% drop in PHC concentration. In the third year, the initial PHC concentration in April 2006 was 54.6 g $kg^{-1}$ PHC. In contrast to the previous year, the PHC concentration dropped over the fall and winter, possibly due to plant and/or microbial activity during the fall of 2005 (after our last sampling). Because the plants were not dug into the soil at the end of 2005, remediation continued after final sampling. In 2006, climatic conditions were conducive to phytoremediation, with moderate temperatures and sufficient rainfall. Excellent plant growth was observed as a direct result of lower phytotoxicity, which can be attributed to remediation during the preceding years. In this final year, approximately 15% remediation was achieved for plots planted with PGPR plants, while the unplanted control showed a drop of approximately 7%.

PGPR effects from site 1 in 2004 were further confirmed on site 2 in year two and three, with a series of small plots and a total of 5 independent trials (Table A2, Appendix A). Changes were smaller than on the larger plots, average remediation with PGPR treated plants of approximately 18% was observed. In 4 out of 5 replicates there was small, yet reproducible improvement in remediation with PGPR treated plants. Reasons for less

remediation in smaller plots compared to site 1 may be the possible bacteria migration from plot to plot, and the smaller plot size forces a number of samples having to be taken from parts of the plot with less plant growth. Nonetheless, with PGPR treatment the average 18% remediation was statistically significant, whereas in plots without PGPR the remediation was not statistically significant. This mirrored the results from site 1, where only remediation with PGPR treated plants was statistically significant.

As the PHC levels assessed in this study have high inherent variability in soil samples, cautious interpretation of the above data is necessary, particularly when considering the remediation data for each year independently. Furthermore, technology transfers, such as this one, are often difficult to execute in the field and the resulting remediation kinetics may be unpredictable (Nedunuri et al., 2000). To account for these two points, we examined the site 1 remediation data for the three year trial as a whole and observed a positive correlation between PHC removal and the length of time that PGPR treated plants were grown, to an extent that could not be described by the analytical and/or sampling variability. The decrease in hydrocarbon concentration in the soil appears to follow first order kinetics over the course of three years (Figure 2.3), typical of petroleum degradation (Roncevic et al., 2005). In contrast, unplanted control soils, which did not receive any plant or PGPR treatments, showed only small decreases in PHC levels (Figure 2.3, Figure 2.4) and the changes were not significant. Non-linear regressions of the decrease in PHC concentration using an exponential decay model (Equation 2.1) showed a trend, with consistency between each year's data cluster and the overall kinetic analysis. In Equation 2.1, the parameter obtained through this analysis, k is the first order rate constant for PHC remediation. Combining the temporal data set throughout the 30 month trial period, provided high statistical significance to this rate constant. Thus, what we obtained was a very robust data set showing consistent decreases in contamination levels over the three year period. With PGPR treated plants, k was 0.027 month $^{-1}$ versus a k of 0.009 month$^{-1}$ without plants. This suggests that it is phytoremediation that is causing the loss of PHC from the soil.

Phytoremediation is a promising alternative to other remedial biotechnologies, due to its potential for increasing the amount of plant biomass available for phytodegradation and

microbial degradation of soil contaminants. This system still has limitations however, when high levels of contaminants hinder plant growth and depress microbial populations. Addition of PGPR addresses these problems by increasing the plant phytotoxicity threshold and greatly enhancing biomass accumulation. Root growth is especially necessary for remediation and if high levels of root biomass can be achieved, this can stimulate both *in planta* and *ex planta* degradation of PHC. Plants increase the degradation rate, particularly if PGPR treatment is used.

In traditional phytoremediation strategies, high molecular weight petroleum fractions (typically F3 and F4) were often resistant to remediation (Huang et al., 2005). Encouragingly, in this field trial, PEPS was successful at remediating PHC, including recalcitrant F3 and F4. Most of the compounds in F3 and F4, such as PAHs, fall outside of the favorable uptake range of 0.5 to 3 log $K_{OW}$ (Schnoor, 2002; Dzantor and Beauchamp, 2002; Hutchinson et al., 2003). These compounds are most likely degraded in the rhizosphere by bacteria, fungi, and PGPR that are nutritionally supported by plant exudates (Schnoor, 2002; Salt et al., 1998; Alkorta and Garbisu, 2001; Dzantor and Beauchamp, 2002; Hutchinson et al., 2003). Thus, here we have shown the successful application of a PGPR-assisted phytoremediation technology for remediation of a heavily contaminated site containing recalcitrant petroleum compounds. Further, we believe the phytoremediation system can be applied broadly at petroleum-impacted sites where other methods have failed.

## 2.6 Acknowledgements

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# Chapter 3

# Effects of petroleum contamination and plant growth promoting rhizobacteria on plant growth and soil microbial communities during phytoremediation of petroleum hydrocarbon impacted soil.

## 3.1 Overview

Petroleum contaminated soils represent a challenge for phytoremediation due to the phytotoxicity of petroleum hydrocarbons (PHC), especially in soils with high contaminant concentrations. To overcome PHC stress on plants, a plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation system (PEPS) was developed in our laboratory. PGPR were used to alleviate plant stress and promote plant growth to accumulate sufficient biomass for successful remediation. Greenhouse and field studies showed that PEPS can effectively remediate PHC from impacted soils. In this study, temporal analyses of field microbial populations aimed to explore how PEPS changed soil microbe community over a period of two years at a site used for the treatment of petroleum refinery sludge (~100 000 mg PHC per kg soil). We used culture-dependent microbial monitoring methods to ascertain the effects that contaminants, plant growth and plant growth with PGPR inoculation had on the native microbial community present in PHC contaminated soils over a two-year period. The effect of time (up to two years), plant growth (unvegetated, vegetated) and bacterial inoculation (– PGPR, + PGPR) were evaluated. Plant growth and remediation were also monitored. Soil samples were analyzed for total bacteria, petroleum-degrading bacteria, hexadecane-degrading bacteria, total fungi, petroleum degrading fungi and 1-aminocycloproane-1-carboxylic acid (ACC)-metabolizing bacteria. Microbial community structure was analysed using Biolog EcoPlates. Overall, remediation of PHC from the soils with PEPS application was superior to plant growth alone or no plant growth. The microbial numbers in the soils with PEPS application were 30-40% higher than those in the soils with plant growth alone. PEPS and plant growth alone resulted in at least one order of magnitude higher microbial numbers than without plant growth. These results implicate that the key mechanism

operating with regard to PEPS treatment of PHC impacted soils. The PGPR appear to drive the development of an extensive root system and thus an active rhizosphere that supports more PHC degraders. However, PEPS does not functionally change microbial population distribution in the soil.

## 3.2 Introduction

The extensive use of petroleum has resulted in a large number of polluted sites, which now require remediation. The clean up of petroleum hydrocarbon (PHC) impacted sites is being addressed by numerous remediation strategies. One strategy is phytoremediation, which is a biological method that is both financially and environmentally appealing. Plants can remediate contaminants directly through uptake, translocation and degradation of toxicants in their tissues (Meudec et al., 2006) and indirectly by stimulating microbial growth and resultant contaminant metabolism in the rhizosphere (Singh and Jain, 2003). Direct plant uptake of many hydrophobic, organic contaminants is slow because of their relatively low water solubility and their large size (Burken and Schnoor, 1998; Meudec et al., 2006; Kaimi et al., 2007) Thus, the indirect route, through increased microbial activity, is thought to be the primary mechanism for remediation of PHC (Euliss et al., 2008).

When plants are grown, increased microbial density and diversity in the rhizosphere is thought to be due to exudation of organic compounds from plant roots, which support and regulate growth of microorganisms (Euliss et al., 2008). With large numbers of microorganisms in the rhizosphere, remediation of PHC, including recalcitrant fractions 3 and 4 (Fraction 3, C>16 to C34, Fraction 4 C34+, F3 and F4 where C is the number of carbon atoms in the molecule), is greatly increased; degraded PHC are in turn made available for plants. Thus, a key mechanism of phytoremediation of PHC is for plants to stimulate the microbial community in the rhizosphere (Cunningham and Ow, 1996; Siciliano et al., 2003).

High concentrations of contaminants impedes phytoremediation fundamentally by preventing plant growth (Huang et al., 2004a; Greenberg et al., 2007). Beneficially, plant-microorganism associations can be further exploited to enhance phytoremediation capacity through the use of plant growth promoting rhizobacteria (PGPR). In general, PGPR affect root proliferation by increasing total root length and branching (Hodge et al., 2009). This provides more surface area for root colonization sites for bacteria and the roots are able to explore larger volumes of soil (Vessey, 2003).

The increased biomass and surface area is then available for remediation of PHC. The particular strains of PGPR used in this study, UW3 (*Pseudomonas* sp.) and UW4 (*Pseudomonas putida*), as well as many other PGPR, are thought to increase plant growth through production of indole-3-acetic acid (an auxin) and to impede synthesis of the plant stress hormone ethylene (Shah et al., 1998; Chang, 2007). Environmental stresses such as PHC, metals and/or pathogens often inhibit plant growth by inducing the production of higher than normal levels of stress ethylene in plants, hence the inhibition of stress ethylene production by PGPR can facilitate plant growth under these conditions (Glick et al., 2007).

As either petroleum degraders or plant growth promoters, microorganisms play critical roles in the phytoremediation of PHC. However, the nature of the soil environment makes it difficult to study these interactions. To date, limited information, if any, is available about the native microbial populations in PHC contaminated soils during long-term PHC phytoremediation trials (Kirk et al., 2005; Maila et al., 2006; Phillips et al., 2006). Greenhouse (Kirk et al., 2005; Phillips et al., 2006) and field (Maila et al, 2006) studies revealed that plant growth enhances the microbial population, and alters the community structure in PHC contaminated soil. As well, the changes that PGPR inoculants exert on microbial communities in contaminated soils in long-term phytoremediation trials are unknown.

The relationships between microorganisms and plants are complex and species-dependent, thus, addition of particular PGPR does not guarantee its growth promoting effects or its survival with a specific host/environment combination (van Elsas and Heijnen, 1990; van Veen et al., 1997). Although PEPS has been shown to remediate PHC contaminated soils, its mechanisms are not entirely clear. It is likely that the plants promote growth of soil microbes and the microbes consume PHC (Cowie et al., 2010). Understanding these mechanisms will help establish better strategies in subsequent remediation practices.

The objective of this research was to explore the roles that microorganisms, including PHC degraders and two PGPR, UW3 and UW4, play in a successful application of PEPS at a petroleum land farm site with concentrations of PHC as high as ~100 g $kg^{-1}$. In addition to

plant growth and remediation monitoring, soil samples were analyzed for total bacteria, petroleum-degrading bacteria, hexadecane-degrading bacteria, total fungi, petroleum degrading fungi and 1-aminocycloproane-1-carboxylic acid (ACC)-metabolizing bacteria. Microbial populations were further assessed functionally using Biolog Eco Plates to create carbon-based microbial community profile (Garland and Mills, 1991; Zak et al., 1994). We posited that, not only would plants increase microbial numbers in comparison to unvegetated soil, but also that addition of PGPR inoculants would induce changes within the rhizosphere that may explain the increase in remediation by PGPR treated plants. The specific objectives of this study were to, in a long-term phytoremediation trial, investigate 1) the influence of plants on bacterial community in contaminated soils, 2) the impact of bacterial inoculants on microorganisms taking part in degradation of PHC and 3) the effects of bacterial inoculants on endogenous soil microbiota.

## 3.3 Materials and Methods

### 3.3.1 Experimental design

Experiments were conducted at an Imperial Oil land farm in Sarnia, Ontario, Canada. The annual average temperature near this site is 8.25 ˚C, the annual precipitation averages 827 mm. Growth seasons are from the end of April to November for cold weather adapted grasses, such as ryegrass and fescue. The land farm was used for disposal of petroleum refinery sludge for over 20 years. PHC concentration at the experimental site was ~100,000 mg $kg^{-1}$. Three treatments were employed in this trial: 1) phytoremediation without PGPR, 2) PEPS application (plant growth with PGPR), and 3) unvegetated controls. Planted treatments were grown at the experimental site in a paired block design along with unvegetated controls. Each plot was ~40 $m^2$ and planted with a mixture of ryegrass (*Lolium multiflorum)*, tall fescue (*Festuca arundinacea)* from Ontario Seed Ltd. (Waterloo, ON, Canada). PGPR used in this experiment were a combination of UW4 *(Pseudomonas putida)* and UW3 (*Pseudomonas* sp.). Methyl cellulose was used to adhere bacterial suspension to the seeds and it along with seed colorant were added using a Hege II liquid seed treater

(Winstersteiger, Australia) as previously reported (Chapter 2, Gurska et al., 2009). Seeds were planted on April 20, 2006 and May 3, 2007.

### 3.3.2 Sampling

The site was tilled prior to planting and beginning of season soil samples were taken on April 12, 2006. Subsequent soil and plant samplings were performed on (* indicates plant sampling as well): May 29*, June 6*, August 13*, September 21*, November 15 in 2006. The site was tilled again in early spring of 2007 and the second planting was performed on April 19 followed by sampling on May 3, June 4, July 3*, Aug 2*, September 5* and October 8 in 2007. Five soil samples per plot were taken using a 20 cm hand-held Edelman auger (Eijelkamp Agrisearch Equipment, Giesbeek, the Netherlands). At each point, ~500 g of soil at 20 cm depth were taken, mixed in a stainless steel bowl, placed in a glass jars and transported to the laboratory for analysis. PHC levels in the soil were analyzed gravimetrically and plant samples were collected and analyzed, as described previously (Chapter 2; Gurska et al., 2009).

The soil samples for rhizosphere and non-rhizosphere microbial analysis were taken on the September 21, 2006 sampling. For rhizosphere soil, the roots from undisturbed plant samples were first shaken by hand to remove all loosely attached soils. The rhizosphere soil was sampled by washing roots in sterile double deionized $H_2O$. The presence of water in weighed samples was accounted for. Non-rhizosphere soils were taken by auger in the vicinity of planted samples, but were taken from areas without plant growth.

### 3.3.3 Quantification of microorganisms

To quantify cultivable bacteria in the soil, 2 g of soil was aseptically placed in 20 mL sterilized 0.85% NaCl (w/v) solution in 50-mL sterile conical tubes and shaken for one hour on a Multi-Mixer (Lab-Line Instruments Inc., Melrose Park, Illinois, USA) at 500 rpm. The soil suspensions were allowed to settle for one hour and the supernatants from these soil extract suspensions were diluted serially, ($10^1$, $10^2$, $10^3$, $10^4$, and $10^5$), prior to bacterial counts. All bacterial counts were performed in triplicate using the plate count method (Kirk et al., 2005).

Culturable, aerobic heterotrophic bacterial cells (total bacterial numbers) were grown and enumerated as previously described (Kirk et al., 2005). Plate counts were performed after 48h at 20 ˚C in the dark. Total fungi, petroleum-degrading aerobic bacteria and petroleum-degrading fungi were grown and enumerated according to Kirk et al., (2005). Plates were incubated at 20˚C for 4 days in the dark before enumerating colonies.

Hexadecane-degrading aerobic bacteria were enumerated by spreading 100 µL of each serial dilution on oil agar medium. The oil agar medium constituted of 990 mL of BH Agar (Kirk et al., 2005) supplemented with ten mL of filter sterilized hexadecane (Sigma Aldrich, Oakville, ON, Canada). Plates were incubated at 20˚C for 4 days in the dark.

DF salt medium with ACC as a sole nitrogen source was used to estimate the number of ACC-utilizing bacteria in soil (i.e. PGPR). The DF salt medium was prepared as described previously (Penrose and Glick, 2003). The final concentration of ACC in DF salt medium was 3.0 mM. From each serial dilution, 100 µL was spread on a plate. Plates were incubated at 20˚C for 2 days in the dark before enumerating colonies.

### 3.3.4 Biolog Plate Inoculation

Community profile of soil microorganisms was assessed using carbon substrate utilization plates (EcoPlate$^{TM}$, Biolog Inc., Hayward, CA, USA), containing 31 carbon sources from six chemical guilds (polymers, carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds), and replicated three times on each 96 well microplate. To prepare the inoculant, a 10 g soil sample (wet weight) was added to 100 mL deionized water in a 250 mL Erlenmeyer flask and shaken for 1 hour using a rotary shaker (80 rpm), then allowed to settle for 1 hour, following which two tenfold dilutions were made. An aliquot (150 µL) of diluent was used to inoculate each well. The plates were incubated in the dark at RT and absorbance (600 nm) was measured every 24 hours over the period of 7 days using Victor $^{3}$V plate reader (Perkin-Elmer, Waltham, Massachusetts, USA). The water blank was subtracted from each OD reading at each time point. Prior to statistical analysis, to reduce biases by different inoculum densities, a standardization of OD values was performed by dividing each OD value by average well colour development (AWCD) of all 31 substrates (Garland and Mills,

1991). The Biolog data based on 7 day incubation readings of Biolog plates after the AWCD standardization or the day with appropriate, fixed OD reading (typically day 5) were used for substrate diversity (H') (Zak et al., 1994). H' was calculated: Shannon's diversity index [H' = - $\Sigma Pi \ln Pi$, where $Pi$= (OD reading of well $i$)/(sum of all wells)].

### 3.3.5 Data analysis

A two-way multivariate analysis of variance (MANOVA) was used to test Biolog data and the two years were significantly different from each other (Hotelling's Trace, $P < 0.05$). One-way MANOVA was used to analyze the effects of plant growth and inoculation with PGPR and plant growth on remediation, carbon substrate utilization and microbial populations. A principal component analysis (PCA) was performed using the carbon sources as variables.

Colony forming units (CFU) were examined for overall treatment effects by ANOVA, followed by a Tukey test (variances equal) or a Games Howell test (variances unequal) to determine whether significant differences occurred between treatments. CFU data were log transformed prior to analysis. All statistical tests were performed using SPSS software 18.0 (SPSS Inc, Chicago, USA).

## 3.4 Results

### 3.4.1 Remediation and plant growth

In 2006, the weather was optimal for phytoremediation (average temperature from May to Sept. of 18.2 ˚C, rainfall of 415 mm). The plants grew well with a prominent increase in growth with application of PGPR (Table 3.1) (Gurska et al., 2009). Higher biomass and better plant ground cover was observed with PGPR application, with the dry plant biomass being approximately 30% higher with PGPR treatment than in untreated plants (Table 3.1). In 2007, the weather was very dry in the first half of growth season with normal rainfall in the second half (average temperature from May to Sept. of 18.5 ˚C, rainfall of 319 mm).

**Table 3.1 Remediation and plant growth on experimental plots at the Sarnia land farm in 2006 and 2007.**

Remediation was monitored on April 12 2006, April 19 2007 and October 8 2007. The average plant growth (dry weight $g/m^2$) throughout the two-year trial was 842.53 (± 210.23, SE, n ≥ 30) for + PGPR plants and 626.42 (± 154.36, SE, n ≥ 30) for – PGPR plants. Plant biomass column contains growth improvement relative to the normalized year average. * indicates significant remediation from the beginning of the 2006 year samples using an ANOVA test, $P < 0.05$, n ≥ 3. # indicates numbers used for remediation collected in April of 2007 (start of second year of monitoring).

| Year | Treatment | Plant Biomass (%) | PHC Start ($g\ kg^{-1}$) | PHC End ($g\ kg^{-1}$) | Remediation (%) |
|---|---|---|---|---|---|
| **2006** | **Unvegetated** | n/a | 10.4±0.90 | #9.21±0.24 | 8 |
| | **– PGPR** | 100±13 | 9.3±0.61 | #8.45±0.58 | 13 |
| | **+ PGPR** | 130.2±17 | 9.9±0.78 | #8.38±0.80 | 15 |
| **2007** | **Unvegetated** | n/a | 9.21±0.24 | 9.43±0.45 | 2.3 |
| | **– PGPR** | 100±14.7 | 8.45±0.58 | 7.26±0.87 | 10 |
| | **+ PGPR** | 136.7±15.0 | 8.38±0.80 | 6.89±0.75 | 18* |

Plants showed drought stress in beginning of July (only 40 mm precipitation), but recovered later in August due to sufficient rainfall. Average plant growth for 2007 was again 37% higher for PGPR treated plants and good PHC remediation was observed in the sites where the PEPS was applied (Table 3.1). A 33% PHC decrease ($P < 0.05$) was observed over the two year period in plots planted with PGPR coated seeds (Table 3.1). Only a 22% and 11% PHC reduction were observed on sites planted with non-PGPR coated seeds and unvegetated plots, respectively (Table 3.1). Preliminary data from plots in 2006 and plant growth data for 2006 was published as supplementary information (Gurska et al., 2009).

### 3.4.2 Heterotrophic bacterial counts

The total bacterial numbers in the soil with the + PGPR over the course of two years was 42.1% higher than that in the soil of – PGPR ($P < 0.17$, Figure 3.1A). The averages of total bacteria number in two planted treatments (+ PGPR and – PGPR) were significantly higher than that in unvegetated soil ($P < 0.05$) (Figure 3.1A). The total bacteria in planted soils remained 1-2 orders of magnitude higher at most sampling points ($P < 0.05$, Figure 3.2A) over the entire two years of phytoremediation at this site.

The first post-planting sampling event occurred on May 29, 2006. Total bacterial numbers in the soil with + PGPR plants were $3.35 \times 10^7$ CFU/g dry weight of soil, and those in – PGPR were $9.55 \times 10^6$ CFU/g dry weight of soil (Figure 3.2A). Numbers of bacteria in the unvegetated soil remained relatively constant over the course of the trial, at approximately $10^5$ CFU/g dry weight of soil (Figure 3.2A). However, both planted treatment areas had higher heterotrophic bacterial numbers by the first sampling time (i.e. within the first growth month) and those levels remained relatively stable for the remaining time of the study (Figure 3.2A).

### 3.4.3 Petroleum and hexadecane degrading bacterial counts

Petroleum-degrading and hexadecane-degrading bacterial numbers followed a similar trend as total heterotrophic bacterial numbers (Figures 3.1B, C and 3.2B, C). Over the two year field trial, the average bacterial numbers in the + PGPR soils were 34.5% greater for petroleum degrading bacteria ($P < 0.16$) and 29.5% greater for hexadecane degrading bacteria ($P < 0.14$) than those in the – PGPR soils. As well, at 8 out of 10 sampling times, the

+ PGPR soils had greater numbers of both petroleum and hexadecane degrading bacteria than in the – PGPR plots. The averages of both petroleum and hexadecane degrading bacterial numbers in two planted treatments (+ PGPR and – PGPR) were significantly higher than in the unvegetated plots ($P < 0.05$) (Figures 3.1B and 3.1C). This was also the case for the individual time points. Soil bacterial numbers of both petroleum- and hexadecane-degrading bacteria increased in the first two months of 2006 field year. The bacterial numbers in planted soils remained high until the end of September of 2006.

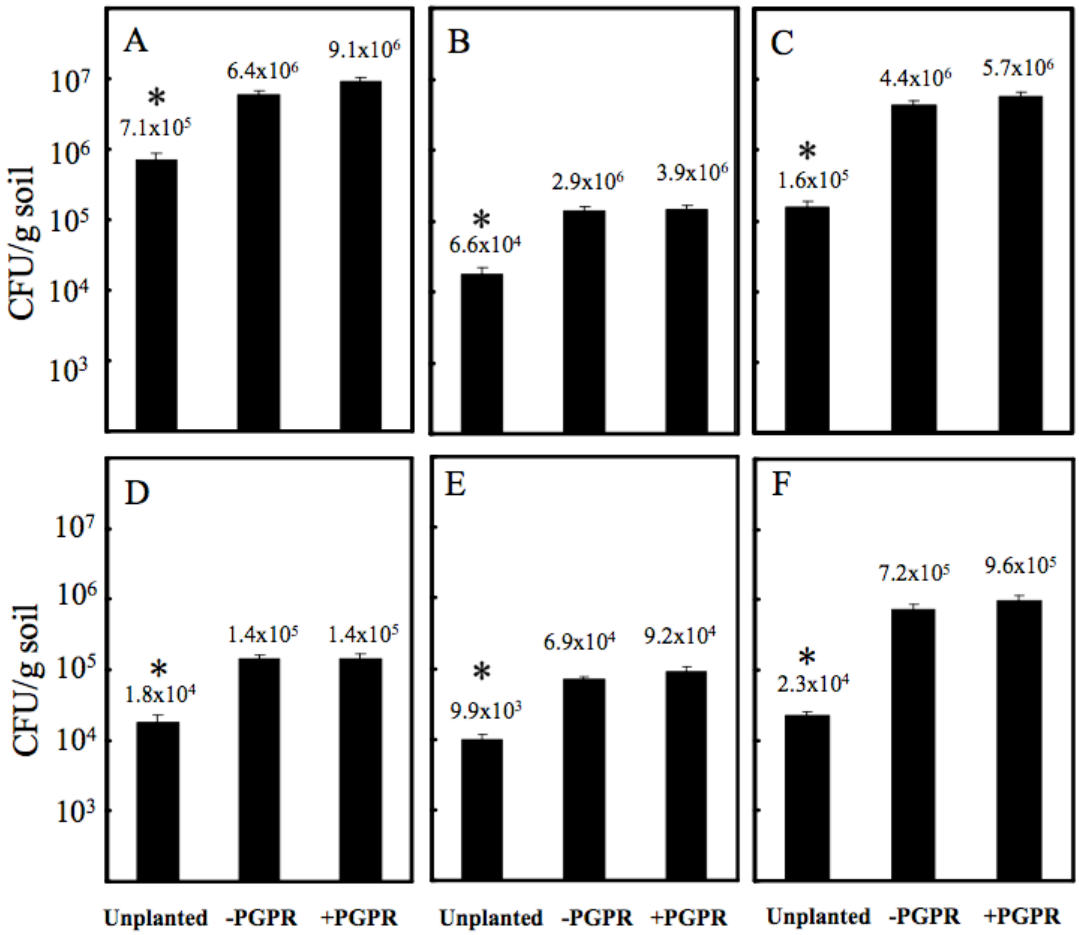



**Figure 3.1 Average microbial numbers over two years of phytoremediation at a PHC landfarm.**

Quantification of microbial groups from land farm soil samples from vegetated and unvegetated plots. Total heterotrophic bacteria (A), petroleum degrading bacteria (B), hexadecane degrading bacteria (C), total fungi (D), petroleum degrading fungi (E) and ACC-utilizing bacteria (F). These graphs display average numbers over the two-year field trial. * indicates difference ($P < 0.05$) between unvegetated treatment and two vegetated treatments. Data are the means ± SE (n=90). Figure modified from Wang, 2008.

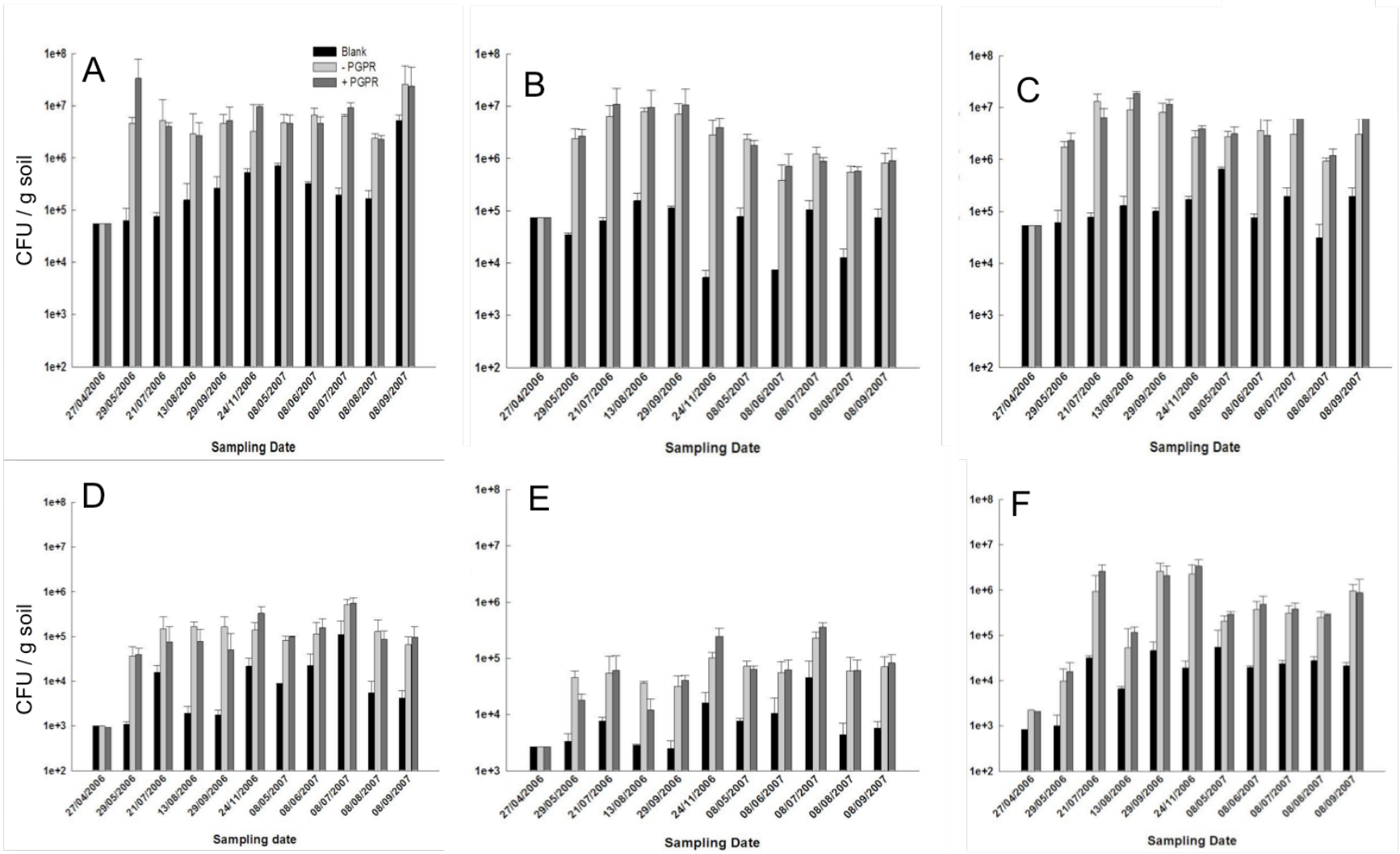
### 3.4.4 Total fungi and petroleum degrading fungi counts

Average total fungi numbers of two planted treatments (+ PGPR and – PGPR) over two year field trial showed similar values (Figure 3.1D). These numbers were approximately two orders of magnitude lower than total bacterial counts (Figure 3.1D and 3.2D). Petroleum degrading fungi specifically were 33.3% higher in + PGPR than those in – PGPR plots ($P < 0.21$) over the course of two years (Figure 3.1E). In both PGPR-treated and -untreated planted soils, higher fungal numbers were found than in unvegetated soils ($P < 0.05$) (Figures 3.1D, E).

Where plant growth was apparent, petroleum degrading fungi and total fungi increased within two months of planting, from approximately $10^3$ to $10^5$ CFU/per gram soil (Figure 3.2D and 3.2E). Over the remaining two years of the field trial, fungal numbers for the planted sites were unchanged. The addition of PGPR at seeded sites increased petroleum fungi numbers compared to – PGPR at 7 of the 10 sampling times (Figure 3.2E). Where there was no vegetation, fungal numbers were found between $10^3$ and $10^4$ CFU/per gram dry weight of soil throughout the field trial (Figures 3.2D and 3.2E). Planted treatments had significantly greater fungal numbers, in general, than the unvegetated treatment throughout the trial ($P < 0.05$) (Figures 3.2D and 3.2E).

**Figure 3.2 Temporal quantification of microbial groups at the PHC landfarm.**

Quantification of microbial groups from land farm soil samples from vegetated and unvegetated plots: total heterotrophic bacteria (A), petroleum-degrading bacteria (B), hexadecane-degrading bacteria (C), total fungi (D), petroleum degrading fungi (E) and ACC-utilizing bacteria (F). These data represent two consecutive years of phytoremediation. * indicates statistically differences ($P < 0.05$) between unvegetated treatment and two planted treatments. Data are the means ± SE ($n = 9$). Figure modified from Wang, 2008.


A
B
C
D
E
F
Blank
- PGPR
+ PGPR
CFU / g soil
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
27/04/2006
29/05/2006
21/07/2006
13/08/2006
29/09/2006
24/11/2006
08/05/2007
08/06/2007
08/07/2007
08/08/2007
08/09/2007
Sampling Date
Sampling date

### 3.4.5 ACC-utilizing bacterial counts

The two-year means of ACC-utilizing bacteria numbers in the soil of the + PGPR plots was 33.3% higher than that in the soil of – PGPR plots ($P < 0.11$, Figure 3.1F). The averages of ACC-utilizing bacteria numbers in two planted treatments (+ PGPR and – PGPR) were significantly higher than those in unvegetated soil ($P < 0.05$) (Figure 3.1F) and were 1-2 orders of magnitude higher than those in unvegetated soils at most sampling times ($P < 0.05$) (Figures 3.1F and 3.2F).

ACC-utilizing bacteria numbers in the planted soils sharply increased at the first sampling time points (May and June, 2006). From an initial count of approximately 850 CFU/per gram of soil, – PGPR increased to $2.62 \times 10^6$; + PGPR increased to $9.23 \times 10^5$; and unvegetated increased to $3.23 \times 10^4$ CFU/per gram dry weight of soil for – PGPR plots (Figure 3.2F). ACC-utilizing bacteria numbers in the soils of + PGPR were higher than those in the soils of – PGPR (8 times out of 10). These could be the PGPR that were applied or enhanced indigenous ACC-utilizing bacteria already present in the soil. Throughout the two-year field study, ACC-utilizing bacteria numbers in planted soils were 1-2 orders of magnitude higher than unplanted soil.

### 3.4.6 Microbial population of microorganisms in rhizosphere and non-rhizosphere soils

Rhizosphere (R) and non-rhizosphere (S) microbe counts in soils were compared and expressed as the R:S ratio (Karthikeyan et al., 2007*)*, *R:S Ratio = R/S*. Soils with increased R:S ratio indicates a positive effect of the rhizosphere on associated microorganism growth compared to microbes not in contact with the rhizosphere. The microbial numbers in the rhizosphere soils and non-rhizosphere soils were quantified using soil samples taken on September 21, 2006 (end of the 2006 season). The microbial numbers of all groups in rhizosphere soils were several times greater than those in non-rhizosphere soils, determined from R/S data (Table 3.2). R/S values for + PGPR plots were significantly higher than – PGPR; 1.5-3 times higher for all microorganisms except for the total fungi. These data suggest PGPR treatment increased rhizosphere microbes compared to untreated.

**Table 3.2 R:S ratio of different groups of microorganisms in the samples with and without PGPR.**

The R:S ratio was defined as the microbial populations ratio between rhizosphere and non-rhizosphere microorganisms using data from Figure 1. All data were significantly different between the + PGPR plots and the – PGPR plots ($P < 0.05$). Table from Wang, 2008.

| Microbial group | – PGPR | + PGPR |
|---|---|---|
| Total bacteria | 2.3±0.24 | 6.7±0.84 |
| Total fungi | 3.9±0.55 | 1.9±0.31 |
| PGPR | 2.8±0.25 | 4.6±0.59 |
| Petroleum degrading fungi | 3.8±0.39 | 7.1±0.67 |
| Hexacecane degrading bacteria | 5.5±0.78 | 8.2±0.72 |
| Petroleum degrading bacteria | 5.4±0.54 | 7.0±0.63 |

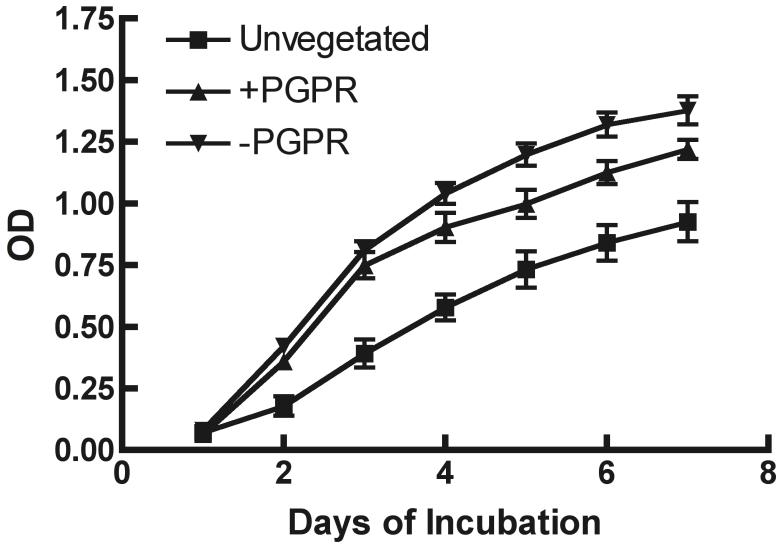
### 3.4.7 Carbon substrate utilization profiles using Biolog EcoPlates

Each Biolog plate contained 31 substrates in triplicate as well as a water control (96 wells in total). Each well containing the dried substrate or water also contained redox dye (tetrazolium) for evaluation of microbial respiration when the substrate is utilized. Following inoculation of each plate with its appropriate dilution of the soil sample, the color development was monitored with a microplate reader, every 24 h for a period of 7 days. The overall color development in the plates was calculated as the mean of all 96 absorbance values (31 substrate and water control on the plate, all in triplicate) (Garland and Mills, 1991) as the average well color development (AWCD). The AWCD in unvegetated samples was lower than those of vegetated soil samples in both the 2006 and 2007 samples (Figure 3.3A, 3.3B). Among vegetated samples, in 2006 the average absorbance of samples with PGPR showed a greater increase than – PGPR samples. However, this difference was not observed again in 2007.

A two-way multivariate analysis of variance (MANOVA) showed that Biolog data in two years were significantly different from each other (Hotelling's Trace, $P < 0.05$). Thus, each year's data was analyzed independently. One-way MANOVA was used to analyze the effects of plant growth and inoculation with PGPR and plant growth on remediation, carbon substrate utilization and microbial populations.

Principal component analysis (PCA) was performed using the BIOLOG data, using the pattern of carbon source utilization of each plate. The data based on 7 day incubation readings of Biolog plates after the AWCD standardization or the day with appropriate, fixed OD reading for PCA. PCA calculates new synthetic variables (principal components), which are linear combinations of the original variables (the substrates). The variation from the original data is depicted with xy coordinates (Ramette, 2007). PCA was performed using the carbon sources as variables. The BIOLOG data were further analyzed by specific compound and by BIOLOG guilds.

A




B

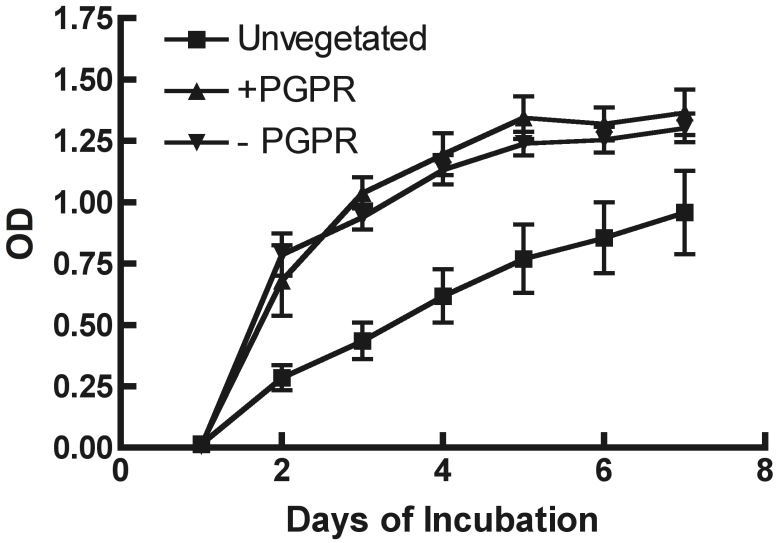



**Figure 3.3 Average well color development in Biolog EcoPlates inoculated with land farm soil samples.**

OD measurements performed at 600 nm. Each data point represents the mean ± SD, n≤5.

A principal component analysis (PCA) was conducted on the 2006 and 2007 end of the season samples separately. The Kaiser-Meyer-Olkin (KMO) measure verified the sampling adequacy for the analysis, (KMO=0.840 for 2006, KMO=0.746 for 2007). Bartlett's test of sphericity $\chi^2 = 801.390$, $P < 0.0001$, indicated the correlations between items were sufficiently large for PCA.

A PCA of soil samples collected at the last sampling of 2006 and 2007 was performed, first by examining day 7 of incubation of the BIOLOG plates followed by analysis at a reading at fixed AWCD to normalize for differences due to inoculum density. PCA based on day 7 indicated a difference among vegetated and unvegetated samples in 2006 but, both – PGPR plots and + PGPR plots sample averages appeared to be clustered (Figure 3.4A). Plot ordination further showed a small difference – PGPR plots and + PGPR plots in 2006. The PCA for 2007 gave a similar pattern of separation for the vegetated versus unvegetated plots, and the separation between – PGPR plots and + PGPR plots was even less evident in 2007 (Figure 3.4B).

To account for differences in starting inoculum density, where high starting microbial numbers dictate increased substrate utilization, PCA analysis was performed at an average AWCD of 0.75 for 2006 and an average AWCD of 0.5 for 2007 (AWCD was much lower for 2007 samples and thus to 0.5 OD was chosen instead of 0.75) (Garland, 1997). This normalization accounts for differences due to inoculum density thus it would account for differences due to different staring numbers of bacteria. In 2006, unvegetated and vegetated – PGPR plots clustered together and the + PGPR plot averages appeared slightly shifted away (Figure 3.4C). The PCA for 2007 showed a clustered pattern for all three samples types (Figure 3.4D).

In 2006, significant decrease occurred in the average absorbance of the amino acid utilization and a significant increase in carbohydrate utilization (Appendix B) in both the – PGPR plots and + PGPR plots. These changes were not as prominent in 2006 and, in fact,

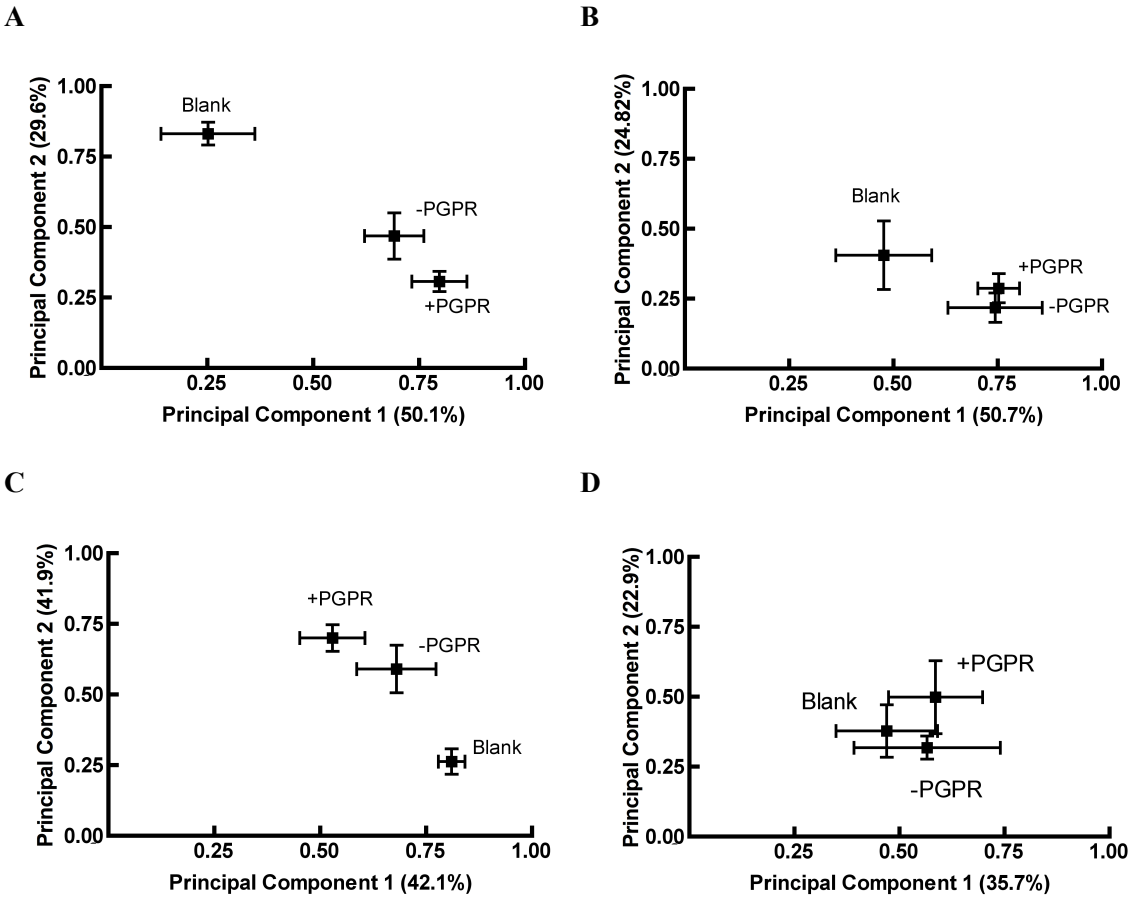



**Figure 3.4 Principal component analysis of microbial community analysis with Biolog.**

Microbial community profiles from blank (unvegetated) soils, soils where plants with PGPR inoculation and no PGPR inoculation in 2006 (A) and 2007 (B) with absorbance measurements of Biolog plates at day 7 of incubation. Variation explained by each component is indicated in brackets. Microbial community profiles in 2006 (C) and 2007 (D) with AWCD values at 0.75 and at 0.5 respectively. In all cases data points represent the means and SE for five or more samples from each soil type.

the utilization of amino acids was higher in vegetated plots compared to unvegetated plots in 2007. Specific substrates which showed lowered utilization in 2006 were L-asparagine, serine, phenylalanine, putrescine, N-acetyl-D-glucosamine, pyruvic acid methyl ester, galacturonic acid, 4-hydroxybenzoic acid, and Tween 40. Several substrates were more highly utilized in vegetated soil than unvegetated: phenylethylamine, lactose, xylose, erytritol, glucose-1-phosphate, ketobutiric acid, 2-hydroxybenzoic acid and cyclodextrine. Some of these trends were also observed in 2007 but overall the data did not show similar substrate utilization. Species diversity, Shannon diversity index (S) was higher in vegetated samples in both 2006 and 2007 (Appendix B). S indices for the utilization of the 31 carbon substrates showed a small difference between vegetated and unvegetated plots, however no differences were detected between the – PGPR plots and the + PGPR plots.

### 3.5 Discussion

Petroleum land farms are unique sites, having accumulated high molecular weight petroleum compounds due to treatment of their soils over time. Phytoremediation may be a viable strategy to remediate PHC impacted soils, but poor soil (low nutrients) and/or water stress, notwithstanding chemical toxicity, often result in poor plant growth (Kirk et al., 2005). Utilizing natural plant-PGPR interactions, enhanced in the PEPS, can reduce stress and increase plant biomass, particularly in the rhizosphere, significantly increasing PHC remediation from soil (Huang et al., 2005; Huang et al., 2004). Monitoring microbial numbers at a PGPR-treated site has provided important insights as to how PEPS influences the resident microbial population, and how these changes ultimately removes PHC from contaminated soils.

Previously at this landfarm, a two year phytoremediation trial reduced PHC contamination by 35% when PGPR were present (Chapter 2; Gurska et al., 2009). Preliminary data for the site in this study suggested that less remediation was evident in small plots (~18%), likely due to more samples being taken in edge of plot area where less plant growth is observed. The plant growth is typically more vigorous on larger fields where the edge effects of less seeding or less watering are not as pronounced as they are in small plots that are affected at their edges. Regardless, significant remediation was realized in PGPR treated plots overall.

The microbial analyses conducted during this trial assessed numbers of total bacteria and fungi, petroleum degrading bacteria and fungi, hexadecane degrading bacteria and ACC-utilizing bacteria. These counts of total microbes showed differences in planted versus unvegetated soils. The microbial numbers in unvegetated soils fluctuated only slightly and within a narrow range throughout two-year field trial. Planted soils however, showed rapid increases in microbial numbers within 2 months of planting but remained consistent thereafter, compared to that initial increase. Planted soils in both the + PGPR and the – PGPR plots had 1-2 orders of magnitude higher numbers of all microorganisms than unvegetated soils at most sampling times and when averaged over the entire two-year period. Plant growth significantly increases the number of total microorganisms including petroleum degraders, supporting the importance of plant growth in phytoremediation of PHC impacted soils (Hutchinson et al, 2003, Olson et al, 2003).

The inoculation of seeds with PGPR improved plant growth by approximately 30% in both years of the trial and resulted in more PHC degradation than in the – PGPR plots, with overall remediation of 33% over the course of two years. Numbers of PHC degraders in soils treated with PGPR were approximately 30% higher than in untreated soils averaged over the course of two year monitoring. PHC degraders, which include members of total bacteria, petroleum degrading bacteria, hexadecane degrading bacteria, petroleum degrading fungi, and ACC-utilizing bacteria, had a higher R:S ratio in the rhizosphere soils of + PGPR than of – PGPR. Additionally, in PGPR treated soils the total number of microorganisms were higher, including those of petroleum degraders. Although there is concern that the addition of PGPR may negatively influence the resident microbial community through competition for nutrients, these results indicate PGPR treatment creates a more robust microbial population, in terms of numbers of PHC degraders and total microbes.

In the past, the UW4 and UW3 PGPR applied to this site have been shown to influence plants (Glick, 2003; Glick et al., 2007), and has resulted in improved plant growth (Cheng et al., 2007; Glick et al., 2007; Greenberg et al., 2007; Huang et al., 2005). If different exudates are stimulated by the application of PGPR, or if actively growing, less stressed plants exude more organic nutrients to the surrounding soils than weak plants (van Elsas et al., 2007), this

would provide additional nutrients for microbial growth surrounding the rhizosphere, resulting in the higher R:S ratios, in + PGPR plots. Increased microbial populations could lead to faster degradation of PHC in the more exudate-rich rhizosphere.

The numbers of PHC degraders do not, however, always correlate with remediation rates of PHC, as a number of laboratory-scale studies have indicated (Phillips et al., 2006; Euliss et al., 2008). A reason for this discrepancy may be the different conditions for identifying PHC degraders in laboratory versus the field. In contaminated soils with high concentrations of PHC, such as land farm soils, PHC degraders would be selected for, creating a veritable race for nutrients. Degraders would outcompete non-degraders and lower their numbers, and the increase in PHC degraders would intuitively increase PHC remediation (Euliss et al., 2008). This potential spike in PHC degraders would biodegrade PHC, as the vastly abundant resource, faster than the minor contaminants. As a result, short-term phytoremediation may be more efficient in the first year and less effective with decreasing contaminant concentrations. As time goes on, there may be a decrease in bioavailability in the contaminants remaining in low concentrations, and this would certainly produce long-lasting, slow remediation rates. This may explain the trend observed in a previous three-year field remediation study on this land farm (Chapter 2; Gurska et al., 2009).

As a stark contrast to the field where a variety of carbon sources are available, laboratory reared PHC degraders are typically provided PHC as a sole carbon source. With the availability of carbon, in plant exudates for example, PHC degraders are not limited to PHC as sole carbon source (Euliss et al., 2008). Finally, culture-dependent methods vary in how representative they are of the bacteria present in the environment; the overall consensus is that only about 1% of bacteria are analyzed by culture based methods and the represent fastest growing microorganisms (Ritz, 2007). The differences existing between field soils and laboratory soils present a viable explanation to the perceived discrepancies between numbers of PHC degraders and remediation rates.

Compared with published greenhouse and growth chamber studies with soil PHC concentrations from 0.5 to 5%, this study reported lower total microbial and petroleum

degrader numbers. The microbial numbers reported here were one to two orders of magnitude lower than a growth chamber study using weathered oiled soil (Phillips et al., 2006), a greenhouse study using artificially spiked soils (Kirk et al., 2005), and a laboratory study in petroleum-refining wastewater irrigated agricultural soils (Li et al., 2007). Variation in these microbial counts are present in the literature, however. Two research groups have reported total bacterial numbers of $10^6$-$10^7$ CFU/g soil total bacterial numbers; (Euliss et al., 2008; Kaksonen et al., 2006). Similar to this study, PHC concentrations in the field were present at a comparable level (Euliss et al., 2008). The microbial counts in these studies were all conducted using plate count methods, and differences in the native microbes, specifically their ability to be cultured, presents a viable concern in regards to variability among collected data in these studies. Further the differing concentration and types of PHC across the studies seem to be one critical factor affecting the microbial populations (Maila et al., 2006). For example in the Sarnia land farm soils, high concentrations of PHC result in only tolerant microorganisms being able to survive, and only those utilizing PHC able to grow well. Conversely, this unfavorable environment for indigenous microorganisms is likely the reason that inoculants are able to thrive when growth of resident inhabitants was impacted (Strigul and Kravchenko, 2006).

Aiding growth of microbes, root exudates create nutrition gradients surrounding root structures in the soil. This produces a corresponding bacterial gradient in the rhizosphere (Corgie et al., 2006), and results in more microorganisms supported than in non-rhizosphere soils (Table 1). A subsequent biodegradation gradient of contaminants like phenanthrene (PHE) in the rhizosphere (Joner et al., 2003) also becomes apparent. In this study, soil samples taken at a distance from the roots (bulk soil) produced fewer total bacteria and PHC degraders (Table 1), and soils taken at the middle site of the rhizosphere and non-rhizosphere soils also showed medium numbers of total bacteria and PHC degraders (data not shown). This may indicate that PHC closer to the roots would be consumed faster, and, as a result, a PHC gradient may exists around roots.

In this study, total fungal populations and fungal petroleum degraders increased with plant growth (Figures 3.1 and 3.2). This is not surprising, as over 40 fungal types or strains have

been found to tolerate growth on crude oil (Davies et al., 1979) and several fungi have been successfully used in the bioremediation of PHC (Yateem et al., 1998). Interestingly, the R:S ratio of total fungi conflicts with that of petroleum degrading fungi. The rhizosphere of + PGPR have higher R:S ratio for petroleum degrading fungi and lower ratio for total fungi than that of – PGPR. This indicates that application of PGPR may lead to the fungal community structure changes.

Fungi may interact synergistically with bacteria on degrading PHC (Merkl et al., 2006). Different from bacteria, that favor neutral pH conditions, fungi favor acidic conditions. During biodegradation, alkanes, the dominant components in petroleum hydrocarbons, are converted by monooxygenases or dioxygenases to alcohols, aldehydes, and fatty acids, which can then be metabolized through β-oxidation (van Beilen et al, 2001). The formation of the acids could lower the soil pH, resulting in the acidic conditions favored by fungi. As well, many root exudates are acidic, including $CO_2$ and amino acids, which can be dissolved in the rhizosphere (Tate et al, 2000), lowering pH by 1–2 units compared to unvegetated soil (Kaksonen et al, 2006). Although the abundance of fungal petroleum degraders typically found in the rhizosphere is only 1 to 10% of their bacterial partners, considering their larger size, fungi could play a significant role in the degradation of PHC. Furthermore, the higher R:S ratios of petroleum degrading fungi in PGPR treated rhizosphere than that in non-PGPR treated (Table 1) might also partly contribute to the faster removal of PHC in PGPR treated soils. It should be noted that Biolog Eco plates do not account for changes in fungal community because fungi cannot reduce tetrazolium (Preson-Mafham et al., 2002). Thus it is likely that if fungal community was more affected than bacterial community this would not be detected and needs to be investigated further.

Biolog analysis is a well-established method for assessing biological diversity through carbon substrate utilization**.** Biolog EcoPlates, contain substrates that are known plant root exudates or that have previously been found to have a high discriminatory power among soil communities and has been used to measure the ability of unidentified microorganisms to exploit different carbon sources (Insam and Goberna, 2004). The resolution of Biolog plates comes from the continuous data collection (growth in response to each substrate) that aids the

distinction between communities. The rate of increase of average well color development (AWCD) in unvegetated plots was lower than those of vegetated soil plots in both 2006 and 2007. Since color development is related to cell density (Preson-Mafham et al., 2002) the increase in AWCD is likely because the number of microbes was higher in vegetated soils. Further, among vegetated plots, in 2006 the average absorbance of samples with PGPR increased more than – PGPR samples; however, this difference was not observed again in 2007. These observations correlate with what was found with microbial counts for total bacteria.

To examine the functional fingerprint of different soil samples using PCA analysis two normalization methods were used to observe effects with and without normalization due to inoculum size. The functional fingerprint generated using the PCA analysis with Biolog EcoPlates revealed the largest difference existed between the unvegetated and vegetated plots and not between the + PGPR and the – PGPR plots, in both 2006 and 2007 samples. PCA indicated distinct qualitative differences among vegetated and unvegetated samples in their use of carbons substrates in both years when inoculum size was not controlled for. In 2006 the difference persisted when PCA was performed on samples normalized for inoculum size, indicating presence of plants change the bacterial community somewhat. In 2007, the difference between vegetated and unvegetated samples was only observed when bacterial density was not accounted for. At the conclusion of 2007 no differences were detected between vegetated and unvegetated samples. Taken together, these findings indicate that the biggest differences in metabolic diversity of microbial populations come from plant growth and not from inoculation with PGPR. Physiological changes in response to a PGPR have previously been detected in tomato (Correa et al., 2007) and maize roots (Padney et al., 1998), however, lack of effects has also been observed (Correa et al., 2007). This conflicting response to inoculants has been attributed to the plant genotype under study (Correa et al., 2007). Taken together, the PCA analysis suggests that UW3 and UW4 do not have a large influence on the functional structure of indigenous microbial community in petroleum-contaminated soils. PGPR influence plant growth and improve root growth and morphology. This increase in plant root biomass likely results in a larger rhizosphere and higher number of

sites for bacterial colonization. Healthier, larger plants likely support more exudation to the rhizosphere thus increasing microbial population. The functional distribution of this community remains unchanged.

There were several differences in utilization of carbon guilds, for instance, amino acids were used less in vegetated than in unvegetated samples in 2006, but this trend was reversed in 2007. The unvegetated soils showed unusually high amino acid utilization in 2006, whereas in 2007 it was quite low. The utilization of amino acids in vegetated samples did not change between 2006 and 2007, suggesting that perhaps the unvegetated data was anomalous in either 2006 or 2007. Further, the utilization of carbohydrates increased in vegetated samples for both 2006 and 2007, though not as prominently in 2007. This is not surprising as the presence of plants would select for carbohydrate utilizing organisms, as root exudates are known to be rich in carbohydrates. With PGPR present, there would be more plant biomass and, therefore, likely more root exudates and perhaps more carbohydrate-utilizing microorganisms than in soils without PGPR. There were some differences in carboxylic acids; this was previously observed with in Biolog analysis of rhizosphere microbes when phytoremediation of PAHs was examined where carboxylic acids were favored relative to non-rhizosphere community (Heinonsalo et al., 2000; Siciliano et al., 2003). The Shannon indices of metabolic diversity in soils were on par with those found in another study with perennial ryegrass and alfalfa and bulk soil (S = 3.3) (Kirk et al., 2005). However, contrary to study by Kirk et al., differences between bulk and vegetated soils in both growing seasons of the study were detected.

Results from this study parallel previous studies showing quantitative and qualitative differences coming from growing plants on contaminated soils (Banks et al., 2003a; Kirk et al., 2005). Based on our findings in analysis of the microbial environment in the – PGPR plots, the + PGPR plots and unvegetated soils, we can conclude that PEPS is an environmentally safe technology, with a benefit to soils coming mostly from plant growth. PEPS was superior in phytoremediation efficiency of PHC from soils. PGPR used in this study (UW3 and UW4) resulted in better phytoremediation of land farm soil by increasing plant biomass. Additionally, more petroleum degraders were observed in the soils planted

with PGPR coated seeds than those in the soils planted with non-PGPR coated seeds. This leads to the more rapid biodegradation of PHC in PGPR treated soils than untreated ones. Rhizodegradation by PHC degraders was shown at this site as a major mechanism of PHC phytoremediation by Cowie et al. (2010), who measured $\Delta^{14}C$ of phospholipid fatty acid (PFLA) biomarkers from the landfarm soil and found that ~80% of microbial PLFA carbon was derived from petroleum hydrocarbons and only approximately 20% was obtained from metabolism of modern carbon sources, likely plant exudates. Thus, almost all of the carbon in the elevated microbe numbers due to plant growth came from PHC. This explains why the PHC is being remediated; it is the source of reduced carbon for the soil microbes.

Carbon substrate utilization results showed that the petroleum-contaminated microbial communities are somewhat impacted by inoculation with PGPR but not more so than by vegetation alone. PGPR inoculants appear to stimulate growth of a microbial community capable of utilizing a similar variety of general carbon sources as the uninoculated, vegetated bacterial community. Thus the differences between uninoculated and inoculated bacterial populations are small, and the PGPR effects likely comes from differences in distribution of microorganisms already present. Thus, the key mechanisms of PEPS of PHC from petroleum land farm soil appear to be due to the added PGPR eliciting a larger and more active rhizosphere that support higher numbers of petroleum degraders. The PEPS, with the application of PGPR as a core procedure, is a promising approach to remediate toxic, persistent and recalcitrant contaminants from soil.

## 3.6 Acknowledgements

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# Chapter 4
# Use of a Plant Growth Promoting Rhizobacteria-Enhanced Phytoremediation System at a weathered petroleum hydrocarbon-contaminated biopile

## 4.1 Overview

Phytoremediation field tests were conducted at a site with low levels of recalcitrant petroleum hydrocarbons (PHC). A plant growth promoting rhizobacteria (PGPR)-enhanced phytoremediation system (PEPS) was used. PGPR are naturally present soil bacteria, which exert positive effects on plant growth through a variety of means. PEPS was previously used on a site with high PHC levels in fine-grained soil. In this study PEPS was used at a site with low levels of highly weathered PHC in coarse-grained soils. PGPR improved plant growth and microbial growth in contaminated soil and lead to increased degradation of PHC in contaminated soils. The contribution of plant exudates to assays of PHC levels was examined and it was found that plants contribute detectable levels of extractable hydrocarbons. Further, recalcitrant biomarkers were used to confirm degradation of PHC at this site.

## 4.2 Introduction

The discovery that contaminants disappear in the vicinity of plant roots has lead to the development of a field of research and development of a biotechnology called phytoremediation (Cunningham et al., 1995; Cunningham and Ow, 1996). Steady efforts have been undertaken to optimize phytoremediation for degradation of organic contaminants such as petroleum hydrocarbons (PHC), polycyclic aromatic hydrocarbons (PAHs), polychlorinated phenols (PCBs) or trichloroethylene (TCE) (Salt et al., 1998) (Huang et al., 2004b; Huang et al., 2005; Gurska et al., 2009) and metals (Alkorta and Garbisu, 2001). The objective in phytoremediation of PHC is to progressively lower contaminant concentrations by uptake and degradation by plants, and by microbial degradation in the rhizosphere, the area immediately surrounding the root zone (Wenzel, 2009). The ultimate goal of phytoremediation is the complete disappearance of the contaminants, or to reach generic targets set by the regulatory bodies such as the Canadian Council of Ministers of the Environment (CCME).

Phytoremediation of PHC proceeds through two primary mechanisms. Plants take up contaminants with certain optimal characteristics, such as log $K_{OW}$ between 1.5-3 (Burken and Schnoor, 1998). Most of the degradation of PHC however is thought to occur in the rhizosphere (Hutchinson et al., 2003; Cowie et al., 2010). Plants exude large amounts of the photosynthate they generate, and this organic carbon serves as substrates for growth of rhizosphere microbial organisms (Domanski et al., 2001; Singer et al., 2003; Dennis et al., 2010). Exudates include amino acids and organic acids, carbohydrates, fatty acids, enzymes, sterols, vitamins, nucleotides and phenols (Buyer et al., 2002). This plethora of compounds shapes the diverse microbial community that is supported by plant root systems.

One of the challenges faced in phytoremediation of PHC is phytotoxicity, resulting from most toxic PHC components such as PAHs (Baek et al., 2004; Alkio et al., 2005; Palmroth et al., 2006). Remediation is dependent on plant growth, and is thus impeded if plants cannot grow due to high levels of toxicants. This setback in phytoremediation can be overcome by exploiting plant microbe interactions. Microbes within the rhizosphere form a complex community and may exert positive, negative or neutral effects on plants

as well as other microbes (Podile and Kishore, 2003). One group of rhizosphere organisms, called plant growth promoting rhizobacteria (PGPR), are known to improve plant growth under stress conditions through a variety of direct and indirect mechanisms (Glick et al., 2007). Notably, they can lower the production of the plant stress hormone ethylene through action of 1-aminocyclopropane-1-carboxylate (ACC) deaminase – an enzyme, which consumes the ethylene precursor, ACC. PGPR have previously been used to lower phytotoxicity of contaminants in both agricultural and phytoremediation applications (Glick, 2003; Podile and Kishore, 2003; Huang et al., 2005; Gerhardt et al., 2009; Gurska et al., 2009)

In previous field studies, we have shown the protective effect of PGPR on plant growth where levels of PHC were high (the approximate starting concentration was 130 g $kg^{-1}$) (Gurska et al., 2009). In this study at a petroleum refinery landfarm for disposal of petroleum sludge, PGPR mitigated negative effects of PHC and increased remediation, presumably through higher biomass production. The PHC found there was highly aged, or weathered, a process known to decrease bioavailability of contaminants (Semple et al., 2003; Semple et al., 2007). Having observed favorable results at this site, the goal was to test the range of applicability of a PGPR enhanced phytoremediation system (PEPS). Amassing evidence suggests that phytoremediation is much less successful with aged contamination than freshly spiked soils, likely due to low bioavailability (Wenzel, 2009). Thus, a site was chosen with PHC concentrations below 10 g $kg^{-1}$ that is composed primarily of weathered, recalcitrant PHC compounds that have remained after many years of natural attenuation, landfarming and biodegradation, and were suspected to be resistant to remediation. The test site also had poor quality, course-grained, gravelly soil. The source of the contamination at this site was from a flare pit, an earthen pit typically located at a petroleum extraction site where waste gases are burned off and liquid waste hydrocarbons are diverted. Such pits may contain a plethora of wastes including salt, lubricating oils and petroleum sludge. In many cases, including at this site, sparse records exist regarding the types and amounts of disposed contaminants, in part due to the age of the pits (Cook et al., 2001). This particular pit was excavated and spread at a treatment site as a biopile. The soil at this site was substandard for plant growth, high in

gravel content and low in organic matter. Such soils result in inhibited plant growth in part due to ethylene levels.

The objective of this research was to determine if phytoremediation is a viable method to remediate highly recalcitrant PHC compounds, and further if PEPS was superior to plant growth alone. We show that PEPS can be used to remediate sites with low, recalcitrant PHC contamination. PEPS increases remediation through modest but consistent increases of plant growth and resultant increases of microbial numbers in the soil. A number of challenges with the quantification of remediation were encountered at this site. In particular, we address challenges associated with measurements of low levels of PHC during this phytoremediation trial and suggest improvements for field evaluations. Further we evaluated contribution of biogenic organic compounds (BOCs) to PHC measurements and used persistent petroleum biomarkers (e.g. hopanes), to evaluate the efficacy of phytoremediation at this site.

## 4.3 Materials and Methods

### 4.3.1 Site description

Tests were performed at a biopile in Alberta, Canada for three consecutive years commencing in May of 2005. The trapezoidal study site had the dimensions of 70 x 70 x 50 x 35 meters (~2800$m^2$) and was further divided into experimental plots (Figure 4.1). The soil was contaminated with below 10 g $kg^{-1}$ of PHC that was associated with a flare pit from petroleum extraction operations. Standard farming practice of fertilizer addition (three times a year using 37-17-0-0 at 100 kg/ha) and irrigation was implemented. Soil at this site was of poor quality, gravelly soil with clay loam/loam texture. The $Mg^{2+}$ and $Ca^{2+}$ values slightly exceeded acceptable guidelines for good plant growth, which should fall between 100-300 mg $kg^{-1}$ for $Mg^{2+}$ and 1000-4000 mg $kg^{-1}$ for $Ca^{2+}$. The elevated $Ca^{2+}$ is consistent with the high salinity often characteristic of old flare pits in western Canada, due to brine water inputs (Rutherford et al., 2005). Organic matter was below the range of 4-15% that is recommended for the type of vegetation used in this study. Salt levels in this soil were low, 1-1.5 dS $m^{-1}$, thus salt would have no negative impact on soil.

Selected chemical and physical soil parameters are listed in Table 4.1. Nutrient and soil texture analysis was performed by Agri-Food Laboratories (Guelph, ON, Canada). Starting PHC and PHC fractions concentrations are listed in the results sections, 4.4.3 and 4.4.4 and its corresponding tables.

**Table 4.1 Soil characteristics at the Turner Valley biopile.**

| Soil Parameter | Value, unit |
|---|---|
| Sand | 37-40% |
| Silt | 33-38% |
| Clay | 25-27% |
| Soil texture | Loam/Clay Loam |
| Phosphorous | 9-28 mg $kg^{-1}$ |
| Potassium | 160-220 mg $kg^{-1}$ |
| Magnesium | 440-600 mg $kg^{-1}$ |
| Calcium | 5550-5950 mg $kg^{-1}$ |
| Sodium | 13-40 mg $kg^{-1}$ |
| Organic matter | 3-3.6% |
| pH | 7.5-7.7 |
| Total Salts (ECe[1]) | 1-1.5 dS $m^{-1}$ |

[1]ECe, electrical conductivity (EC) of soil samples, which are a measure of the soil salinity, expressed in dS/m (deci Siemens per meter).

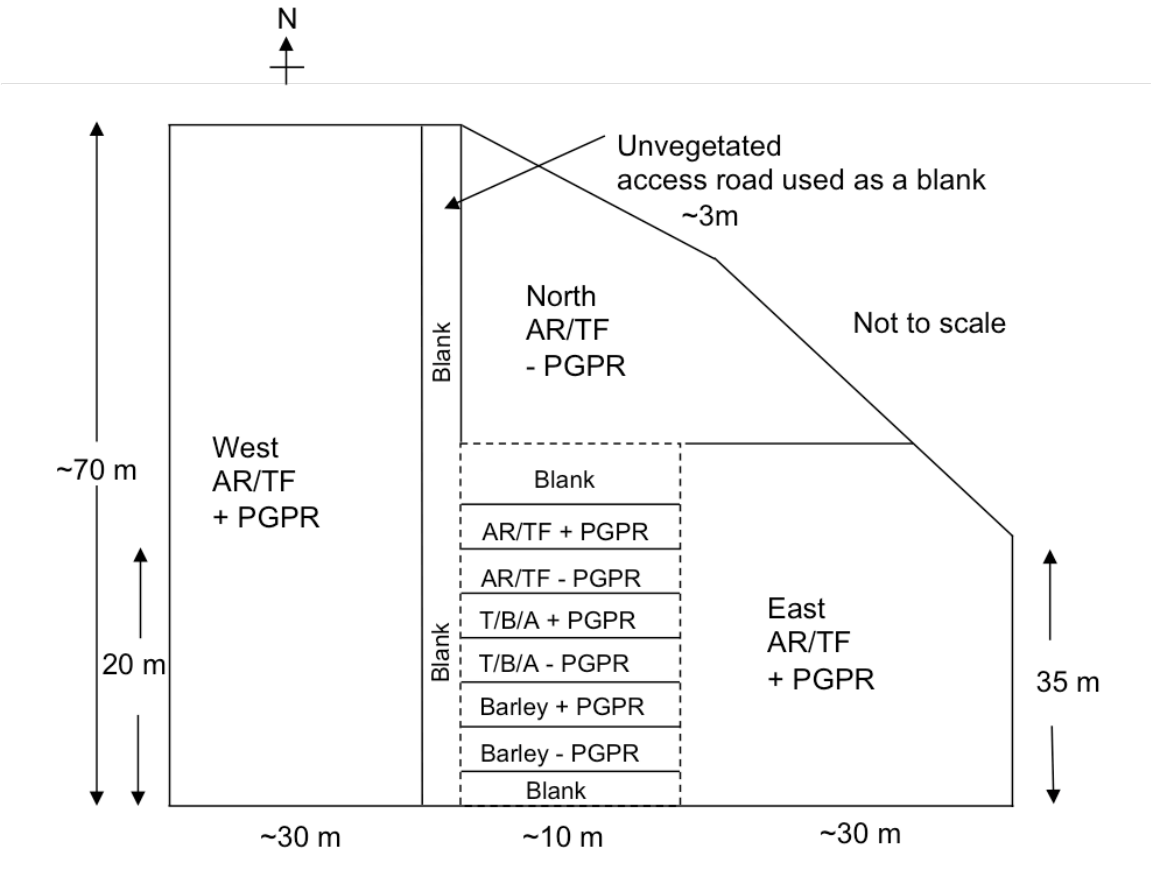



**Figure 4.1 Site plan and planting scheme.**

In 2005 the West and East plots were planted with annual rye (AR) and tall fescue (TF) grass mixture with PGPR, the North plot was planted with AR/TF – PGPR. A series of small plots with AR/TF mixture, timothy/brome/alfalfa (T/B/A) and barley, with and without PGPR, were planted. The East + PGPR plot was not tilled in 2006 and was overseeded with PGPR treated AR/TF seed. In 2007, all sites except the blank were tilled and replanted as per 2006. The blank area was approximately 3 m wide in all years.

### 4.3.2 Soil remediation using PEPS

PEPS consisted of land-farming and sunlight exposure (aeration/photooxidation) prior to planting and growth of plants with PGPR. The physical soil treatment was performed by tilling of the soils prior to planting. This facilitated the exposure of a new layer of soil to light and air. Plants were allowed to grow for the entire plant growth season (May to September). The control area consisted of soil that was not vegetated and not tilled, except at the beginning of season in 2006 (discussed later in the text).

Plant species used in the current study were previously tested in the greenhouse (Huang et al., 2003; Huang et al., 2004a; Huang et al., 2005) and in the field (Gurska et al., 2009). Tall fescue (*Festuca arudinacea*) and annual ryegrass (*Lolium multiflorum*) were chosen due to their extensive root production, suitability to local Alberta climate and previously determined germination rate under PHC stress (Huang et al., 2005). Every year, the grasses were planted as a mixture. Small test plots for new plant species were tested and those included triticale (*Triticosecale* var. Bunker), barley (*Hordeum vulgarae*), fall rye (*Secale cereale*), forage mixture of timothy/brome/alfalfa (T/B/A, 35% Richmond timothy (*Phleum pratense*), 35% BromePro$^{TM}$ Blend, 30% AC Grazeland alfalfa). All seeds were bought from Ontario Seed Co., Waterloo, Ontario, Canada (annual ryegrass, tall fescue, fall rye), Cribit Seeds, West Montrose, Ontario, Canada (barley, triticale) and PICKSEED, Alberta, Canada (forage mixture).

### 4.3.3 Site preparation and seeding

In 2005 planting was performed on May 16. In addition to large plots, a series of small plots were set up to test several plant species at this site; in 2005 those were triticale, fall rye and barley.

In 2006, planting was performed on May 8. The West + PGPR plot and the North – PGPR and the unvegetated blank plots were tilled extensively to mix the soil. The East + PGPR plot was over-seeded but not tilled (Figure 4.1). The soil from test plots was lifted and moved to a soil treatment facility where two small 5 x 10 m biopiles were constructed, one of 30 cm depth and one of 60 cm depth. Each biopile was divided in half, with one half planted with PGPR treated seed and the second half with untreated

seed; the biopiles were planted with a mixture of tall fescue and annual ryegrass. On the main site where soil was lifted (see above text), test plots were set up with the mixture of two grasses (annual ryegrass and tall fescue), forage mixture of timothy/brome/alfalfa and barley alone. Also in 2006, a section of the West + PGPR plot was planted with timothy/brome/alfalfa with and without PGPR (each plot was approximately 10 by 30 meters, Figure 4.1).

In 2007 planting was performed on May 30, 2007. The entire site except the blank area and the T/B/A area were tilled. T/B/A was overseeded with new seed with the appropriate treatment. Grasses were replanted as a mixture as described above. In 2007, small plots consisted of tall fescue and annual ryegrass mixture, forage mixture and barley planted as a single plant species. In 2007 the small 30 cm and 60 cm biopiles were tilled and planted with the same plant species (tall fescue/annual ryegrass mixture) PGPR inoculation of seeds.

PGPR used in this study were two strains of *Pseudomonas putida* (UW4) and *Pseudomonas* sp. (UW3). Both strains produce indoloacetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase; these characteristics are believed to make UW3 and UW4 suitable plant growth promoters (Glick et al., 1999). The bacterial inoculants were prepared as previously described (Gurska et al., 2009). Final bacterial density was $1 – 5 \times 10^6$ CFU per seed, depending on the plant species used. A methyl-cellulose polymer coating was used to aid in PGPR adhesion to the seeds, increasing number of bacteria present on the seeds during planting. A coloring agent (Color Coat Blue, Becker Underwood, Saskatoon, Saskatchewan, Canada) was added to distinguish treated and untreated seed. In all cases seeds were dried and stored for a maximum of 30 days prior to sowing.

### 4.3.4 Soil sampling and chemical analysis

Soil samples were collected using an Edelman auger (Eijelkamp, Agrisearch Equipment, Giesbeek, the Netherlands) from the top 30 cm of soil and stored at $4^oC$ in glass jars until further analysis. To monitor depletion of PHC from the soil, samples were collected at the beginning and at the end of every season in a grid pattern. Chemical analyses to determine PHC concentrations in the soil were either gravimetric in-house methods, or

accredited commercial laboratory methods with gas chromatography with flame ionization detector (GC-FID) measurements of CCME fractions 1-4 as well as gravimetric methods.

CCME fractions F1-F4 are defined as follows: F1 has a carbon range of C6-C10, F2 has a carbon range C>10-C16, F3 has a carbon range of C>16-C34, F4 has a carbon range of C>34 to C50+). Total PHC (this is the sum of CCME F1-F4 obtained from the same chromatogram as each individual PHC fraction) was also obtained. In-house gravimetric analyses did not employ silica cleanup, however the accredited commercial laboratory did use this method. Silica cleanup is used to remove polar biogenic organic compounds (BOC) that would erroneously increase the final PHC numbers. The cleanup method used by commercial laboratories was the *in situ* silica clean up method, which is less effective than silica column (British Columbia Ministry of Water, 2004). This process removes compounds that are more polar than PHC and may be derived from biological sources such as plants, but also compounds from petrogenic sources that have been partially metabolized or photooxidized. According to the standardized CCME protocol followed by accredited laboratories, sufficient rinsing of the silica gel column should minimize these losses (CCME, 2001b). Soil sampling was performed at each time point, unless otherwise specified.

The GC-FID analyses to obtain CCME F1-F4 were performed by certified independent laboratory (ALS Canada Ltd in Calgary, AB and ALS Canada Ltd in Waterloo, ON) according to CCME protocols (CCME, 2001a). The gravimetric method in our laboratory was an ultrasonic method. Briefly, air-dried soil samples (2 g) were extracted 3 times by ultra-sonication for 50 min into total 20 mL of 1:1 hexane/acetone mixture (EPA, 1998). Extracts were dried by completely evaporating the solvent under a stream of nitrogen gas. The amount of extracted PHC was determined by weighing the dried extracts. Three data sets, F3 and $PHC_{TOTAL}$ from independent laboratory and $PHC_G$ from our laboratory were evaluated. The three year gravimetric data was fitted to a first-order decay model according to the equation:

$$C = C_0 \exp(-kt) \qquad \textbf{(Equation 4.1)}$$

where C is the concentration (g $kg^{-1}$), *t* is time (months), $C_0$ is the initial concentration, and *k* is the kinetic rate constant ($month^{-1}$).

Two other sets of data of PHC levels were obtained through collaboration with laboratories from Environment Canada (Dr. Zhendi Wang) and ExxonMobil Biomedical Sciences Inc. (Dr. Roger Prince and Dr. Mark Lampi) and those methods are discussed in 4.3.5.

### 4.3.5 Measurements of plant biomarkers and PHC biomarkers

In year three, analyses to determine the contribution of biogenic and petrogenic compounds to the PHC analyses was initiated. Several samples from planted and unplanted soils were analyzed for biogenic material. A total of six samples were analyzed, two from each treatment: planted at the beginning of the season, planted at the end of the season and unplanted at the end of the season. Cost prohibited additional sample analyses. This work was performed by Zhendi Wang at Environment Canada, Ottawa, Canada according to the following protocol. The PHC contaminated samples were first air dried, and stored at -20$^{o}$C. Approximately 10 g of air dried soil sample was mixed and ground with anhydrous sulphate and surrogates spikes were added. Samples were extracted overnight with n-hexane/acetone mixture (1:1, v:v) with a Soxhlet apparatus. Total solvent extractable materials (TSEM) were determined by taking 1 mL of the final extracts and drying with gentle stream of nitrogen. To determine PHC content, an aliquot (900 ul) of the final extract was mixed with hexane (up to 1 mL) and assayed with gas chromatography with flame ionization detector (GC-FID). Determination of *n*-alkanes, PAHs, petroleum biomarkers and biogenic organic biomarkers was performed using a gas chromatography-mass spectrometry (GC-MS).

Petroleum biomarkers were analyzed by at the ExxonMobil Biomedical Sciences Inc. facilities. Beginning and end of the year samples were analyzed for 2006 and 2007 planting year. These years were year two and year three of the three-year project. Analyses was performed on soil samples collected from plots planted with plants + PGPR and on unvegetated control soil. Each treatment was analyzed in triplicate according to the following protocol. For analysis of conserved petroleum biomarkers soil was dried by mixing with $Na_2SO_4$ then 10 g of soil was extracted with methylene chloride. Gravimetric

oil content was obtained by weighing of the extracts. Gas chromatography coupled with mass spectrometry was used to analyze individual groups of hydrocarbons and total hydrocarbon (PHC) in selected ion monitoring (SIM) mode and total ion mode respectively. PHC normalized to hopanes was obtained from gas chromatograms not from the gravimetric oil content. The particular ranges of individual hydrocarbons that were examined were the hopanes and sterene biomarkers as well as the persistent PAHs, chrysenes. Detailed methods and principles of the analysis can be found here (Prince et al., 2003).

### 4.3.6 Biomass analyses

Plant biomass was measured by isolating a square of soil, 20 cm in depth, with as little of disturbance of roots as possible. Plant growth was normalized for the area sampled for each time point. Plants including roots were isolated from the soil and washed with water to remove all soil particles adhering to the roots. Roots and shoots were separated and blotted dry to obtain fresh weight. To obtain dry plant weight, plant samples were dried for 2 days in an oven at 40$^{o}$C and re-weighed.

### 4.3.7 Chlorophyll-*a* fluorescence

Alterations in the health of the photosynthetic apparatus of plants were measured by chlorophyll-*a* fluorescence induction using Pulse Amplitude Modulated (PAM) fluorometer (PAM-101, Walz, Effeltrich, Germany). To carry out fluorescence measurements using the PAM, samples taken from the field were transported to laboratory in a cooler on ice and dark adapted for approximately 30 minutes. Plants were exposed to weak, pulsed red light (< 1 μmol $\cdot m^{-2} \cdot s^{-1}$) to acquire the $F_o$ value (minimal fluorescence). This light was not strong enough to promote photosynthesis but stimulated background fluorescence only. Saturating light (~3000 μmol $\cdot m^{-2} \cdot s^{-1}$, 600 ms) from a halogen lamp was applied to measure the $F_m$. The maximal activity of PSII was calculated as $F_v/F_m = (F_m-F_o)/F_m$ (Maxwell and Johnson, 2000). Plants were then exposed to actinic light (fluence rate of 70 μmol $\cdot m^{-2} \cdot s^{-1}$) and to saturating light pulses to obtain steady state fluorescence ($F_t$) and maximal fluorescence during steady state photosynthesis ($F_m$') respectively. Values that were obtained from PAM fluorescence with the actinic light were Yield ($F_m$'-$F_t/F_m$'), qP [($F_m$'-$F_t$)/($F_m$'-$F_o$')] and qN [($F_m$-

$F_m$')/($F_m$-$F_o$)]. Yield is the effective quantum yield of PSII under steady state conditions. qP and qN are coefficients of photochemical electron transfer and non-photochemical (heat) fluorescence quenching respectively (Maxwell and Johnson, 2000).

### 4.3.8 Quantification of microorganisms

Microbial quantification was performed by taking 2 g of each soil sample jar and placing in 20 mL sterilized 0.85% NaCl (w/v) solution in 50-mL sterile conical tubes under sterile conditions. The soil suspensions were shaken for one hour on a Multi-Mixer (Lab-Line Instruments Inc., Melrose Park, Illinois, USA) at 500 rpm. The soil suspensions were allowed to settle for one hour and the supernatants from these soil extract suspensions were diluted serially, $10^1$-$10^5$, and used for the microorganism quantification assays. All bacterial counts were performed in triplicate using the plate count method (Kirk et al., 2005).

Culturable, aerobic heterotrophic bacterial cells (total bacterial numbers) were grown and enumerated as previously described (Kirk et al., 2005). Plate counts were performed after 48 h at 20 ˚C in the dark. Total fungi, petroleum-degrading aerobic bacteria and petroleum-degrading fungi were grown and enumerated according to Kirk et al., 2005. Plates were incubated at 20˚C for 4 days in the dark before enumerating colonies. Hexadecane-degrading aerobic bacteria were enumerated by spreading 100 µL of each serial dilution on oil agar medium. The oil agar medium constituted of 990 mL of BH Agar (Kirk et al., 2005) supplemented with ten mL of filter sterilized hexadecane (Sigma Aldrich, Oakville, ON, Canada). Plates were incubated at 20˚C for 4 days in the dark.

### 4.3.9 Statistical analysis

Statistical analyses were performed using SPSS 18 (SPSS Inc., Chicago, USA). One-way ANOVA along with Tukey's post test was performed for microbial analysis and for remediation analysis. PAM measurements were analyzed by a t-test. Degradation rates for each plot were calculated by assuming first-order kinetics. Degradation curves were fitted using nonlinear regression by Systat Software (Systat Software, Point Richmond, CA) to Equation 4.1.

## 4.4 Results

### 4.4.1 Plant growth

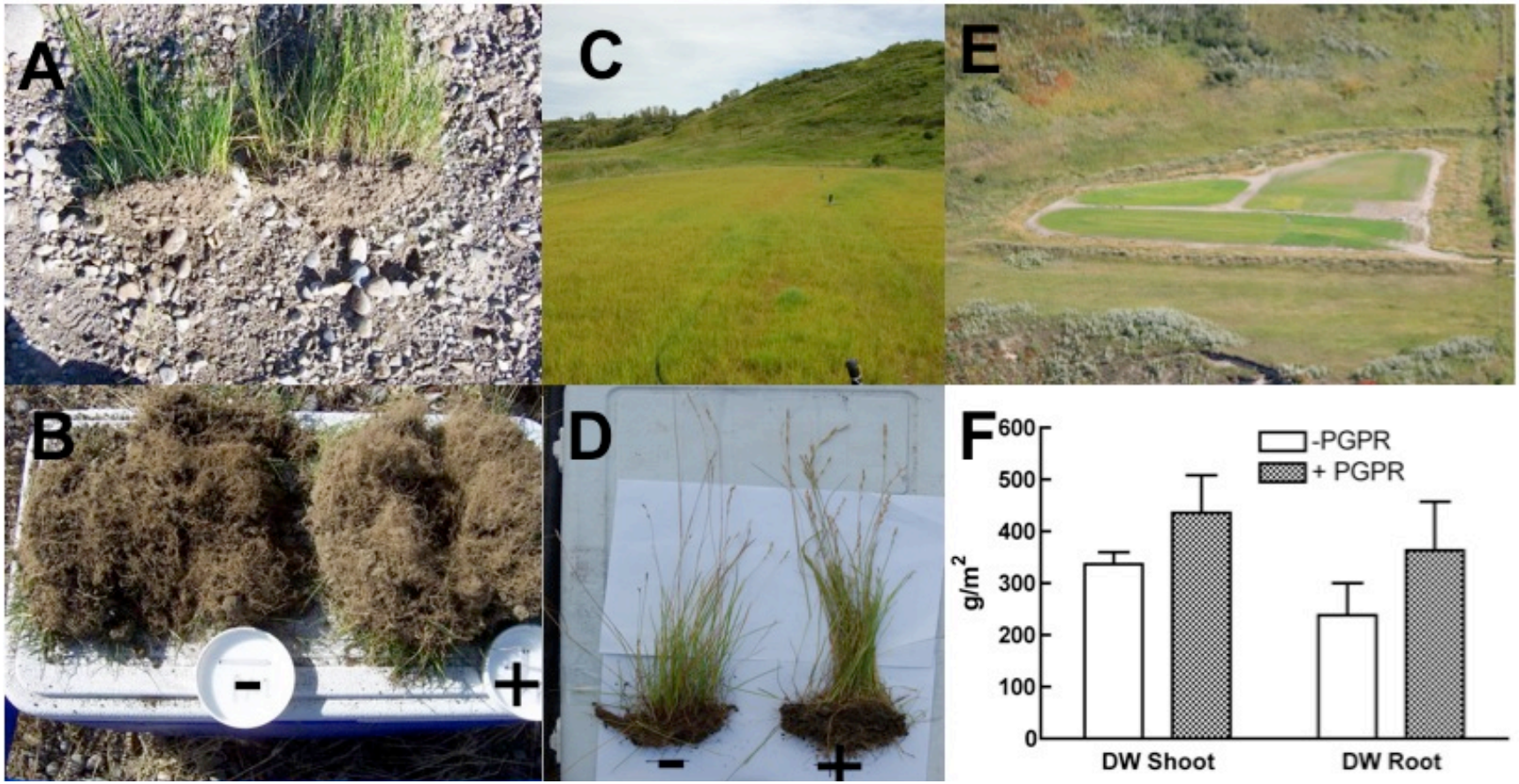
In 2005, the main sections of the site were planted with annual rye and tall fescue and the plants grew well, with the PGPR treated plants performing modestly better (Figure 4.2A, B). An irrigation system was put in place due to insufficient precipitation during the summer (Figure 4.2C). This consisted of a hose and a sprinkler system, and thus resulted in better areas of plant growth closer to the sprinkler heads. In 2006 the main segments of the site were tilled and replanted with annual rye and tall fescue, and good growth was observed again.

Plant growth measurements, especially roots were highly variable as previously observed in other field trials (Gurska et al., 2009). Roots were difficult to separate from the soil without breaking the fragile tissues. This was especially difficult at this site due to the poor quality of this soil; gravelly, firmly packed and nutrient deficient soil. Plant samples were taken at the end of each season. These measurements showed similar trends each year and representative data from West + PGPR and North – PGPR are shown (Figure 4.2F). A close up of plant growth in the field shows small noticeable differences in plant growth in – PGPR and + PGPR treatments (Figure 4.2D). These improvements due to PGPR were also evident in the dry weights of the plants (Figure 4.2F).

Likely some of + PGPR and –PGPR differences at this site were obscured by irregularities of this site, e.g. the placement of the – PGPR plot resulted in large quantities of pooled precipitation at this site, thus increasing growth and high variability of plant measurements. Nonetheless, differences in plant growth due to PGPR were apparent (Figure 4.2F); plant growth increased by approximately 30-50% dry weight with PGPR treatment when plant growth was examined in small and large plots.

**Figure 4.2 Plant growth measurements and photographs.**

A. Shoots of rye and fescue mix in September 2005 (– PGPR to the left and + PGPR to the right). B. Shoots of rye and fescue mix in September 2005 (– PGRP to the left and – PGPR to the right). C. Plant growth on West + PGPR plot in July 2006 with the sprinkler irrigation system. D. Plant growth or rye and fescue in October 2007 (– PGPR to the left and + PGPR to the right) E. Sky pictures over the site mid season 2007. Shows superior growth on West + PGPR plot and North – PGPR plot. East + PGPR plot grew poorly. F. Tall fescue and rye mixture growth from West + PGPR field and North – PGPR field in July 2006. Data are average ± SE (n = 7).


A
C
E
B
D
F
-
+
-
+
600
500
400
300
200
100
0
g/m²
-PGPR
+ PGPR
DW Shoot
DW Root

### 4.4.2 The effect of PGPR on plant photosynthesis

PAM fluorometry measurements were performed in 2005 to evaluate the effect of PGPR on photosynthesis. PAM fluorometry was performed on annual ryegrass and tall fescue, barley and triticale plants (Table 4.2). Although rye and fescue plants responded well to the PGPR treatment, these differences were only moderately reflected in the PAM parameters: $F_v/F_m$ values were only shifted higher in PGPR plants; Yield was also higher in plants with PGPR treatment after 60 days of growth. qN, a sign of plant stress decreased in PGPR plants at 150 days relative to the – PGPR plants. PGPR significantly improved $F_v/F_m$ in barley and significantly improved $F_v/F_m$ and Yield in triticale (Table 4.2). Overall, there were small but consistent increases in $F_v/F_m$ and Yield, especially after 60 days of growth. When measurements from all plant types taken at 60 days were averaged, the positive effects of PGPR on qN, $F_v/F_m$ and Yield indicated strong PGPR effects.

**Table 4.2 PAM fluorometry measurements.**

PAM parameters obtained from annual rye and tall fescue plants, barley and triticale plants in 2005 after 60 of growth and from annual rye and tall fescue after 150 days of growth. Triticale and barley plants matured and were dry prior to 150 days of growth. Each measurement is an average of 5 readings ± SD. * indicate + PGPR numbers significantly different than their – PGPR equivalent for each plant species, determined with a t-test, $P < 0.05$.

| Growth [days] | Plant species/ PGPR | $F_v/F_m$ | Yield | qP | qN |
|---|---|---|---|---|---|
| 60 | R/F – | 0.65 ± 0.02 | 0.50 ± 0.03 | 0.90 ± 0.04 | 0.32 ± 0.04 |
| | R/F + | 0.68 ± 0.03 | 0.54 ± 0.03 | 0.91 ± 0.03 | 0.31 ± 0.04 |
| 150 | R/F – | 0.73 ± 0.06 | 0.58 ± 0.07 | 0.94 ± 0.06 | 0.43 ± 0.08 |
| | R/F + | 0.72 ± 0.03 | 0.57 ± 0.03 | 0.93 ± 0.04 | 0.36 ± 0.03 |
| 60 | Barley – | 0.67 ± 0.02 | 0.58 ± 0.03 | 0.96 ± 0.02 | 0.23 ± 0.03 |
| | Barley + | 0.71 ± 0.03* | 0.60 ± 0.03 | 0.94 ± 0.02 | 0.26 ± 0.06 |
| 60 | Triticale – | 0.66 ± 0.02 | 0.55 ± 0.02 | 0.95 ± 0.02 | 0.29 ± 0.05 |
| | Triticale + | 0.71 ± 0.01* | 0.60 ± 0* | 0.93 ± 0.01 | 0.25 ± 0.05 |
| Ave at 60d | All – PGPR | 0.658 ± 0.021 | 0.542 ± 0.4 | 0.935 ± 0.04 | 0.283 ± 0.06 |
| | All + PGPR | 0.700 ± 0.03 | 0.583 ± 0.04 | 0.927 ± 0.03 | 0.234 ± 0.05 |

### 4.4.3 In-house gravimetric PHC analyses

Remediation in the first year of the study (2005) was superior for all of the PGPR treated plots compared to plots that did not receive PGPR (Table 4.3). This was the case for both the large planted areas as well as the small experimental plots. This correlated with the observed increased plant growth and improved photosynthetic performance in PGPR treated plots. In year one, to increase sample size, both in-house and commercial laboratory gravimetric analyses were used.

Over the three-year period of this project, gravimetric PHC ($PHC_G$) analysis performed in our laboratory demonstrated significant remediation (Table 4.4). From the onset to the end of the three year trial, – PGPR, and + PGPR decreased by 36% and 56% respectively (Table 4.4). Significant remediation occurred on all three planted fields in 2006. The three year in-house gravimetric remediation data was also fitted to first-order decay model and the rate constant *k* was obtained (Table 4.4). The *k* constant was 0.028 $month^{-1}$ for + PGPR and for – PGPR, *k* was 0.019 $month^{-1}$, summarizing the improved performance of plants + PGPR.

The unvegetated control plot did not show significant remediation between beginning and end of each season. When the unvegetated soil was monitored for loss of $PHC_G$ over the course of the trial, it decreased by 2%, 11% and 2% in years one through three, respectively; this is equal to 14% $PHC_G$ loss in the unvegetated soil in three years. There was however, a significant loss in PHC concentration (ANOVA, $P < 0.05$) between fall of 2005 and spring 2006 due to soil tilling and mixing during site preparation in the spring of 2006 which redistributed PHC between plots (Table 4.4); PHC decreased from 8.46 to 4.88 g $kg^{-1}$. The soil from the remediated, vegetated area was redistributed to the blank area and the soil from the blank area was redistributed the vegetated plots, concurrently increasing their concentration. Thus, the calculation of the *k* constant for the unvegetated area could not be completed as a result of this large PHC drop due to site mixing that happened outside of the growing season.

**Table 4.3 Percent remediation in 2005.**

Remediation obtained after 150 days of growth. Remediation in large plots is also presented in Table 4.4. Remediation percentile in small plots was measured by comparison to blank at the beginning of the year using the in-house gravimetric method (composite sample, 8.60 g $kg^{-1}$).

| Plot type | Plant type | PGPR | % Remediation | Difference (%) |
|---|---|---|---|---|
| Large | Rye/Fescue | - | 12 | - |
| | | + | 17 | + 5 |
| Small | Rye/Fescue | - | 11 | - |
| | | + | 23 | + 12 |
| | Barley/Rye | - | 17 | - |
| | | + | 29 | + 12 |
| | Triticale | - | 13 | |
| | | + | 18 | + 5 |
| Average | - PGPR | | 13 | - |
| | + PGPR | | 22 | + 9 |

### 4.4.4 Accredited commercial analytical laboratory F3 and total PHC analysis

The accredited commercial analytical laboratory performed analysis of CCME fractions, F1-F4 and the $PHC_{TOTAL}$ (total of all fractions added together). Only F3 (the carbon range of C>16-C34) exceeded the regulatory thresholds for F3 fraction in coarse-grained soils (300 mg $kg^{-1}$). Notably, analytical difficulties arose throughout this study. During the 2006 growing season, mid study, the commercial laboratory made a change to their PHC extraction method, which resulted in a 20% increase in extraction efficiency and rendered these numbers unusable for our study; thus the numbers for F3, $PHC_{TOTAL}$ are absent from the row for Fall 2006 (Table 4.4). Following this result, new soil samples from this time point could not be acquired. All subsequent soil samples for the remainder of the field trial were requested to be analyzed using the original method.

Fraction and $PHC_{TOTAL}$ data was obtained from accredited commercial laboratory. In 2005, $PHC_{TOTAL}$ remediated by 48% and 38% for the – PGPR and + PGPR plots respectively. In year one, F3 was remediated by 39% and 46% for + PGPR and – PGPR respectively. In May 2006, the method change by the independent laboratory did not allow for comparison of beginning and end of year samples, however samples were again collected in early 2007 (January 2007). Comparing the $PHC_{TOTAL}$ and F3 measurements it appeared that there was no decrease in $PHC_{TOTAL}$ or F3. In 2007 only + PGPR samples showed a decrease in $PHC_{TOTAL}$ (15% decrease) and a 13% decrease in F3. In summary, remediation determined from these data slowed in year two and three.

Overall cumulative remediation for the $PHC_{TOTAL}$ analysis for the three year period for – PGPR and + PGPR treatments was 48% and 54% respectively (cumulative values subtract the PHC concentration at the end of three years from starting concentration). These results were mirrored by the F3 numbers with 46% and 52% remediation for – PGPR and + PGPR treatments respectively. The correlation of F3 and $PHC_{TOTAL}$ is not surprising, as the $PHC_{TOTAL}$ and F3 are derived using the same method.

The apparent slowed remediation in year two, the lack of correlation with gravimetric data and variability observed in F3 and $PHC_{TOTAL}$ data, together with the sudden method change by the commercial laboratory prompted more investigation of the PHC analysis methods. Three avenues were investigated: analytical error, a possible lack of sufficient

microbial growth needed for remediation, and plant biogenic compounds accumulated in the soil that may obscure PHC decreases. As well, alternative analysis methods employing persistent PHC biomarkers were used to estimate degradation.

Duplicate soil samples from year two and three were sent to a second commercial laboratory for F1-F4 analysis. Figure 4.3 displays results of this analysis. In year two (month 13 and month 18) remediation was still somewhat evident but less so in year three. Months 1 and 6 are samples from the first commercial laboratory analysis (that are also presented in table 4.4) and are graphed in Figure 4.3 along side numbers from the duplicate samples for remediation comparison. According to this second set of F3 data points, analysis in year two indicated that some remediation may be occurring. In year three there was no observable remediation based on this duplicate F3 fractions analysis and this correlated with the previous findings from a commercial laboratory.

**Table 4.4 Remediation over the three year trial.**

$PHC_G$ is the gravimetric in-house analysis. Where no end of the season data were available, the measurements from the beginning of next year were used to calculate remediation. If standard deviation is not indicated for F3 and $PHC_{TOTAL}$, only one sample was taken. In all other cases n = 2 for F3 and $PHC_{TOTAL}$ and n ≥ 5 for $PHC_G$.

| Site | Year | | *k* | $PHC_G$ | % Remediation | F3 | % F3 Remediation | $PHC_{TOTAL}$ | % Total PHC |
|---|---|---|---|---|---|---|---|---|---|
| – PGPR | 2005 | Spring | 0.019 | 6.46±1.70 | 13 | 2.8 | 46 | 4.0 | 48 |
| | | Fall | | 5.65±1.30 | | 1.5 | | 2.1 | |
| | 2006 | Spring | | 6.5±0.67 | 34 | 2.2 ± 0.4 | 0 | 3.1 ± 0.1 | 0 |
| | | Fall | | 4.31±0.89[#] | | - | | - | |
| | 2007 | Spring | | 3.87±0.90* | 0 | 2.3 ± 0.4 | 0 | 3.3 ± 0.1 | 0 |
| | | Fall | | 4.15±0.99* | | 2.5 | | 3.4 | |
| + PGPR | 2005 | Spring | 0.028 | 9.3±1.28 | 19 | 3.0 ± 1.0 | 39 | 4.3 ± 0.2 | 38 |
| | | Fall | | 7.5±2.50 | | 1.8 ± 0.7 | | 2.6 ± 0.1 | |
| | 2006 | Spring | | 6.4±1.40* | 25 | 2.3 ± 0.6 | 0 | 3.3 ± 0.1 | 0 |
| | | Fall | | 4.8±0.80*[#] | | - | | - | |
| | 2007 | Spring | | 3.9±0.05* | 0 | 2.3 ± 0.5 | 13 | 3.4 ± 0.1 | 15 |
| | | Fall | | 4.1±0.07* | | 2.0 ± 0.5 | | 2.9 ± 0.1 | |
| Blank | 2005 | Spring | – | 8.6±0[1] | 2 | - | - | - | - |
| | | Fall | | 8.46±0.85 | | - | | - | |
| | 2006 | Spring | | 4.88±0.31*^ | 11 | 1.8 ± 0.3 | 0 | 2.6 ± 0.1 | 0 |
| | | Fall | | 4.35±0.47* | | - | | - | |
| | 2007 | Spring | | 4.03±0.33* | 2 | 1.9 ± 0.1 | 0 | 2.8 ± 0.1 | 0 |
| | | Fall | | 3.96±0.31* | | 2.4 | | 3.3 | |

[1] Composite blank collected at the time of planting. [#] significantly different than spring of current year ($P < 0.05$); * significantly different from time 0 ($P < 0.05$); ^ significantly different than fall of previous year ($P < 0.05$). For 2007 spring measurements the numbers for F3 and $PHC_{TOTAL}$ were collected in January 2007, which would effectively make it before spring 2007. "-" indicates no data was available for this indicated sampling date.

[2] *k*, degradation rate constant ($months^{-1}$)

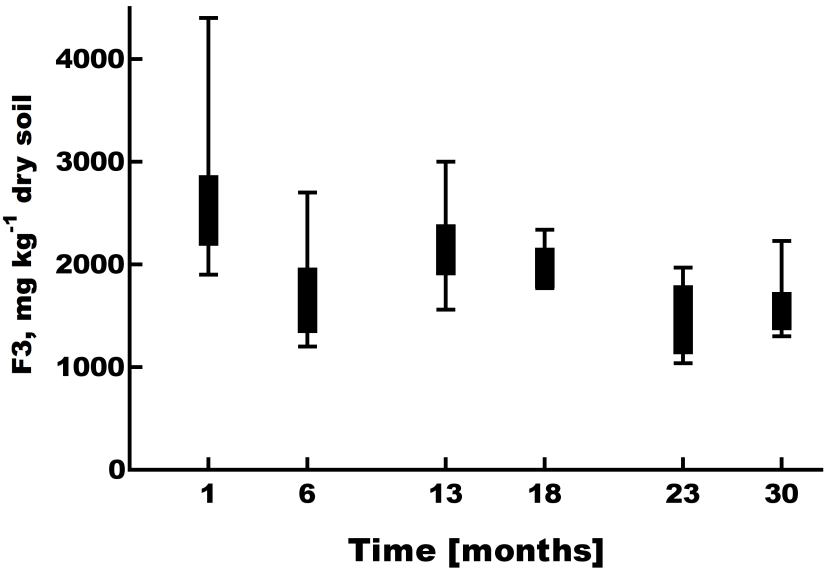



**Figure 4.3 Analysis of CCME fraction 3.**

F3 data analysis was performed on a subset of + PGPR samples by a second commercial laboratory. Time in months is indicated on a linear scale. Month one indicates first sampling prior to planting. Month 1 and 6 data are from the original commercial lab analysis while months 13, 18, 23, 30 is from the duplicate samples sent in year 2 and 3 of the field trial.

### 4.4.5 Microbial analysis

Microbial analysis was performed to ensure that sufficient microbial growth occurred in these substandard, gravely soils. Numbers of total bacteria, petroleum degrading bacteria, hexadecane degrading bacteria and total fungi were determined. In May 2007, total bacterial numbers in the soil of vegetated plots were five to eight times higher than unvegetated plots for – PGPR and + PGPR respectively. Plots + PGPR plots were ~160% higher than that in the soil of plots – PGPR (Figure 4.4A). Total bacteria at the end of the season in October increased in all plots relative to the numbers in May and the PGPR treated plots remained higher in microbial numbers than the – PGPR plot. The PGPR treated plots increased four-fold, whereas the unvegetated soils and non PGPR treated soil showed only a twofold increase. Overall the bacterial numbers in + PGPR soils were threefold higher than total bacterial numbers in soil – PGPR and 16 times higher than in unvegetated soils.

The changes in hexadecane-degrading bacterial numbers showed a different trend than total bacterial numbers (Figure 4.4B). At the onset of the season, the average hexadecane degrading bacterial numbers in the + PGPR samples were lower than those from – PGPR soil samples (+ PGPR was significantly (ANOVA, $P < 0.05$) lower than – PGPR). This trend remained unchanged at the end of the season. Although PGPR treated soil showed a one and a half fold increase in hexadecane-degrading microbes from May to October whereas in the non-PGPR soils hexadecane-degraders decreased. The blank had a nearly three-fold increase from May to October.

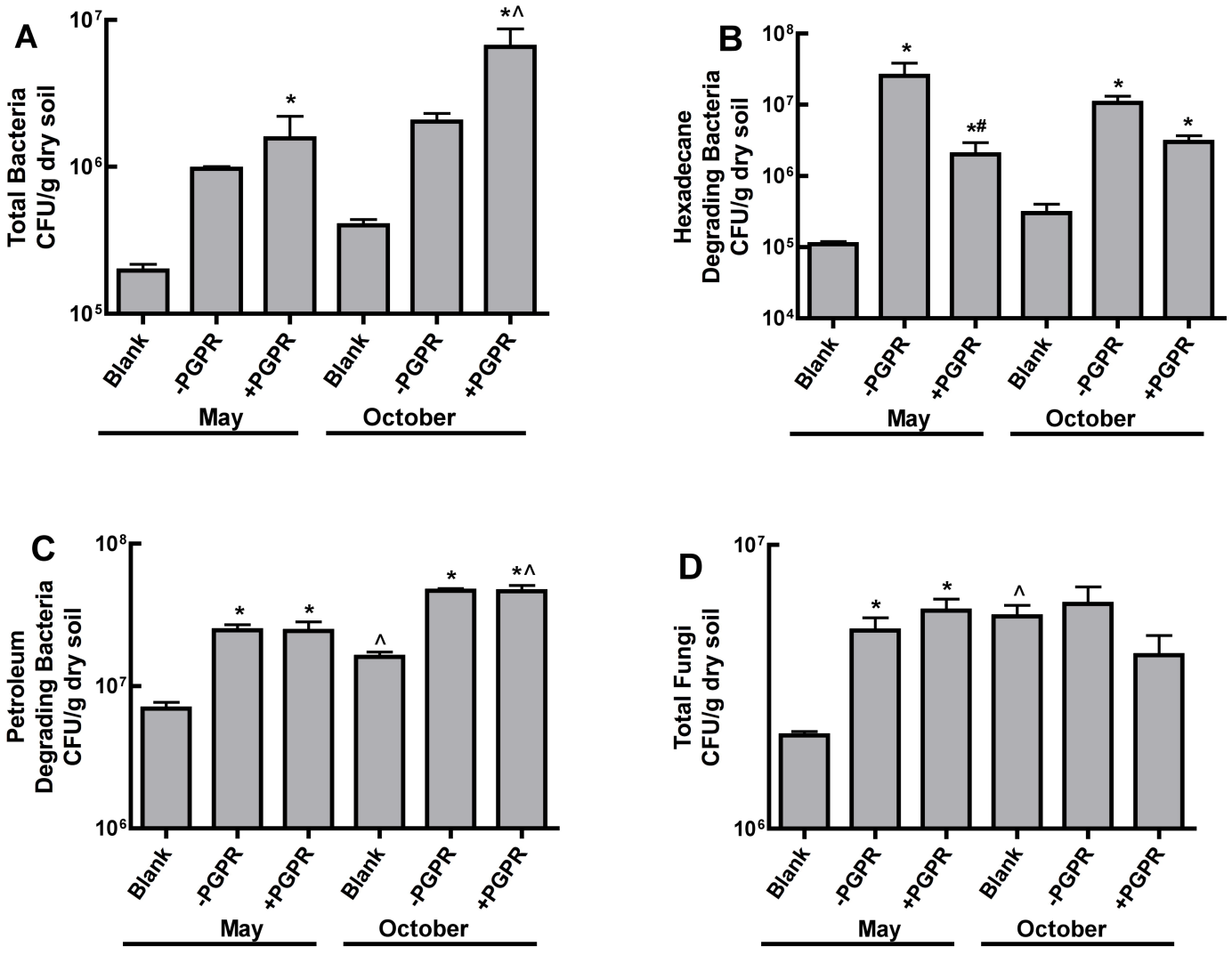
The petroleum-degrading bacterial numbers in vegetated plots were significantly higher than unvegetated soils (ANOVA, $P < 0.05$), this trend resembled the total bacterial numbers. This was consistent when comparing – PGPR plots or + PGPR plots. The numbers of petroleum degraders was significantly higher (ANOVA, $P < 0.05$) for planted segments in comparison to beginning of the season (Figure 4.4C). All samples showed approximately twofold increase between May and October.

Total fungal numbers for each treatment (– PGPR and + PGPR and unvegetated soils) all fell within $10^6$ and $10^7$ CFU/g dry soil (Figure 4.4D). For total fungi, numbers of planted treatments (both + PGPR and – PGPR) were significantly higher (ANOVA, P <

0.05) than unvegetated soils. The blank fungal numbers increased significantly at the end of the season relative to beginning of the season, but vegetated soils did not increase. All microbial numbers, except the fungal numbers remained higher in vegetated plots than unvegetated plots in the spring and fall of monitoring.

**Figure 4.4 Microbial analyses.**

Quantification of microbial groups from land farm soil samples of +PGPR plots (West + PGPR, East + PGPR), – PGPR plot (North – PGPR) and one unvegetated site (Blank). Data were collected in May and October in 2007. Total heterotrophic bacteria (A), hexadecane degrading bacteria (B), petroleum degrading bacteria (C) and total fungi (D). * indicates bars significantly different than blank at the corresponding time point, ^ indicates bars significantly different than its site equivalent in May versus October, and # indicates samples significantly from – PGPR at the corresponding time point. Significance tested with $P < 0.05$. Data are the means ± SE ($n \geq 3$).


A
Total Bacteria CFU/g dry soil
$10^7$
$10^6$
$10^5$
*^
*
Blank
-PGPR
+PGPR
Blank
-PGPR
+PGPR
May
October
B
Hexadecane Degrading Bacteria CFU/g dry soil
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
*
*#
*
*
C
Petroleum Degrading Bacteria CFU/g dry soil
$10^8$
$10^7$
$10^6$
*
*
^
*
*^
D
Total Fungi CFU/g dry soil
$10^7$
$10^6$
*
*
^

### 4.4.6 The effects of plant growth on accumulation of biogenic organic compounds (BOC)

The possibility that fluctuations in F3 measurements can be attributed to accumulation of biogenic material was investigated. Plant exudate deposition varies widely, both in quantity and quality, with plant species and developmental stage (Curl and Truelove, 1986). The changes in biogenic material deposited in the soil would be reflected in the amount of biogenic material that must be removed by the silica gel cleanup. Increase in biogenics that results in overloading of the silica clean up exceeding of the capacity of silica gel, may result unusual variability between samplings and increased PHC values. Silica cleanup removes any biogenic organic compounds (BOC) that could falsely raise PHC content. This process removes compounds that are more polar than PHC and derived from biological sources such as plants. The methods used to examine remediation year to year proved variable, hence the contribution of BOC and PHC to the final concentrations of PHC, F3 and F4, were analyzed in the final year of the trial. A subset of samples from planted and unplanted soils (from the beginning and at the end of 2007 season) was analyzed for biogenic and petrogenic material. This analysis was performed by Zhendi Wang at Environment Canada, Ottawa, Canada.

The overall quality of PHC in the samples was examined. The oils in samples were severely weathered and degraded, evidenced by no *n*-alkanes being detected. Further, a group of highly persistent alkylated PAHs, the chrysenes, showed a distribution towards more highly branched chrysenes, which is an indication of highly weathered and degraded material.

Gravimetric measurement of the PHC content were measured in the form of the total solvent extractable material (TSEM) values, extracted with n-hexane/acetone (1:1, v:v). TSEM values for the soil samples were 3.3-4.5 mg/g (air dried weight). PHC numbers were also generated using these extracts with GC-FID. The ratio of PHC to TSEM was between 58-76%, which indicates that some high molecular compounds and polar potentially biogenic compounds are present (Table 4.5). Resolved peaks in the chromatograms accounted for only approximately 2% of the total PHC, while an

unresolved complex mixture accounted for approximately 98% in most samples (Table 4.5); this also indicated highly weathered material.

The total alkylated PAHs and Total EPA priority PAHs decreased by 21% and 29% respectively between May and October of 2007 (Table 4.5). However, F3 decreased only decreased by 5% and F4 did not appear to decrease.

**Table 4.5 PHC content in selected 2007 samples.**

PHC content in 2007 soil samples from May and October planted (+P) and unplanted (-P) samples. Total solvent extractable material (TSEM) with n-hexane/acetone (1:1, v:v). Each number represents mean ± SD. PHC, petroleum hydrocarbons. Determined using the TSEM with the GC-FID. Resolved Pk is the measurement of resolved peaks in GC chromatograms. UCM, unresolved complex mixture.

| | TSEM (mg $g^{-1}$) air-dried wt | PHC (mg $g^{-1}$) | Resolved /PHC (%) | UCM /PHC (%) | PHC /TSEM (%) | Total Alkylated PAHs (ng $g^{-1}$) | Total EPA priority PAHs (ng $g^{-1}$) | F3 (mg $kg^{-1}$) | F4 (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **May + Plants** | 3.9 ± 0 | 2.54 ± 0.45 | 2.3±0 | 97.7±0 | 65.2±10 | 4929±633 | 92.5±15 | 1725±162 | 551±153 |
| **Oct + Plants** | 3.15±0.21 | 2.08±0.08 | 2.3±0.14 | 97.7±0.14 | 66.65±2.2 | 3901.5±310 | 66±15 | 1635±106 | 643±19 |
| **Oct – Plants** | 4.05±0.64 | 3.03±0.4 | 1.45±0.78 | 98.55±0.78 | 74.6±1.41 | 5571±658 | 67.05±12.7 | - | - |



**Table 4.6 BOC content in selected 2007 samples.**

Biogenic compounds in 2007 soil samples from May and October planted (+P) and unplanted (-P) samples.

| | Fatty acids (C10-C30) (mg $kg^{-1}$) | Fatty alcohols (C16-C30) (mg $kg^{-1}$) | Sterols mg $kg^{-1}$ | Total Biogen. (mg $kg^{-1}$) | Theoretical Biogen. contrib. to F3 (%) |
|---|---|---|---|---|---|
| **May + Plants** | 9.65±3.75 | 5.74±3.1 | 2.17±1.5 | 17.55±8.27 | 1.05±0.57 |
| **Oct + Plants** | 16.60±1.13 | 8.43±1.56 | 5.85±1.34 | 30.85±1.77 | 1.9±0.01 |
| **Oct – Plants** | 12±0.42 | 3.24±0.6 | 0.52±0.06 | 15.75±1.06 | - |

Total biogenic compounds in these samples were mainly contributed from fatty acids and fatty alcohols (Table 4.6). The contribution was dominated by even-carbon-fatty acids and alcohols (individual data not shown). The contribution of the sterols was dominated by vascular plant C29 sterols ((β-sitosterol and stigmasterol) and β-amyrin (Table 4.6). 5β-Cholestan-(3a+3β)-ol, 5a-Cholestan-3β-ol, 5a-Cholestan-3-one, desmosterol and friedelin were absent in all samples. Notably stigmastanol, β-amyrin, α-amyrin were completely absent from unplanted samples. In planted samples, in the spring stigmastanol was 0.09(0.05) mg $kg^{-1}$ and increased to 0.14(0.02) mg $kg^{-1}$ in the fall, β-amyrin was 0.4(0.33) mg $kg^{-1}$ in the spring and increased to 1.38(0.30) mg $kg^{-1}$ in the fall and α-amyrin was 0.17(0.11) mg $kg^{-1}$ in the spring and increased to 0.54(0.09) mg $kg^{-1}$in the fall (number in the brackets indicate standard deviation, n = 2). Stigmasterol and β-sitosterol were present in very low amounts in unplanted samples in the fall (less than 0.1 mg $kg^{-1}$ and 0.4 mg $kg^{-1}$ respectively). In planted samples stigmasterol was at 0.31(0.19) mg $kg^{-1}$ in the spring and at 0.71(0.08) mg $kg^{-1}$ in the fall while β-sitosterol was at 1.11(0.8) mg $kg^{-1}$ in the spring and at 2.76(0.73) mg $kg^{-1}$ in the fall. Cholesterol also doubled in planted samples from 0.10(0.04) mg $kg^{-1}$ in the spring to 0.25(0.06) mg $kg^{-1}$ in the fall and it was 0.12(0.03) in the unplanted samples in October. The overall biogenic contribution had nearly doubled for samples that were planted. Thus, biogenic material was likely interfering with the CCME PHC analyses performed by the analytical labs.

### 4.4.7 Persistent PHC biomarker analysis

A subset of samples closely corresponding to the samples sent for biogenic analysis in section 4.4.6, was also analyzed for persistent PHC biomarkers at ExxonMobil Biomedical Sciences Inc. Samples were extracted with methylene chloride and analyzed using GC-MS in selected ion monitoring (SIM) mode to detect common PHC components, PAHs (e.g. benz[a]antracene, chrysene), and the hopanes and sterene biomarkers. PHC analysis was performed in total ion mode.

All samples had elemental sulfur present, characteristic of brine water used to extract PHC products or a result of other sulfur wastes deposited into the flare pit (Rutherford et al., 2005). This was consistent with the available history of the site. There were no detectable phenanthrene, methylphenanthrenes, dimethylphenanthrenes or

trimethylphenanthrenes, dibenzothiophene, methyldibenzothiophenes, dimethyldibenzothiophenes or trimethyldibenzothiophenes, pyrene, benzo[a]pyrene or benzo[e]pyrene. There were no *n*-alkanes but some pristine and phytane (data not shown). Similar to other findings, this data indicates highly weathered material.

The GC-MS m/z = 191 (mass to charge ratio of 191) traces used to measure hopanes and other triterpanes, did not appear as characteristic petroleum compounds because the characteristic doublets of many peaks were not present (Figure 4.5). The chrysenes were examined and also appeared degraded. Figure 4.5C, D and E displays the chrysenes normalized to the C2-chrysene.

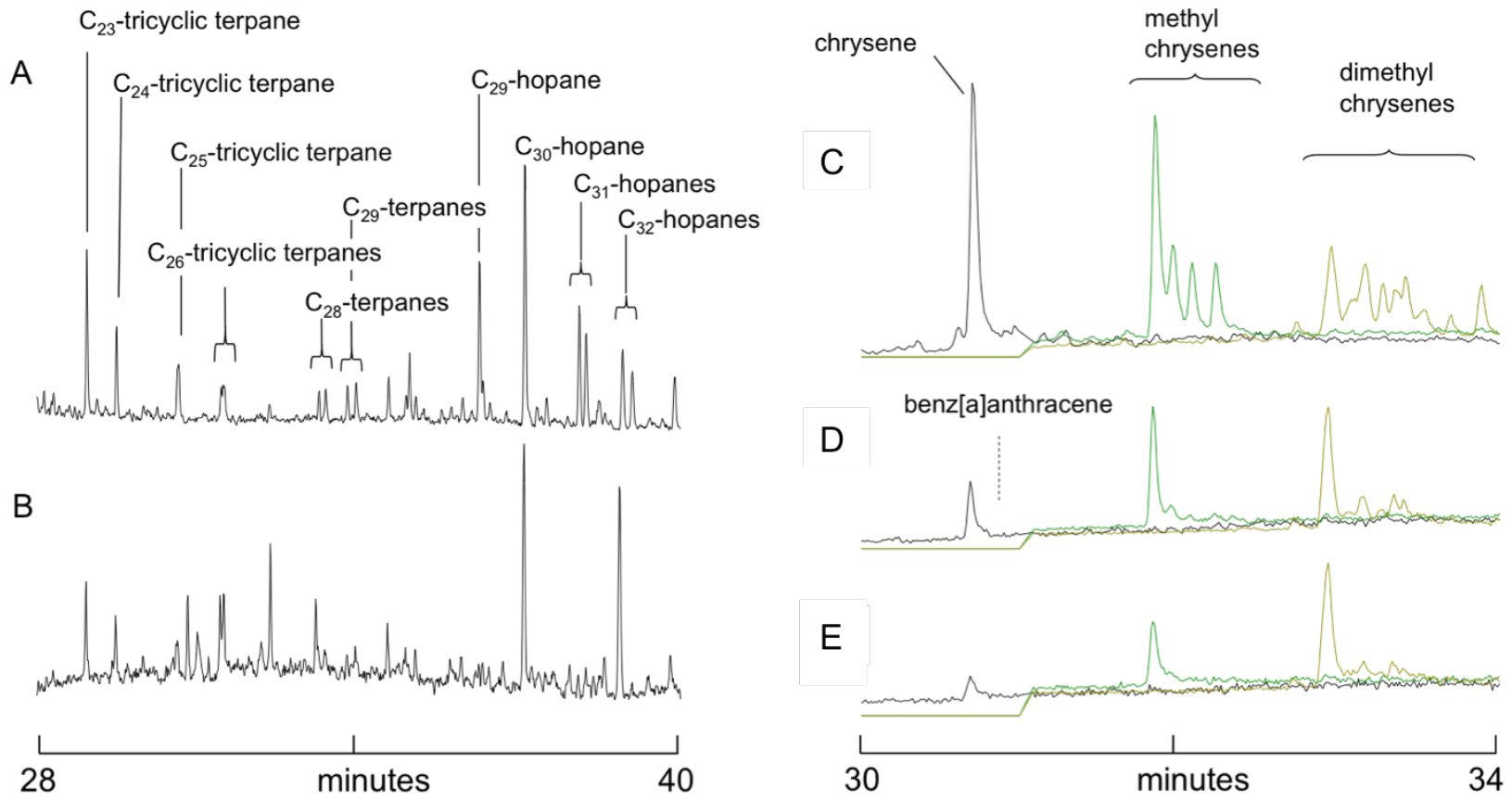



**Figure 4.5 Characteristic GC-MS peaks.**

Terpane and hopane peaks present in Alaska North Slope crude oil (A) do not appear in the site soil (B). Characteristic GC-MS chrysene peaks present in Alaska North Slope crude oil (C) appear degraded in site soil with no plants (D) and ever more so in planted soil (E). SIM in m/z = 228, 242, 256 mode. Chrysene chromatogram was normalized to C2-chrysenes.

Gravimetric oil content was extracted with methylene chloride in this analysis. This was in contrast to hexane-acetone (1:1, v:v) used previously in the in-house method and methods used by the accredited commercial laboratory. PHC, measured as methylene chloride extracted gravimetric PHC, decreased in planted samples but not in unplanted samples, in both year two and year three of the trial (Figure 4.6A, D).

Hopanes were used as a conserved internal marker. Normalizing PHC loss to hopanes accounts for abiotic loss of contaminants with the assumption that hopanes are not significantly degraded (Howard et al., 2005). When PHC levels measured by GC-MS were normalized to total hopanes, no remediation was observed in either planted or unplanted samples (Figure 4.6B,E). However, when the C2-chrysenes were normalized to total hopanes, they decreased in both 2006 and 2007, and more so in the planted samples than in unplanted samples (Figure 4.6C,F). C2-chrysenes were degraded by 40% in year one and by 26% in year two in vegetated samples, whereas in the unvegetated blank they decreased by 10% and 14% respectively.

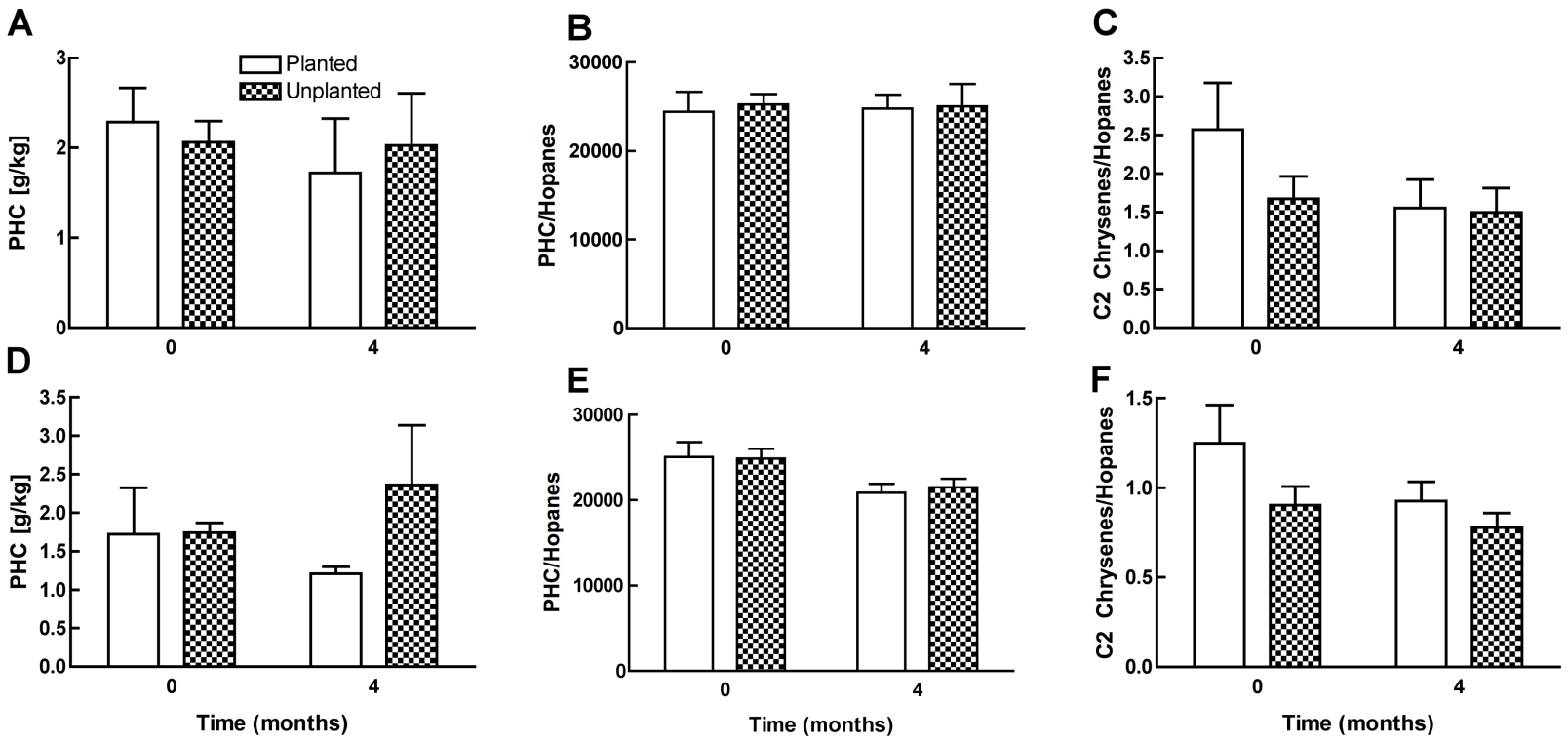



**Figure 4.6 GC-MS quantification of PHC and its components.**

Quantification of 2006 samples: gravimetric oil measurements (A), PHC normalized to hopanes obtained from GC-MS (B) and C2-chrysene degradation normalized to hopanes (C) obtained from GC-MS. Quantification of 2007 gravimetric oil content (D), PHC normalized to hopanes obtained from GC-MS (E) and C2-Chrysene degradation normalized to hopanes obtained from GC-MS (F). White columns represent planted samples and checkered columns represent unplanted samples. Bartlett's test indicated that variances were unequal ($P < 0.05$), thus non parametric test for significance were performed. The Kruskal-Wallis test indicated that medians did not vary significantly ($P < 0.05$).

## 4.5 Discussion

Phytoremediation applications have been very successful for PHC remediation in the greenhouse but poor soil quality, unfavorable weather conditions and contaminant toxicity have hindered the transfer of this technology to remediate PHC in the field. PGPR have previously been used to improve plant growth in greenhouse phytoremediation (Huang et al., 2004b; Huang et al., 2005) and in a full-scale remediation at a PHC refinery landfarm (Gurska et al., 2009). However, the full range of applicability of PGPR-enhanced phytoremediation has not been tested. Here, over the course of three years, it was shown that PEPS remediated weathered PHC in soils and the analytical methods used to assess PHC remediation in the field were further explored. Hindrances commonly encountered while executing phytoremediation and its assessment will be discussed.

### 4.5.1 PGPR improved plant growth and photosynthetic performance

PHC-tolerant plant species were grown on a site contaminated with low levels of PHC. Poor quality soil necessitated fertilizer use. The addition of PGPR improved plant growth and photosynthetic parameters compared to plants without PGPR. Although the PGPR effect on growth was not as pronounced as previously observed at a highly contaminated petroleum landfarm site (Gurska et al., 2009), plant roots at this site consistently appeared larger than when plants did not receive PGPR at this site. The advantage to phytoremediation in having greater root biomass is the increased root surface area that can support higher numbers of soil microbes and those in turn degrade PHC (Hutchinson et al., 2003). Increases in plant mass should result in faster rates of xenobiotic degradation and sequestration by plants (Trapp et al., 2004). PGPR enhanced phytoremediation circumvents stress responses in plants allowing for better growth in contaminated and poor quality soils (Glick et al., 2007).

In plants grown in contaminated soils, photosynthesis has been shown to be inhibited by exposure to polycyclic aromatic hydrocarbons (PAHs), photomodified PAHs, creosote (Huang et al., 1997; Marwood et al., 2000; Marwood et al., 2001) and PHC (Kvesitadze et al., 2006). At this site, the negative effects to photosynthesis in PHC contaminated soil were minimal. Likely due to low levels of PHC, this was reflected with only a modest impact on

photosynthesis. Changes in triticale and barley plants with added PGPR confirm a modest improvement in photosynthetic parameters even with low PHC stress. Similar to Gurska et al., 2009 (Chapter 2), the annual rye and tall fescue mixture did not show significant changes in terms of photosynthetic parameters, however qN, (non-photosynthetic quenching), which can also be used as a sign of environmental injury, was lowered in ryegrass and tall fescue plants at the end of the season when PGPR were used. These small changes in PAM parameters are correlated with good plant growth overall and no visible damage to plants. Photosynthetic data suggests that although PGPR may improve photosynthetic performance, the changes are small enough that they are not likely a result of direct influence of PGPR on the photosynthetic machinery. This is consistent with the modest PGPR effect on growth and is likely the result of plants not being detrimentally stressed. To summarize, plant growth and photosynthetic performance were moderate without PGPR and elevated with the use of PGPR. These results indicate that optimal growth conditions for phytoremediation were met at this study site.

### 4.5.2 Gravimetric PHC analyses

Phytoremediation using annual ryegrass and tall fescue was successfully performed over a period of 30 months, with the gravimetrically extracted PHC, $PHC_G$, concentration was lowered by 56% and 36% for + PGPR and – PGPR respectively. The increased remediation due to PGPR was further reflected in the percent remediation in test plots for year one, where all of the PGPR treated plots showed increased degradation. Remediation based on gravimetric analysis was also observed in 2005 and 2006, although decrease ceased in year three. This is consistent with what was previously observed at the petroleum landfarm where remediation slowed over the course of the trial (Gurska et al., 2009 and Chapter 3).

To further evaluate phytoremediation performance at this site, data were fit to a first order kinetics equation. First order kinetics are typically used to quantify PHC degradation rates in bioremediation (Roncevic et al., 2005). The nonlinear regressions of the decrease in PHC concentration was conducted using an exponential decay model (Equation 4.1), where $k$ is used as a descriptor of the system's performance (Nedunuri et al., 2000). For the vegetated

plots, the *k* for + PGPR treated plants is notably higher than where the plants did not receive PGPR treatment. These data suggest that phytoremediation would be an acceptable remediation strategy for sites with lower levels of recalcitrant PHC. The *k* constant for + PGPR plots was 0.028 $month^{-1}$ and – PGPR, *k* was 0.019 $month^{-1}$. These values fall closely to a *k* of 0.027 $month^{-1}$ previously obtained at PHC-contaminated landfarm with PGPR treated plants, this despite the differences in starting PHC concentrations and opposing farming practices, such as watering and fertilizing between the two sites.

### 4.5.3 Fraction 3 and $PHC_{TOTAL}$ analysis

High molecular weight petroleum fractions such as F3 are often resistant to remediation (Huang et al., 2005). Most of the compounds in F3 such as PAHs, would likely fall outside of the favorable plant uptake range of 0.5 to 3 log $K_{OW}$ (Schnoor, 2002; Dzantor and Beauchamp, 2002; Hutchinson et al., 2003) and thus are most probably degraded in the rhizosphere by bacteria, fungi and PGPR, which are nutritionally supported by plant exudates (Salt et al., 1998; Alkorta and Garbisu, 2001; Hutchinson et al., 2003). Encouragingly, in a previous study at a petroleum landfarm PEPS was successful at remediating 38% of F3 over the course of 30 months. In the present study F3 remediation was observed in year one, however the results thereafter were variable and indicated that remediation was reduced. Changes to the F3 and $PHC_{TOTAL}$ numbers correlated, which was not surprising as they were derived from the same sample chromatogram by the independent laboratory. The F3 and $PHC_{TOTAL}$ numbers did not correlate with the gravimetric data, however. As larger differences may be obscured by analytical variability, only values from the beginning and end of the three year period should be considered (Gerhardt et al., 2009). Over the course of three years, F3 decreased by 52% and 46% for + PGPR and – PGPR respectively, showing a modest but consistent increase in remediation due to PGPR treatment.

### 4.5.4 Soil microbial numbers

One possible explanation for the slowed remediation in year two and three, was insufficient microbial numbers present to spur degradation of PHC. Bacterial numbers were monitored beginning in 2007; two years after PGPR were first introduced to the site. The total bacteria

numbers in planted soils were higher than those in unvegetated soil at the beginning of the season in 2007 before planting. At the end of the season, the unvegetated soils and – PGPR total microbial counts increased only by approximately twofold. However, the PGPR treated plots increased by four-fold. Similar to the previous study (Gurska et al., 2009), at a PHC contaminated landfarm, total bacterial numbers in soils with PGPR treated plants remained higher than in soils with untreated plants; an association posited to be responsible for increased degradation when PGPR are present (see Chapter 3). The changes in hexadecane-degrading bacterial numbers showed a different trend than total bacterial numbers. At the onset of the season, the hexadecane degrading bacterial numbers for the + PGPR soil were lower than those from – PGPR soil samples and this trend remained for the fall sampling. The petroleum-degrading bacteria numbers at the onset of 2007 were higher for the – PGPR plot and the + PGPR plot than the control plot; this start of season trend resembled the total bacterial numbers. The petroleum degraders increased significantly from beginning to end of season in all planted treatments.

At the beginning of the season, the fungal counts in the planted areas were higher than unvegetated soils; a finding that is similar to the petroleum degrading bacterial counts. Previous studies showed the importance of fungi in phytoremediation of PHC due to their ability to tolerate low pH, drought and low nutrient conditions (Merkl et al., 2005). The fungal numbers at this site were between approximately $10^6$ and $10^7$ CFU/per gram weight of soil, higher than previous studies where fungal numbers fell between $10^3$ and $10^4$ CFU/per gram dry weight of soil (see Chapter 3). Likely, fungal growth was more impacted at the highly contaminated site and the lower PHC levels at this site allowed for increased fungal growth. High microbial and fungal numbers are a prerequisite for remediation of PHC compounds (Alkorta and Garbisu, 2001; Beauchamp and Dzantor, 2002; Merkl et al., 2005).

Substrate availability, such as PHC, will dictate microbial growth (not considering plant derived nutrients as a source of carbon), and thus degradation. When substrate concentrations are low, the bacteria will not grow, and effectively less PHC will be degraded. Thus, higher PHC levels might be degraded faster and more completely than when low levels of PHC are present (Trapp et al., 2004). Presence of PGPR has been shown to increase the number of

microorganisms, which facilitated the degradation of PHC in the aforesaid ways (see Chapter 3) and this increase due to PGPR was observed here in total microbial numbers.

### 4.5.5 BOC, PHC and PAHs measurements

The accumulation of biogenic organic compounds (BOC) was evaluated in the final year of the trial. These compounds may be plant-derived and may be undistinguishable from PHC compounds during analysis. Remediation of F3, total PHC and PAHs was evaluated at the same time as BOC in soil samples. Between May and October 2007, the total alkylated PAHs and total EPA priority PAHs decreased by 21% and 29% respectively. However F3 decreased by only 5% and F4 or $PHC_G$ did not appear to decrease. Thus, despite components of F3, such as PAHs, demonstrating degradation this was not reflected in the F3 numbers. One possible explanation was the increase in BOC measured in the samples, which nearly doubled between May and October for planted samples. This combined with the remediation of specific alkylated PAHs and EPA priority PAHs suggest that some of the compounds included in the analysis of F3 and F4 are in fact of biogenic origin. At first look, the biogenics account for less than 2% of the F3 number. However, the current method used to measure biogenics only detects a small portion of plant exudates. If undetected compounds are also extracted in the analysis, this could result in an even higher elevation of F3 and PHC values. For example, flavonoids have been shown to comprise as much as 37% of plant secondary metabolites exudates in *Arabidopsis thaliana* and they were not measured in the biogenic analysis (Narasimhan et al., 2003). The biogenics measured in this study nearly doubled, and if this increase is representative of what is occurring with undetected biogenics, the remediation of PHC may be much greater than what is perceived currently.

Some of the biogenic compounds deposited into the rhizosphere by plants may be polar compounds, such as organic acids and alcohols, sulfur compounds, phenols, and tannins. These may be removed during sample preparation, usually through a silica gel cleanup (CCME, 2001b). The inefficiency of removal of BOCs from PHC contaminated soil samples has been recognized by the industry and recommendations for improvement have been made (National Council for Air and Stream Improvement, 2005). Previous research with biogenic

hydrocarbons from pine needle compost and dried grass has been shown to require more than one silica cleanup and the dried grass continued to inflate PHC concentrations even after three silica gel applications at levels of 2,600 mg $kg^{-1}$ extracted with Freon-113 (TPHCWG, 1998). In those cases where the co-extracted vascular plant materials is not properly removed, it would inflate the PHC concentrations reported (Stout and Wang, 2007). The CCME methodology used by certified laboratories specifies that only a single silica gel cleanup is permitted, which if biogenic deposits in the soil at the end of the year are considerable, may overload the silica column capacity. The overloading of silica column, especially in the fall when biogenic concentration is expected to be high would likely result in underestimate of remediation, more likely to be evident at sites with low contamination such as this one. Increasing effectiveness of silica clean up would likely reveal that more remediation is occurring.

Silica cleanup efficiency may also be ineffective due to poor technique or the nonpetroleum compounds may have a similar structure to petroleum compounds and behave similarly thus escaping cleanup. The silica gel cleanup CCME standards allow for *in situ* (silica added to extracted PHC in solvent) or *ex situ* (extracted PHC in solvent passed through a column); however *in situ* method is often preferred as it is less labor intensive. Preliminary evidence from multiple sources indicates that *in situ* silica cleanup is inferior to column silica cleanup in removing polar hydrocarbons (British Columbia Ministry of Water, 2004). Further performing in silica cleanup with even low levels of acetone in the solvent (< 5%) will allow organic material to escape the silica clean up (British Columbia Ministry of Water, 2004); the polar solvent will occupy silica binding sites; this is problematic because the samples analyzed in this study and the general protocol in commercial laboratories, following the standardized CCME protocol, contains acetone (samples are extracted with 1:1 hexane:acetone mixture). Lastly, non-polar plant biogenics will not be removed by the silica clean up (British Columbia Ministry of Water, 2004). All of these factors render the silica cleanup similarly ineffective to gravimetric analysis without silica cleanup. Both the gravimetric analysis without clean up and the commercial laboratory data are likely inflated by biogenics accumulating throughout the plant growing season.

### 4.5.6 Degradation measurements using hopanes as an internal biomarker

Alternative analysis methods that employed conserved internal PHC biomarkers were used to estimate degradation. The general biodegradation trend is as follows: *n*-alkanes > BTEX and other monoaromatic compound > branched and cyclo-alkanes > PAHs > biomarker terpanes and steranes (Wang and Christensen, 2006). It should be noted that smaller PAHs are more susceptible to biodegradation than larger PAHs and an increase in alkylation level in the same PAH series decreases susceptibility to microbial attack. Most PHC components are biodegradable, however a few are resistant to biodegradation and as such are often used as conserved internal markers in the PHC mixture. This allows the comparison of the easily biodegraded compound to those select recalcitrant compounds. Petroleum biodegradation has been extensively studied and a number of internal conserved markers such as 17α(H)21β(H) hopane have been used. Presumably the use of conserved biomarkers, correct for abiotic loss of individual PHC compounds in a field study as the biomarker would be lost as well as the PHC. Historically, pristane and phytane have been used; those isoprenoids were found to be biodegradable and thus their use for monitoring of compounds much more resistant to biodegradation, such as PAHs, is not suitable (Howard et al., 2005). Secondly, hopanes were thought to be conserved. Those too have been shown to be susceptible to biodegradation, although at a much slower rate than pristine and phytane (Howard et al., 2005). Another PHC group relatively resistant to degradation are the C2-chrysenes. C2-chrysenes are four ring PAHs with an alkyl substitution at the 2 position on the ring. The alkyls are generally methyl and ethyl groups. Using the total hopanes as a conserved biomarker demonstrated that C2-chrysenes were degraded when PEPS was employed. However, when PHC measurements were normalized to total hopanes there were no differences between planted and unplanted samples despite PHC decrease in both years in planted samples. This might be because some degradation of hopane is occurring as it has previously been documented (Huesemann et al., 2003). Preferential disappearance of C2-chrysenes suggests that degradation of persistent PAHs is occurring. The lack of degradation in PHC when normalized to hopanes suggest that either 1) only a small proportion of PHC is degraded, 2) BOC is extracted with PHC and falsely inflating the PHC values or 3) hopanes

may be degrading at the same rate as other components of the PHC mixture. Full investigation, including use of other highly conserved biomarkers would be needed to evaluate these possibilities.

It should be noted that the solvent used for extraction in analysis of hopanes and PHC in this particular analysis was dichloromethane (DCM). DCM is a non-polar solvent which would likely extract plant biogenic compounds such as waxes that would not be extracted with the previously extracted hexane:acetone solvent mixture. Accumulation of plant material extracted by acetone could contribute to the lack of degradation evident with planted sample.

### 4.5.7 Challenges with PHC measurements

A number of considerations with PHC quantification during phytoremediation have become apparent throughout this study. A close monitoring of PHC analytical methodology must be performed as any changes in protocol may obstruct any meaningful degradation due to phytoremediation. For example, the 20% increase in PHC extraction efficiency observed in the fall of 2006 appeared as an increase in PHC. Further, changes of laboratory facilities during phytoremediation trials are not recommended as laboratories differ in implementation of approved methodology. A possible reason for difficulty in obtaining accurate remediation data is the implementation of the silica method. Depending on the amount of dilution of the extract, as well as the initial concentration of PHC and the degree of weathering, silica gel columns could become overloaded and become inefficient in removal of polar compounds. The current method states that silica clean-up must only be performed once (CCME, 2001b), but this may not be sufficient.

To summarize, addition of PGPR increased phytoremediation at a site with low levels of highly weathered PHC. PEPS increased growth of plants and improved photosynthesis, and its application resulted in overall higher remediation in PGPR treated plots. Gravimetric analysis and $PHC_{TOTAL}$ and F3 measurements indicated that remediation occurred in year one and slowed in subsequent years, although PAHs continued to be degraded. Further investigation revealed that biogenic compounds might be the confounding PHC

measurements. Further investigation to determine optimal assays to detect degradation should be performed and the importance of proper silica clean up should be emphasized. Biogenic compounds that accumulate throughout the phytoremediation trial likely mask remediation and possibly the effect of PGPR increasingly over time.

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# Chapter 5
# Impacts of newly isolated PGPR from Northwest Territories on plant growth and phytoremediation in PHC-contaminated soils

## 5.1 Overview

Plant growth promoting rhizobacteria (PGPR) were isolated from Northwest Territories (NWT) soils. Three newly isolated PGPR were identified as *Serratia grimesii*, *Pseudomonas frederiksbergensis* and *Pseudomonas marginalis*. To determine the ability of these PGPR to improve plant growth in contaminated growth media, they were tested first on Pro-Mix general purpose growth medium spiked with creosote (0.5% v/w). Following that, the PGPR were tested on petroleum hydrocarbon (PHC) impacted soil from NWT. Three grass species were tested: creeping red fescue (*Festuca rubra* L), slender wheatgrass (*Elymus trachycaulus trachycaulus*) and tall fescue (*Festuca arundinacea*); the first two being native to NWT, while the third is a demonstrated PHC phytoremediator and has been shown to have positive growth effects when inoculated with PGPR. PHC remediation of the two impacted soils using PGPR-assisted phytoremediation was tested using the newly isolated PGPR. Of the two PHC impacted soils, one was also salt impacted (500 to 800 mg $kg^{-1}$, designated biocell soil), while the other (designated tank 53 soil) had low levels of salt. Single isolated PGPR increased plant growth in Pro-Mix spiked with creosote. When tested on impacted soils from NWT, mixtures of two and three bacteria enhanced plant growth, while single bacterial species used individually did not have a large effect on plant growth. Of the two soils used in these experiments, there was more plant growth inhibition in the salt impacted soil (biocell soil) and the PGPR effect was more apparent in biocell soil than in tank 53 soil.

## 5.2 Introduction

Upstream oil and gas production often results in contamination of terrestrial ecosystems with petroleum hydrocarbons (PHC). Common contamination sources include flare pits used to store and/or burn produced fluids at well sites, drilling fluids (e.g. diesel invert), pipeline

spills and wells that are no longer operational. In many cases the sites are located in remote areas (Alberta Environment, 2001; Rutherford et al., 2005). In Canada, much of the upstream oil and gas processing takes place in western and northern regions of the country, including the Northwest Territories where many spills are associated with the oil/gas sector (14%) and transportation (7%) ( Northwest Territories Department of Environment and Natural Resources, 2011). Two primary obstacles facing site remediation work in these areas are the difficulties of reliable access and the uncompromising environment. Access to remote sites is often limited to only a few months of the year, and even then heavy equipment required for many remediation strategies is difficult and expensive to transport. For remediation to be performed off site, or *ex situ*, equipment for excavation and transport of soil is still required, which suffers from similar high cost and high transport difficulties. Due to these challenges, there is a need for *in situ* remediation approaches to address such sites.

Phytoremediation addresses the inaccessibility of large equipment and transport costs. It is an *in situ* process, and thus does not require transportation of the soil to be remediated. This method makes use of plants to assimilate and degrade contaminants, and is both low in cost and easily maintained. It is thus an excellent option for remote regions. One obstacle with use of phytoremediation at PHC-impacted sites is the inability to grow plants when contaminant levels are high. This may often be exacerbated by sub-optimal weather conditions such as low precipitation and drought, flooding or lack of nutrients in the soil. To overcome these setbacks, plant growth promoting rhizobacteria (PGPR) have been used. These microbes act indirectly, by modulating plant hormone levels, or directly, by providing nutrients to the plant (Glick et al., 2007). PGPR-enhanced phytoremediation has been shown to be successful at other petroleum-contaminated sites, including those that were heavily PHC contaminated (Gurska et al., 2009), and many PGPR species have been shown to improve plant growth in response to environmental injury (Podile and Kishore, 2003).

The importance of proper native plant selection to maintain the delicate northern Canadian ecosystem has long been recognized, and the use of native vegetation is preferred by both scientists and regulatory bodies (Adams and Lamoureux, 2005). Plants selected for phytoremediation must be PHC tolerant to facilitate their growth at impacted sites, have deep

root systems, be adaptable to or come from local climates, be easily planted and maintained, and have fast growth rates (EPA, 2000). The two plant species chosen for this study were creeping red fescue (*Festuca rubra* L) and slender wheatgrass (*Elymus trachycaulus trachycaulus*); both are native to Northwest Territories (United States Department of Agriculture, 2010). Additionally, tall fescue (*Festuca arundinacea*) was chosen for growth comparison in the lab as it has extensively been used for remediation of PHC. As soil microorganisms play vital roles in the preservation of ecosystem biodiversity, any changes in the structure and function of microbial communities may influence the ecosystem (Derry et al., 1999). Proper selection of native plant growth promoting microorganisms may be also critical. Thus, native PGPR species were isolated from NWT soils to avoid introduction of bacterial species that may not be native to those regions. PGPR isolation was performed based on selection of soil bacteria that have the ability to use 1-amino-cyclopropane-1carboxylate (ACC) as a sole nitrogen source. To do so they use the enzyme ACC deaminase, which consumes the plant ethylene (plant stress hormone) precursor, effectively lowering ethylene levels in plants under stress (Penrose and Glick, 2003). This trait along with other plant growth promoting mechanisms allows PGPR to increase plant growth in highly contaminated soils (Gurska et al., 2009).

Native isolated PGPR were tested for improvement of plant growth of native and non-native grass species. The remediation of PHC from NWT soils was tested using a combination of newly isolated PGPR and native plants, including mixtures of PGPR. Further, PHC remediation in two types of soil was examined; a salt impacted and salt unimpacted soil. It was observed that although salt negatively impacted plant growth, it did not inhibit PHC remediation. Often PHC and salt are present together at oil and gas extraction sites; salt is a by-product of the petroleum extraction processes, which utilize brines. Thus, phytoremediation is a viable option for addressing remediation requirements in remote regions where other remediation methods may be inaccessible.

## 5.3 Materials and Methods

### 5.3.1 Isolation of PGPR

Bacteria were isolated according to Penrose and Glick (2003) with modifications outlined below. Environmental soil samples from planted areas were collected and stored at 4$^{o}$C prior to isolation. A sample of 1 g of near-root and rhizosphere soil was transferred into Tryptic Soy Broth (TSB, 30 g/L Fisher Scientific, Ottawa, Ontario, Canada) and agitated for ~24 h at 100 rpm, at room temperature (22 ± 2$^{o}$C, RT). An aliquot of this suspension was transferred into fresh TSB and incubated for 24 h at 100 rpm, at RT. An aliquot of this suspension was transferred to DF salt media (+ $(NH_4)_2SO_4$, + glucose; DF media made as per Penrose and Glick (2003) and shaken for 24 h at 100 rpm, at RT. An aliquot was centrifuged and the pellet was washed with DF salt medium without $(NH_4)_2SO_4$, and then resuspended in this medium. The inoculum was allowed to grow in DF salt media without $(NH_4)_2SO_4$ and with ACC as nitrogen source for 48 hours or until the solutions appeared cloudy. A loopful of this growing culture was streaked on a DF agar plate (1.5% Bacto-Agar, Difco Laboratories, Detroit, MI, USA) with 0.5 M ACC (Calbiochem-Novobiochem Corp., La Jolla, CA, USA) as the sole source of nitrogen and incubated at 30$^{o}$C for 3 days. Colonies were selected and grown in liquid DF salt minimal media with 0.5 M ACC. An aliquot of this culture was transferred to TSB, from which bacterial stocks were made for further analysis (18% glycerol). Bacterial strains were tested for ACC deaminase activity according to the protocol by Penrose and Glick (2003) with the following modifications. Following growth in the DF media with ACC, bacterial cell pellets were suspended in 0.1 M Tris-HCl, pH 8.0 prior to addition of toluene. Indole acetic acid production assay followed the protocol by Khalid et al. (2004) and siderophore activity was determined as described in Alexander and Zuberer (1991). Identification of each putative PGPR strain, chosen based on ACC deaminase activity, was performed based on 16S RNA sequencing (~500 bp were sequenced) by Accugenix Inc, Newark, DE, USA. Sequences obtained from Accugenix were used to perform a search using BLAST database to confirm the identity of isolates. The PGPR identified as (*Serratia grimesii* [NWT2-3], *Pseudomonas frederiksbergensis* [NWT6] and *Pseudomonas marginalis* [NWT4]) were used for seed treatments prior to planting (see

section 5.4.1 for % identity of isolates). A previously tested, UW4 *Pseudomonas putida*, was used as a positive control for improvement of plant growth.

### 5.3.2 Growth in artificial media spiked with creosote

As an initial screening of growth promotion by isolated bacteria, seeds of tall fescue and fall rye were inoculated with each bacteria then grown in general purpose plant growth medium, Pro-Mix 'BX' (Plant Products Co. Ltd., Brampton, ON, Canada) spiked with creosote (0.5% v/w). Tall fescue (*Festuca arundinacea*), annual rye (*Lolium multiflorum*), and fall rye (*Secale cereale*) have previously been shown to be effective remediators and have shown improvement in growth and remediation in response to PGPR (Huang et al., 2004a; Huang et al., 2005; Gurska et al., 2009). All seeds were purchased from Ontario Seed Ltd., Waterloo, ON, Canada. Bacterial inoculum was prepared by growing each strain (500 μL frozen stock in 100 mL of TSB [50 $g \cdot L^{-1}$, Fisher Scientific, Ottawa, Ontario, Canada]) for 24 h at RT with agitation. The bacterial culture was centrifuged (5000 rpm, 20 min) and re-suspended in autoclaved deionized water to obtain a final OD of 1.5-2. Seeds were inoculated with each strain of bacteria by soaking the seeds in bacterial suspension for 15 min and air drying for 1 hour prior to planting. Control plants were soaked in water. The seeds were sown in creosote-spiked Pro-Mix and control Pro-Mix in plastic four cell plant plug trays for Pro-Mix (Jack van Klaveren Co., St. Catherines, ON, Canada). Single bacterial species were used for inoculation to determine individual effects of the bacteria. The plants were grown in the greenhouse under seasonal lighting conditions and were harvested after 30 days of growth. To determine dry weight, shoots were cut off and dried for 2 days at 40$^{o}$C in an oven and re-weighed.

### 5.3.3 Growth in soil impacted with PHC

Plant selection for growth in petroleum contaminated soils from Northwest Territories was as follows. Tall fescue has been used in our laboratory and has been shown to be a good phytoremediator of PHC. Red fescue has previously been used to re-vegetate disturbed sites in northern Canada (Wishart, 1988; Martens and Younkin, 1989). Slender wheatgrass has also been widely used for rehabilitating mine soils, oil-drilling sites and wildlife habitat

(Smith, 1963; Brown and Johnston, 1978; Chambers, 1989). Slender wheatgrass is also tolerant of saline and alkaline soils (Pearen et al., 1997). Both red fescue and slender wheatgrass are native to NWT (United States Department of Agriculture, 2010). Red fescue and tall fescue have been shown to be effective phytoremediators (Huang et al., 2004b; Phillips et al., 2006) and slender wheatgrass is a hydrocarbon tolerant species (Frick et al., 1999b). Mixtures of two strains of PGPR and three strains of PGPR were used to treat seeds and the mixtures were as follows: NWT4 and NWT6 (designated NWTMix2), and NWT4, NWT6 and NWT2-3 (designated NWTMix3). In total there were six treatments (UW4, NWT4, NWT6, NWT2-3, NWTMix2, NWTMix3) and two controls (plants – PGPR, unvegetated soil).

Plant growth experiments were conducted in the greenhouse for 60 days. Biomass accumulation (roots and shoots) and total remediation were assessed. Plant seeds (3 g) treated with or without PGPR as described above were sown in six cell multicell trays, each cell containing approximately 1 kg of soil. All plants were watered once per day using tap water. After 30 days of growth, all pots were watered weekly with tap water (100 mL per pot) containing 100 g $kg^{-1}$ of 20-20-20 fertilizer (Plant Products Co. Ltd., Brampton, ON, Canada), in addition to the daily watering regime. At 60 days, plants were harvested for fresh weight and dry weight analyses. Shoots and roots were separated and dried for 2 days after which weight of the sample was determined. A 60 day experiment was performed with all treatments. Replicate experiments were performed with mixtures of bacteria only (Table 5.1).

The petroleum-impacted soils from the Norman Wells site were from a land treatment facility. Prior to analysis, soil samples were homogenized and characterized (Table 5.2, Agri-Food Laboratories, Guelph, Ontario). Both soils were slightly nutrient-deficient with respect to $K^+$. Tank 53 soil exceeded typical guidelines for growth in silt and silt loam soil with respect to concentrations of $Mg^{2+}$ (505.2 mg $kg^{-1}$) and $Ca^{2+}$ (538.3 mg $kg^{-1}$). Biocell soil, exceeded these guidelines for $Ca^{2+}$ (5422 mg $kg^{-1}$), $Na^+$ (287.36 mg $kg^{-1}$) and $Cl^-$ (339.2 mg $kg^{-1}$). F3 levels were 1774 mg $kg^{-1}$ and 2020 mg $kg^{-1}$ for tank 53 and biocell soils respectively, and thus exceeded the Canada-Wide Standards of 800 mg $kg^{-1}$ for fine grain soils.

**Table 5.1 Experimental design used for tank 53 and biocell soils.**

Tall fescue, slender wheatgrass and creeping red fescue were used. The – PGPR denotes plant seeds treated with reverse osmosis purified water. Mix2 is NWT4, 6 and Mix 3 is NWT2-3, 4, 6. All treatments were performed in trial I. If treatment was not repeated in trial II, this is indicated by "-".

| Plant Species | Bacterial Treatment | Trial I | Trial II |
|---|---|---|---|
| Tall fescue | - | + | + |
| | UW4 | + | - |
| | NWT4 | + | - |
| | NWT2-3 | + | + |
| | NWT6 | + | - |
| | Mix2 | + | + |
| | Mix3 | + | + |
| Wheatgrass | - | + | + |
| | UW4 | + | - |
| | NWT4 | + | - |
| | NWT2-3 | + | + |
| | NWT6 | + | - |
| | Mix2 | + | + |
| | Mix3 | + | + |
| Red fescue | - | + | + |
| | UW4 | + | - |
| | NWT4 | + | - |
| | NWT2-3 | + | + |
| | NWT6 | + | - |
| | Mix2 | + | + |
| | Mix3 | + | + |

**Table 5.2 Agronomic and petroleum analyses of tank 53 and biocell soil.**

| Parameter | Tank 53 | Biocell |
|---|---|---|
| PHC (g $kg^{-1}$) | 3.79 | 4.04 |
| F2 (mg $kg^{-1}$) | 95.2 | 111.4 |
| F3 (mg $kg^{-1}$) | 1774 | 2020 |
| F4 (mg $kg^{-1}$) | 1013.4 | 1170 |
| Total PHC (g $kg^{-1}$) | 2.99 | 3.30 |
| F4G (g $kg^{-1}$) | 3.19 | 3.84 |
| pH | 7.58 | 7.44 |
| Total Salts, ECe (dS $m^{-1}$) | 3.12 | 3.88 |
| Organic Matter | 3.64 | 4.3 |
| Phosphorous (mg $kg^{-1}$) | 18.8 | 13 |
| Potassium (mg $kg^{-1}$) | 66.6 | 190.6 |
| Magnesium (mg $kg^{-1}$) | 505.2 | 280.4 |
| Calcium (mg $kg^{-1}$) | 538.3 | 5422 |
| Sodium (mg $kg^{-1}$) | 51.2 | 287.4 |
| Chloride (mg $kg^{-1}$) | 47.2 | 339.2 |
| SAR | 0.12 | 0.7 |
| CEC (MEQ/100g) | 32.6 | 30.8 |
| Sand % | 33 | 44.8 |
| Silt % | 51.4 | 38.4 |
| Clay % | 15.6 | 16.8 |
| Classification | Silt loam | Loam |

PHC determined gravimetrically following a hexane:acetone (1:1, v:v) extraction. F2 is the fraction of C>10 to C16, F3 is the fraction of C>16 to C34 and F4 is the fraction of C>34 to C50+.
Petroleum fractions (F2-F4) and Total PHC were determined using GC-FID. F4G is the gravimetric heavy fraction. SAR is Sodium absorption ratio, CEC is the cation exchange coefficient, MEQ is milliequivalent. ECe, electrical conductivity (EC) of soil samples, is a measure of the soil salinity, expressed in dS/m (deci Siemens per meter).

### 5.3.4 Petroleum hydrocarbon analysis

PHC content was analyzed immediately upon receipt and homogenization of the soil samples (referred as t0 samples). Every trial had a control treatment without plants, which was watered and fertilized in the same manner as the vegetated samples. The t0 samples and unvegetated control samples had a similar PHC content. To obtain remediation values, soil samples were analyzed for PHC levels at the end of the 60 day growth experiments in the greenhouse.

Where indicated, petroleum concentrations in the soil were determined gravimetrically as described in Chapter 2. Petroleum fractions F1-F4 (F1 fraction contains the carbon range C6 to C10, F2 fraction C>10 to C16, F3 fraction C>16 to C34, F4 fraction is C>34 to C50+) and gravimetric analysis (F4G) where indicated were performed by an accredited commercial laboratory (ALS Laboratory Group, Waterloo, ON, Canada) according to guidelines by Canadian Council of Ministers of the Environment (CCME, 2001a).

### 5.3.5 Statistical analysis

One-way ANOVA ($P < 0.05$) was used to test significance of creosote screening tests in Pro-Mix, plant growth in PHC impacted soils and PHC remediation.

## 5.4 Results

### 5.4.1 Bacterial Isolation from Northwest Territories

Potential PGPR with detectable ACC activity were isolated and designated NWT4, NWT6, NWT2-3. Isolates were sequenced (Accugenix Inc, Newark, DE, USA) and their partial 16S RNA sequence was compared to the Accugenix database. The sequences were also used to search the Genbank database for closest matches using the Blastn algorithm. Closest matches to bacterial 16S RNA sequences are listed in Table 5.3. In two cases, NWT4 and NWT6, the ID provided by Accugenix was confirmed by Genbank sequence search. In one case, NWT2-3 was identified as *Serratia grimesii*, however this species did not come up in the Genbank sequence search.

**Table 5.3 PGPR isolate characterization.**

Genbank ID, similarity and PGPR properties such as siderophore activity, ACC deaminase activity and IAA production for the isolated bacterial species.

| Isolate | # of Nucleotid. Sequenced | Accugenix Match | Accugenix % Similarity | Genbank Accession number (homologs) | Genbank Similarity | Siderophore Activity[a] | ACC deaminase activity [μmol α-KA/mg protein/h][b] | IAA activity [μg/mL/$A_{600}$][c] |
|---|---|---|---|---|---|---|---|---|
| UW4 | - | - | | - | - | ½+ | 4.75 | 1.08 |
| NWT4 | 522 | *Pseudomonas marginalis* | 100% | AF311387.1 | 99% | ½+ | 8.53 | 0.38 |
| NWT6 | 524 | *Pseudomonas frederiksbergensis* | 100% | NR_028906.1 | 100% | +++ | 2.2 | 0.33 |
| NWT2-3 | 528 | *Serratia grimesii** | 99% | - | - | + | 1.53 | 0.42 |

[a] activity measured semi-quantitatively on CAS-agar plates, as appearance of orange halo on a blue plate.
b activity of cell free extracts prepared after growth in DF salt minimal medium plus ACC (n = 3, ± SE).
c IAA secreted into the growth medium after 48h of incubation in DF salts medium (n = 9, ± SE), normalized to bacterial density at 600 nm (A600), measured with the addition of tryptophan.
*NWT2-3 was identified as *Serratia grimesii* from the Accugenix database, but was not confirmed in the Genbank sequence search. NWT was identified as *Serratia* sp. from Genbank.

### 5.4.2 PGPR growth promotion on creosote spiked Pro-Mix growth medium

Plant growth was decreased by 50-60% when Pro-Mix was spiked with 0.5% (v/w) creosote. Growth of plants in creosote increased when newly isolated bacteria were used to inoculate the seeds (Table 5.4). Coinciding with the shoot growth, fall rye roots after 10 days of growth were longer than control plants exposed to 0.5% creosote, and were densely packed at the bottom of the growth cells when inoculant treatments were used (Figure 5.1). Rye/fescue plants grew more slowly than fall rye and the roots did not reach the bottom of the growth cell at the end of the experiment (images not shown), however growth improvement was also observed for shoot biomass of rye/fescue plants (Table 5.4).

**Table 5.4 Plant biomass on creosote spiked Pro-Mix.**

Pro-Mix was spiked with 0.5% creosote (v/w). Each value is an average of 2 measurements, +/- SE. Numbers without SE are n = 1. Fresh weight (FW) and dry weight (DW) of shoots were collected after 10 days of growth. Plant growth was measured in g/growth cell. Significance tested using one-way ANOVA ($P < 0.05$) and none detected.

| | | Fall Rye | | Rye/Fescue | |
|---|---|---|---|---|---|
| **PGPR** | **0.5% Creosote** | **FW [g]** | **DW [g]** | **FW [g]** | **DW [g]** |
| **None** | **-** | 5.47 | 0.56 | 3.34 ± 0.09 | 0.26 ± 0.03 |
| **None** | + | 3.31 ± 0.71 | 0.34 ± 0.06 | 2.14 ± 0.11 | 0.17 |
| **NWT4** | + | 4.38 ± 0.90 | 0.44 ± 0.10 | 2.50 ± 0.04 | 0.20 ± 0.01 |
| **NWT6** | + | 4.07 ± 0.92 | 0.41 ± 0.08 | 2.24 ± 0.11 | 0.20 ± 0.03 |
| **NWT2-3** | + | 3.70 ± 0.51 | 0.37 ± 0.04 | 2.27 ± 0.04 | 0.18 ± 0.01 |
| **UW4** | + | 4.24 ± 0.81 | 0.40 ± 0.07 | 1.75 ± 0.07 | 0.14 |

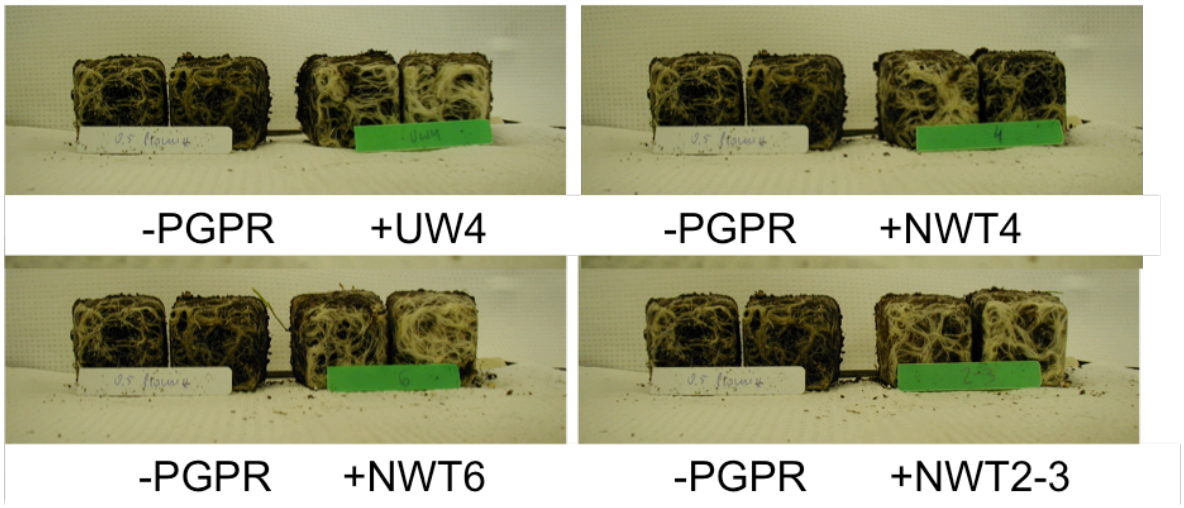


**Figure 5.1 Fall rye roots after 10 days of growth in creosote spiked Pro-Mix.**

The seeds were sown in creosote-spiked Pro-Mix and control Pro-Mix in plastic four cell plant plug trays for Pro-Mix. Creosote was spiked at a concentration of 0.5% (v/w).

### 5.4.3 The effects of soil type on plant growth

Two PHC impacted soils from NWT were used: biocell soil was salt impacted, and tank 53 was not salt impacted. Tank 53 soil exceeded typical agricultural guidelines for plant growth in silt and silt loam soil with respect to concentrations of Mg and $Ca^{2+}$, while the biocell soil exceeded these guidelines for $Ca^{2+}$, $Na^{+}$ and $Cl^{-}$ (Table 5.2). All other soil parameters were similar in the two soils. PHC concentrations were similar for both soil types and were approximately 4 g $kg^{-1}$. The salt impacted biocell soil inhibited growth in uninoculated plants by 41%, 36% and 11% (fresh weight shoots) for red fescue, wheatgrass and tall fescue, respectively (Figure 5.4), when compared to the tank 53 soil after 60 days. Fresh weight of roots was 43% lower in red fescue grown in the biocell soil in comparison to tank 53 soil, but was not impacted in either wheatgrass or tall fescue relative to tank 53 soil (Figure 5.4). Overall, tank 53 soils were less toxic to the uninoculated plants based on fresh weight (Figure 5.4, Figure 5.5).

### 5.4.4 PGPR effects on plant growth in PHC-impacted NWT soils

During the first 30 days of growth, grasses treated with PGPR treatments showed improved growth compared to those without PGPR (Figure 5.2). Both wheatgrass and red fescue grew better when inoculated with mixtures of two or three bacteria, rather than those inoculated with individual PGPR species, or control plants without PGPR. Growth of tall fescue was improved only when it was inoculated individually with the NWT2-3 isolate. Overall, using PGPR mixtures, wheatgrass and red fescue consistently showed a stronger positive response to PGPR than tall fescue (Figure 5.2).

After 60 days of growth, PGPR treated plants were larger than the untreated plants. However, plant growth was limited by pot size, and the PGPR treated plants filled the existing space much faster, leaving no room to grow. Consequently, plants without PGPR could increase in size at a higher relative rate than PGPR treated plants during the latter part of the trial, as they still had room to grow. Conversely, in some cases, an obvious PGPR effect was not observed at 35 days (e.g. red fescue, Figure 5.2 C, D) but the difference between plants with and without PGPR became quite pronounced at 60 days (Figure 5.4, Figure 5.5). The biggest differences were consistently obtained between

PGPR treated plants and untreated plants for both wheatgrass and red fescue (Figure 5.4A, C). Tall fescue plants without PGPR treatment grew better than untreated wheatgrass and red fescue plants. In general, tall fescue plants accumulated more biomass (note the scale difference in Figure 5.4 and Figure 5.5)

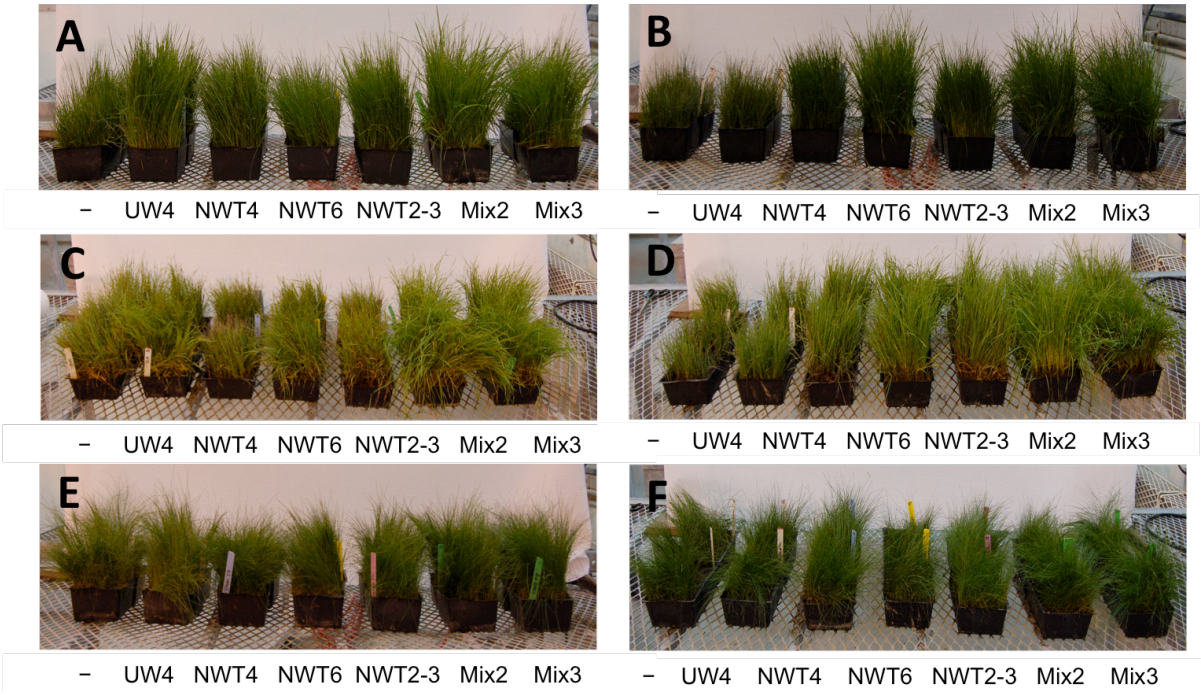


**Figure 5.2 Plant growth at 35 days of trial I.**

Tall fescue on tank 53 soil (A) and in the biocell soil (B), wheatgrass in tank 53 soil (C) and in the biocell soil (D). Red fescue in tank 53 soil (E) and in the biocell soil (F). "–" indicates no PGPR used. Mix2 is a mixture of two PGPR, NWT4, NWT6. Mix3 is a mixture of three PGPR, NWT2-3, NWT4, NWT6.

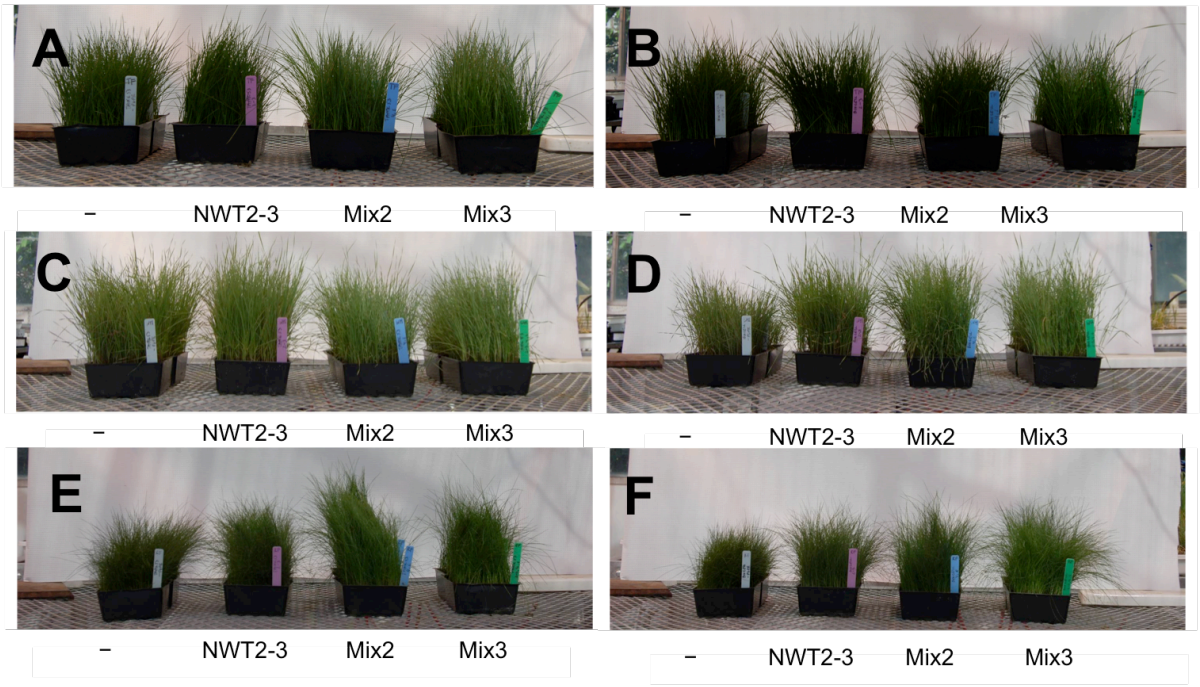


**Figure 5.3 Plant growth at 30 days of trial II.**

Tall fescue in tank 53 (A) and biocell soil (B), wheatgrass in tank 53 (C) and biocell (D) soil, red fescue in tank 53 (E) and biocell (F) soil. "–" indicates no PGPR used. Mix2 is a mixture of two PGPR, NWT4, NWT6. Mix3 is a mixture of three PGPR, NWT2-3, NWT4, NWT6.

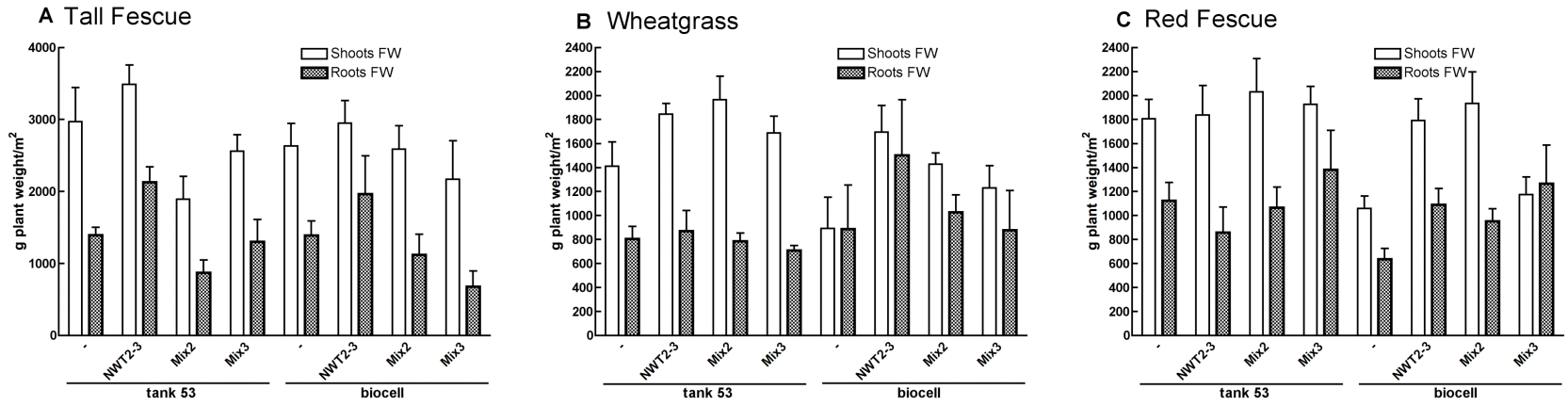


**Figure 5.4 Fresh weight of plants grown in PHC contaminated soils.**

Tall Fescue (A), wheatgrass (B) and red fescue (C) grown on tank 53 and biocell soil. Data combined from Trial I and Trial II. "–" indicates no PGPR used. Mix2 is a mixture of two PGPR, NWT4, NWT6. Mix3 is a mixture of three PGPR, NWT2-3, NWT4, NWT6. Plants were grown in the greenhouse.

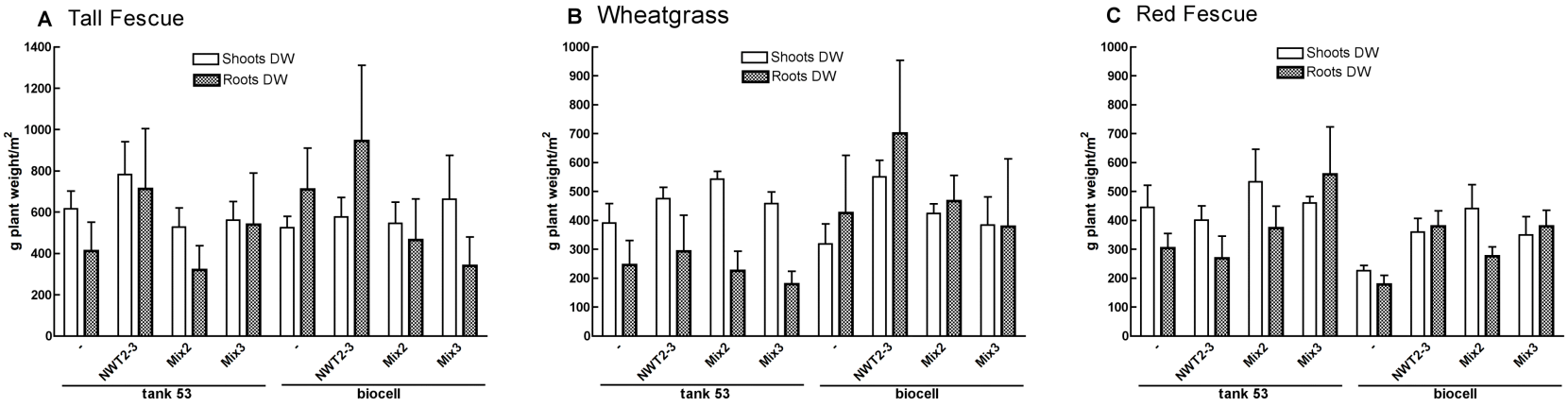




**Figure 5.5 Dry weight of plants grown in PHC contaminated soils.**

Tall Fescue (A), wheatgrass (B) and red fescue (C) grown on tank 53 and biocell soil. Data combined from Trial I and Trial II. "–" indicates no PGPR used. Mix2 is a mixture of two PGPR, NWT4, NWT6. Mix3 is a mixture of three PGPR, NWT2-3, NWT4, NWT6. Plants were grown in the greenhouse.

Plant growth of treated and untreated plants were compared, and results from trial I indicated that bacterial mixtures increased plant biomass to a greater extent than individual bacterial species inoculants (Figure 5.2). Of the bacterial treatments, NWT2-3 and combinations of two or three PGPR (NWTMix2 or NWTMix3 respectively) consistently elicited a positive growth response. Those three treatments were chosen to be repeated in trial II. Data for trial I and II replicates of the PGPR treatments (NWT2-3, NWTMix2, NWTMix3) indicated increases in plant fresh and dry weight in red fescue and wheatgrass. PGPR caused a larger increase in red fescue and wheatgrass fresh weight of shoots, and this was not as apparent in fresh weight of roots (Figure 5.4). Tall fescue showed increased biomass accumulation with NWT2-3 only (Figure 5.4, Figure 5.5). Improvements with PGPR inoculation were more evident on the biocell soil than on the tank 53 soil, with all plants and treatments. It appeared that tall fescue may be inhibited by the mixtures of two bacteria (NWT4 and NWT6) and this effect was more pronounced on biocell soil.

### 5.4.5 In-house gravimetric PHC analysis

All samples were analyzed using a gravimetric method for analysis for PHC ($PHC_G$). In biocell soil, $PHC_G$ remediation varied between 1.7% and 10.8% with an overall average of 6.9% relative to unvegetated control. In tank 53, soil remediation varied between 0% and 10.63% with an overall average of 1.51% relative to unvegetated control.

**Table 5.5 Remediation in biocell and tank 53 soils measured by in-house laboratory.**

Each gravimetric measurement was performed in duplicate on a composite sample from 2 pots. Errors represent standard deviation. Remediation calculated relative to biocell unvegetated control (3.9 ± 0.1 g $kg^{-1}$) and tank 53 unvegetated control (3.6 ± 0.2 g $kg^{-1}$). RF, red fescue; TF, tall fescue; WH, tall wheatgrass. Plants were grown in the greenhouse for 60 days.

| Plant Type | Bacterial Treatment | Remediation (%) | |
|---|---|---|---|
| | | tank 53 | biocell |
| RF | - | 0 | 8.8 ± 2.9 |
| | 2-3 | 5.13 ± 2.8 | 6.9 ± 5.6 |
| | 4,6 | 2.36 ± 2.8 | 10.8 ± 0 |
| | 4,6,2-3 | 0 | 6.0 ± 5.4 |
| TF | - | 10.63 ± 3.0 | 9.1 ± 5.7 |
| | 2-3 | 0.52 ± 2.7 | 7.1 ± 0 |
| | 4,6 | 1.18 ± 5.3 | 7.5 ± 2.7 |
| | 4,6,2-3 | 0 | 6.0 ± 5.3 |
| WH | - | 3.86 ± 2.8 | 7.1 ± 2.8 |
| | 2-3 | 3.37 ± 2.8 | 6.0 ± 2.7 |
| | 4,6 | 2.88 ± 2.8 | 1.7 ± 2.6 |
| | 4,6,2-3 | 2.32 ± 2.7 | 6.0 ± 2.7 |
| All | All | 1.51 ± 2.8 | 6.9 ± 2.8 |

Given the low initial PHC levels, and large variability in the dataset, using the results of gravimetric analysis to differentiate between treatments is difficult. Thus, a meaningful way to evaluate the data is to compare remediation with and without plants, and without differentiating between PGPR treatments. The overall average remediation (all PGPR strains and untreated controls) for tank 53 and biocell soils was 1.5% and 6.9% respectively, relative to unvegetated control when $PHC_G$ values were evaluated.

### 5.4.6 Remediation of PHC fractions F3 and F4

Remediation of Fraction 3 (F3) and Fraction 4 (F4) was determined from accredited analytical commercial laboratory data. Averages of overall remediation in biocell and tank 53 soil by tall fescue, red fescue and wheatgrass from trial I and II (In trial II, only biocell soil was analyzed by the commercial laboratory) are summarized in Table 5.6. There were no differences between + PGPR and – PGPR, thus individual plant and ± PGPR data is not presented here. Instead, data from all planted treatments was pooled to assess if remediation of PHC was occurring relative to unplanted treatments. F3 of both the biocell and tank 53 soils remediated by 34%. F4 was remediated by 35% and 26% for biocell and tank 53 soil, respectively. Gravimetric PHC levels which were determined by the commercial laboratory, the F4G or here referred to as $PHC_{F4G}$ resulted in 22% remediation of biocell and 28% remediation of tank 53 soil. The commercial $PHC_{F4G}$ employs the silica gel clean up discussed in detail in Chapter 4, while the in-house $PHC_G$ does not. Silica gel cleanup would remove some biogenic material from the samples. Gravimetric analysis values $PHC_{F4G}$ obtained from the commercial laboratory reflect the values we obtained with the in-house gravimetric analysis. Remediation values are somewhat lower for the in-house analysis, most likely due to lack of silica clean up in the in-house method. Also the values for $PHC_{F4G}$ fell in the range of 3.2 g $kg^{-1}$ for both biocell and tank 53 (Table 5.6), while for $PHC_G$ performed in-house, they were 3.9 g $kg^{-1}$ and 3.6 g $kg^{-1}$ (Table 5.5). This difference can be attributed to the silica clean up in $PHC_{F4G}$.

**Table 5.6 PHC levels in biocell and tank 53 soils measured by commercial laboratory.**

Each average represents selected soil samples from red fescue and tall fescue and wheatgrass, from trial I for tank 53 soils (n = 9 for planted, n = 5 for unvegetated soils) and from trial I and II for biocell soils soils (n = 19 for planted, n = 9 for unvegetated soils). Plants were grown in the greenhouse for 60 days.

| | | PHC fraction (mg $kg^{-1}$) | | | |
|---|---|---|---|---|---|
| **Soil** | **Treatment** | **F3** | **F4** | **Total** | **F4G** |
| biocell | – Plants | 1679±146 | 1010±73 | 2768±231 | 3167±321 |
| | + Plants | 1066±226 | 652±151 | 1762±382 | 2480±1001 |
| | % Remediation | 37 | 36 | 36 | 22 |
| tank 53 | – Plants | 1774±160 | 1013±147 | 2990±421 | 3194±564 |
| | + Plants | 1177±228 | 754±112 | 1979±385 | 2309±462 |
| | % Remediation | 34 | 26 | 34 | 28 |

## 5.5 Discussion

Phytoremediation is a viable option for remote regions of Canada where other remediation methods may be too costly or invasive. However, with a shorter growing season and the inability to regularly fertilize or irrigate remote sites, it would be beneficial to have other methods to ensure good plant growth and remediation. PGPR enhanced phytoremediation has previously been shown to increase plant growth under unfavorable conditions such as toxicity or poor soil quality. To date, PGPR used in this approach have been isolated from Ontario (Penrose and Glick, 2004) and thus may not pass the regulatory standards to be used in a sensitive environment such as those in NWT. Further, the use of native plant species with these PGPR would be desirable. Here we have isolated new species of PGPR from NWT territories soil, tested them with plants native to those regions and have shown improvement in plant growth. Further, we have shown that plants chosen were successful in remediation of PHC from experimental soils, both salt impacted and salt unimpacted. Phytoremediation with PGPR, used to lower fertilizer needs and increase remediation, would be superior to other phytoremediation options for remote regions, and would fulfill the need for environment specific bacteria and environment specific plants.

### 5.5.1 PGPR performance

PGPR isolated from the Northwest Territories (NWT) were identified as *Serratia grimesii* (isolate NWT2-3), *Pseudomonas frederiksbergensis* (isolate NWT6) and *Pseudomonas marginalis* (isolate NWT4). All three species are not considered pathogens, and are listed under biosafety level 1 by the American Tissue Culture Collection. Members of the genus *Serratia* have been reported to have many plant growth promoting strains (Ashelford et al., 2002; Podile and Kishore, 2003; Somers and Vanderleyden, 2004). *Pseudomonas marginalis* was originally isolated from plant-derived foodstuff, particularly endive. Specific strains of this species were implicated in bacterial diseases, such as bacteria head rot of broccoli (Pajot and Silue, 2005), parsnip (Hunter and Cigna, 1981), and carrot (Godfrey and Marshall, 2002); mostly implicated in macerative diseases of vegetable crops. However, *P. marginalis* was also isolated from a nitro-aromatic contaminated site and was demonstrated to metabolize 2,4-dinitrotoluene

intermediates (Snelix et al, 2003), thus pathogenicity is not suspected. *Pseudomonas frederiksbergensis* was first isolated from soil of a former coal gasification site in Frederiksberg, Copenhagen, Denmark. Interestingly, this particular bacterial species has been demonstrated to degrade phenanthrene (Andersen et al., 2000). It has also been isolated from the roots of *Brassica napus* and found to have full disease suppressive activity against *Fusarium culmorum* (a plant pathogen) (Johansson and Wright, 2003). Although strains identified here may not necessarily have the same characteristics as those described in the literature, as these metabolic capabilities are strain dependent (Glick et al., 1999), the prevalence of growth promoting or degradation characteristics in other investigated strains suggests that the newly identified strains may aid in phytoremediation applications.

Mixtures of PGPR performed better in trial I. The grounds for using a mixture of bacteria is that different PGPR may complement each other, as most isolates generally do not possess all of the possible plant growth promoting traits (Koo et al., 2010). Koo et al. isolated 374 strains of bacteria from plants grown in metal and PHC contaminated soils and only one hundred sixty two strains had multiple plant growth promoting traits such as IAA production, siderophore production or ACC activity (Koo et al., 2010). In fact, these authors found that there was a negative correlation between IAA producers and ACC deaminase producers. Thus, combining strains may result in an additive or even synergistic effect on plant growth improvement. However, as observed in tall fescue performance in the current study, a single bacterial species may be equally capable of improving growth, especially if strains in the mixture are not compatible with each other. It may be beneficial to test for growth promotion prior to using new strains on particular plant species and results are often plant and microbe dependent.

### 5.5.2 Plant species

Two NWT endogenous plants were chosen, tall wheatgrass and red fescue as well as tall fescue, which had previously responded well to PGPR. Tall wheatgrass and red fescue proved to be amenable to PGPR treatment; in most treatments the two species responded well to isolated bacteria, showing a substantial increase in growth of shoots and roots. Tall fescue, when comparing plants without PGPR, accumulated more biomass than

wheatgrass and red fescue plants; tall fescue is a most robust of the grass species. However, when PGPR was added, it only responded to NWT2-3 isolate and its growth appeared to be somewhat inhibited by NWT4 and NWT6. Tall fescue and red fescue were demonstrated to phytoremediate in previous trials (Huang et al., 2004b; Phillips et al., 2006) and slender wheatgrass is a hydrocarbon tolerant species (Frick et al., 1999b). Remediation was observed with all three plant species. Thus, if the use of indigenous plants is important, both red fescue and slender wheatgrass can be used in conjunction with PGPR.

Less frequently than the other two plant species, PGPR improved the growth of tall fescue. This particular plant species has been demonstrated to be petroleum stress tolerant and consequently had high accumulation of biomass in experiments here (e.g. approximately 60% and 110% in tank 53 and 190% and 150% in biocell soil higher than wheatgrass and red fescue, respectively). Further, the mixtures of bacteria were not compatible with tall fescue, but NWT2-3 did appear to improve plant growth. Interestingly, tall fescue did not show an improvement in growth to the positive control strain, UW4 in either biocell or tank 53 soil, although this has been shown in other types of soil previously (data not shown). Plant-microbe interactions may be host specific, with some bacterial species having wider or narrower host potential; tall fescue may not be compatible with the other bacterial strains (NWT6, NWT4) of the mixture (Podile and Kishore, 2003).

### 5.5.3 Growth improvement with PGPR on different soil types

PGPR improved both shoot and root growth in the two types of soil. The effect was more prominent on the biocell than tank 53 soil, however tank 53 soils frequently had a positive PGPR response with NWT2-3. Although both soils had low PHC (~4 g $kg^{-1}$), biocell soil was also salt impacted. This increased salt content in biocell soils likely resulted in higher stress to plants. The increased stress in plants, leading to increased production of the plant stress hormone ethylene, could be neutralized by the PGPR enzyme, ACC deaminase. ACC is an immediate precursor to ethylene, and the increased amount of ACC can be degraded by the PGPR and used as a nitrogen source, effectively lowering the amount of ethylene in plants under stress (Glick et al., 2007). The lack of

effect on tank 53 soil may be attributed to this mechanism; when stress ethylene is not produced this may result in a lack of PGPR response on soils that are moderately or not toxic to plants. This response to combined salt and PHC stress is significant, since many PHC contaminated sites are also contaminated by salt. Remediation of PHC in the presence of salt may otherwise prove difficult because high salt concentration may inhibit microbial growth and subsequent degradation (Rhykerd et al., 1995) or may decrease PHC solubility and bioavailability (Margesin and Schinner, 2001). Here, increased salt levels did not impede degradation of PHC, likely because endogenous soil microbes that were involved in degradation of PHC in these soils, were salt adapted (Margesin and Schinner, 2001).

### 5.5.4 PHC remediation

Remediation was observed in tank 53 and biocell soils. Interestingly, although average plant growth was better in tank 53 soils, remediation was better in biocell soil (1.5% and 7% remediation for tank 53 and biocell, respectively). This is expected, as the trial period of 60 days was very short. Conceivably, this may have been a result of the bioavailability of contaminants present in the soil. Although both soils contained low levels of PHC, which did not exceed 5 g $kg^{-1}$, the contamination present in biocell soil was more bioavailable and thus degraded more. Some remediation was observed in tank 53 soil when examining the fraction data from an independent laboratory; remediation of F3 and F4 fractions was also slightly more pronounced in biocell soil, although differences were not significant.

The difference in remediation between the tank 53 and biocell soils could be the result of differences in soil structure or composition of the microbial community (Wang et al., 2006). In contrast to the biocell soil, tank 53 soil may have been lacking the favorable conditions required for microbial communities to thrive and promote PHC remediation. The tank 53 soil may not have a comparable suite of microbes for efficient degradation of PHC in a 60 day period. Interestingly, the presence of salt did not impede either growth or remediation. These results solidify the need for a greenhouse trial prior to attempting phytoremediation at a large scale, especially on sites where low levels of recalcitrant compounds are present.

In general, when analyzing PHC extracts gravimetrically, remediation values may be underestimated. Organic compounds of non-petrogenic origin, such as biogenic compounds from plants, may be co-extracted with petroleum products and thus increase detectable levels of hydrocarbons in soil samples. The in-house gravimetric analysis does not employ any clean up procedures such as a silica gel clean up, that remove compounds of plant origin. This was evident when comparing values obtained by the gravimetric analyses to values obtained from the independent laboratory. Further, given the low initial PHC levels, and large variability in the dataset, the results of gravimetric analysis to differentiate between treatments is difficult. Thus, a meaningful way to examine the data is to compare remediation with and without plants. The overall remediation as measured gravimetrically, irrespective of plant species or PGPR treatment was 1.5% for tank 53 soil and 7% for biocell soil.

### 5.5.5 Remediation with and without PGPR

The PGPR isolated showed improvements in plant growth in the greenhouse, however no changes in remediation were observed. This is likely the result of 1) size of the trial vessels 2) optimal growth conditions in the greenhouse; both of which did not allow for full differentiation between treatments. As shown here, with the biocell soil, PGPR-plant interactions were exaggerated where growth conditions were adverse (such as higher salt concentration in biocell soil). In the field, the protective effect of PGPR would likely result in an increase in plant growth that is needed to see increased remediation. Because there are numerous biotic and abiotic stressors in the field that are not encountered in greenhouse experiments, the PGPR effect is likely to be more pronounced than in the greenhouse. In the greenhouse, although after 60 days PGPR treated plants may have still been larger, their growth was limited by pot size and roots have reached the bottoms of the soil containers. As PGPR treated plants may have filled the existing space much faster, leaving no room to growth, the untreated plants could increase in size at a higher relative rate than PGPR treated plants during the latter part of the trial, as they still had room to grow.

### 5.5.6 General conclusions

Increased plant growth was observed with all three plant species when PGPR were used, especially when combinations of two or three of the NWT PGPR were used. Plant growth resulted in ~ 15% phytoremediation in the 60 day experiments, a level of remediation anticipated for a 60 day greenhouse trial. Based on this project good plant growth in the field was expected. Non-native species, such as tall fescue may be used to evaluate efficacy of phytoremediation, however if they are not native to the site environment they should be avoided. The native species used with native bacteria can be used together to mitigate the stress of growing on PHC and salt contaminated soils and likely their performance would be enhanced by PGPR.

## 5.6 Acknowledgements

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I would like to particularly acknowledge Dr. Bernie Glick for professional expertise provided for this chapter.

# Chapter 6

# Gene expression of *Secale cereale* (fall rye) grown in petroleum hydrocarbon (PHC) impacted soil with and without plant growth promoting rhizobacteria (PGPR).

## 6.1 Overview

Phytoremediation employs plants to sequester, degrade and transform contaminants. This remediation technology depends on sufficient plant growth, often not achievable with high contaminant concentrations. One way to improve plant growth on impacted soils is by using plant growth promoting rhizobacteria (PGPR). PGPR are naturally occurring soil microbes that stimulate plant growth through variety of means. We examined what genetic changes occurred in a grass species *Secale cereale* treated with PGPR, *Pseudomonas putida* PGPR (UW4), grown in petroleum hydrocarbon (PHC) impacted soil. UW4 promoted plant growth on the PHC impacted soil. Using differential display polymerase chain reaction (ddPCR), six genes were identified based on their altered expression as an effect of PHC exposure and plant PGPR treatment. The changes in levels of expression of selected genes were measured using quantitative PCR (qPCR).

## 6.2 Introduction

Global industrialization has led to enormous strain on our ecosystems as both organic and inorganic contaminants continue to be released into the environment, including soils. As a result there is now a large impetus towards environmentally conscious and cost-effective remediation practices, to aid in the restoration of contaminated terrestrial sites. One technology that has emerged is phytoremediation; the use of plants to sequester, degrade or transform contaminants (Alkorta and Garbisu, 2001). This approach has been widely heralded as an economical and ecologically responsible technique, and has been used to remediate multiple contaminants including metals and organics (Salt et al., 1998; Alkorta and Garbisu, 2001; Banks et al., 2003b). Phytoremediation has been used successfully for remediation of organic contaminants such as petroleum hydrocarbons (PHC) and polycyclic aromatic hydrocarbons (PAH) (Huang et al., 2004b; Huang et al., 2005; Gurska et al., 2009; Heinonsalo et al., 2000; Robinson et al., 2003).

In phytoremediation of PHC, the predominant theories are that organic contaminants are degraded indirectly, by microorganisms supported by root exudates or directly following plant uptake, mainly through roots or to a lesser capacity through leaves (Cunningham and Ow, 1996; Hutchinson et al., 2003). The majority of degradation occurs in the rhizosphere (the area surrounding the root zone), as plants can only take up organic compounds of a certain hydrophobicity and size; optimally with the octanol-water partition coefficient, log $K_{OW}$, between 1 and 3.5 (Burken, 2003). Once in the plant, contaminants go through a process of biotransformation that closely resembles the mammalian liver and is thus referred to as the 'green liver' model (Burken, 2003). Contaminants are transformed by introduction of a functional group that increases solubility or reactivity; conjugated with polar molecules such as sugars or glutathione; and finally compartmentalized to the vacuole or the apoplast (Burken, 2003).

Physiologically, plants stressed by PHC exposure have stunted growth, inhibited germination, decreases in photosynthetic pigments, diminished nutrient assimilation, and poor root and aerial organ growth (Gurska et al., 2009; Peña-Castro et al., 2006). Unhealthy plants may have limited ability to degrade and sequester the toxicants within plant tissues and lower release of nutrients into the rhizosphere, which is needed to

sustain the PHC-degrading microorganisms. Thus, an important and little investigated manner of increasing the effectiveness of phytoremediation is to alleviate stress to the plant.

One way to increase plant growth is by inoculating plants with plant growth-promoting rhizobacteria (PGPR). PGPR occur naturally in soils; living near the plant in a free-living association encouraged by the rich source of nutrients in the form of exudates such as amino acids, monosaccharides and organic acids (Lynch, 1990). In return, PGPR offer direct and indirect benefits to the plant, such as providing necessary nutrients or displacing pathogens respectively (Penrose and Glick, 2003). It has been found that PGPR can relieve several environmental stresses, including those brought on by organic pollutants. They can promote growth of plants exposed to organics contaminants, which is crucial for successful phytoremediation (Huang et al., 2004a, 2004b).

In this study, a strain of *Pseudomonas putida* (UW4) was used as an inoculant applied to seed coats prior to planting. UW4 is a producer of a phytohormone, indoleacetic acid (IAA), auxin which acts to enhance various stages of plant growth (Glick et al., 1998). Uptake of IAA by the plant allows it to stimulate plant cell proliferation and elongation. IAA also upregulates 1-aminocycloproane-1-carboxylic acid (ACC) synthase, an enzyme responsible for the conversion of S-adenosylmethionine (SAM) to ACC. ACC is the immediate precursor for the plant stress hormone ethylene (Glick et al., 2007). The resulting surplus in ACC is exuded for bacterial use. Bacterial strains that possess ACC deaminase can use ACC as a source of nitrogen, effectively lowering the outside concentration of ACC and stimulating further exudation; the end result is ultimately lowering ethylene levels and thus reducing plant stress effects (Glick et al., 2007).

In several studies it has been shown that genetic changes in plants caused by PGPR with a functional ACC deaminase gene are associated with plant growth, cell division, proliferation, down-regulation of genes involved in an ethylene-induced stress response when plants were inoculated with a PGPR (Timmusk and Wagner, 1999; Hontzeas et al., 2004). Further, another study examined changes in gene expression in Bermuda grass, in PHC contaminated soils and found most changes to be related to mechanical or anoxic stress due to PHC (Peña-Castro et al., 2006). No study, however, has attempted to

elucidate the genetic changes in plant gene expression evoked by PGPR and PHC exposure. Understanding how plants adapt to organic toxicants on a molecular level may result in a more cohesive view of the overall changes in response to PHC. Gene expression studies on plants grown with PGPR on PHC-contaminated soils may lead to insights into PHC toxicity, the ability of plants to acclimate, how PGPR increase plant growth under PHC stress, and to the mechanisms of PHC degradation.

Thus, gene expression changes in a cereal, fall rye (*Secale cereale*), after 10 days exposure to PHC, with and without the addition of UW4 were examined. To assess these changes differential display polymerase chain reaction (ddPCR) was employed. This technique enables the evaluation of both novel and identified genes, thus presenting the opportunity to discover novel genes induced in response to PHC (Diener et al., 2004; Akhtar et al., 2005). Changes in gene expression were monitored in *S. cereale* leaf and root tissues. Multiple, differentially expressed genes were found, with six genes shedding light on PGPR improving plant growth in PHC exposed plants. The magnitude of changes in expression was confirmed with quantitative PCR (qPCR). Changes in shoots and roots differed in magnitude, and in some cases trends were opposite between these two types of tissue. The implications of gene changes evoked by PHC and PGPR treatment are discussed.

## 6.3 Materials and Methods

### 6.3.1 Plant Growth

Fall rye (*S. cereale*, Ontario Seed Ltd., Waterloo, ON, Canada) seed were inoculated with *P. putida* UW4 (Glick, 1995; Shah et al., 1998) by soaking the seeds in bacterial suspension for 15 min and air drying for >1 hour prior to planting. Water was used for seed coating of control plants. Bacterial inoculum was prepared by growing UW4 (500 μL frozen stock in 100 mL of TSB (50 $g \cdot L^{-1}$, Fisher Scientific, Ottawa, Ontario, Canada)) for 24 h at RT with agitation. The bacterial culture was centrifuged (5000 rpm, 20 min at 4 $^{o}$C) and re-suspended in autoclaved deionized water to obtain a final OD of 1.5-2. The seeds were sown in PHC-contaminated soil and control soil in plastic four cell plant plug trays (Jack van Klaveren Co., St. Catherines, ON, Canada). Contaminated soil characteristics were as previously described in Gurska et al. (2009) (Chapter 2). Control soil was obtained from uncontaminated land approximately 20 meters from the site. Plants were grown in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA) with lighting conditions with approximate ratio of PAR:UVA:UVB (~100:10:1), typically found in the mid-latitudes in the USA (Gerhardt et al., 2008) and a 16 h light/8 h dark photoperiod . Plants were harvested 10 days after seeding. Shoots and roots were rinsed in deionized water, separated and immediately flash frozen in liquid nitrogen. Frozen tissues were stored at -80 $^{o}$C until further use.

### 6.3.2 Pulse Amplitude Modulated (PAM) fluorometry

Alterations in the health of the photosynthetic apparatus of plants were measured by chlorophyll-*a* fluorescence induction using Pulse Amplitude Modulated (PAM) fluorometer (PAM-101, Walz, Effeltrich, Germany). To carry out fluorescence measurements using the PAM, samples were dark adapted for approximately 30 minutes. Plants were exposed to weak, pulsed red light (< 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$) to acquire the $F_o$ value (minimal fluorescence). This light was not strong enough to promote photosynthesis but stimulated background fluorescence only. Saturating light (~3000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 600 ms) from a halogen lamp was applied to measure the $F_m$. The maximal activity of PSII was calculated as $F_v/F_m = (F_m-F_o)/F_m$ (Maxwell and Johnson, 2000). Plants were then exposed

to actinic light (fluence rate of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and to saturating light pulses to obtain steady state fluorescence $F_t$ and maximal fluorescence intensity ($F_m$ and $F_m$') respectively. Values that were obtained from PAM fluorescence with the actinic light were Yield, qP and qN. Yield is the effective quantum yield of PSII under steady state conditions. qP and qN are coefficients of photochemical electron transfer and non-photochemical (heat) fluorescence quenching respectively (Maxwell and Johnson, 2000).

### 6.3.3 RNA Extractions

Two RNA extraction techniques were evaluated. In the first, RNA isolation was carried out according to protocol previously used for *Lemna gibba* (Akhtar et al., 2005) and *Daphnia magna* (Diener et al., 2004) using TRI reagent (Sigma-Aldrich, Oakville, ON, Canada). The second method was a CsCl gradient extraction (Davis et al., 1986). The TRI reagent method proved to be inferior to CsCl with respect to RNA quality from plant roots and thus the CsCl gradient extraction employed as the method of choice. All buffers and dilutions were made with DEPC-treated water (1% DEPC, mixed for ≥ 1 hour at RT then autoclaved) or commercially purchased RNAse free water (Wisent Inc., St. Bruno, Quebec, Canada). All glassware was made RNAse free by baking at 200 $^oC$, ≥ 4 hours. In the TRI Reagent method (Sigma Chemical Company, St. Louis, MO, USA), plants were grown for 10 days, harvested directly into TRI reagent then homogenized for 30 s using a motorized glass mortar and pestle. The homogenate was transferred to a 1.5 mL RNAse-free microcentrifuge tube (Diamed Lab Supplies Inc., Mississauga, ON, Canada), and centrifuged at 12,000 rpm for 15 min at 4 $^oC$. The upper aqueous phase supernatant was transferred to a new 1.5 mL microcentrifuge tube with 500 μL of 100% isopropanol (Sigma-Aldrich, Oakville, ON, Canada ) and incubated at room temperature for 10 min then centrifuged at 12,000 rpm for 10 min at 4 $^oC$. Supernatant was removed by aspiration, the RNA pellet was washed with 75% ethanol (Sigma-Aldrich, Oakville, ON, Canada) and subsequently centrifuged at 7,500 for 5 min at 4 $^oC$. Ethanol was aspirated off and the pellet was air-dried and reconstituted in DEPC water.

For RNA extractions with CsCl, plant tissue was flash frozen in liquid nitrogen and ground in a mortar and ground to a fine powder with a pestle. The resulting powder was added to 10 mL of GIT buffer, containing 4M guanidine isothiocyanate (Sigma-Aldrich,

Oakville, ON, Canada), 3M NaOAc (Sigma-Aldrich, Oakville, ON, Canada) pH 6.0 and 8.35 mL β-mercaptoethanol (100%, Sigma-Aldrich, Oakville, ON, Canada) per liter of buffer. This suspension was filtered through two layers of MiraCloth (EMD Chemicals (Calbiochem), Gibbstown, NJ, USA) then centrifuged at 15,000 rpm in a Sorvall RC 5B Plus centrifuge with a SS034 rotor, at 4 $^{o}$C. The supernatant was layered onto 3.3 mL of 5.7 M CsCl (Sigma-Aldrich, Oakville, ON, Canada) in a 30 mL ultracentrifuge tube (Beckman Coulter Inc., Mississauga, ON, Canada). Samples were centrifuged at 30,000 rpm at 10 $^{o}$C for 23 hours in a Beckman-Coulter Optima L-90K Ultracentrifuge using a SW-41Ti rotor. The supernatant was removed and pellet was washed with 70% ethanol then resuspended in 500 μL suspension buffer (1:5 3M NaOAc pH 5.5 to water). 1 mL ice cold 100% ethanol was added to the suspended RNA and followed by greater than one hour of incubation at -80 $^{o}$C. Samples were centrifuged at 14,000 rpm at 4 $^{o}$C for 1 hour in a microcentrifuge. Supernatant was discarded and pellet was washed with 1 mL of 70% ethanol then centrifuged again at 14,000 rpm at 4 $^{o}$C for 30 min. The supernatant was removed and pellets were air dried for approximately 15 min by inverting microcentrifuge tubes on a sterile, RNase-free surface. The pellets were then resuspended in 50 to 200 μL RNAse-free water. All RNA samples were stored at -80 $^{o}$C until needed. Concentration of RNA was measured spectrophotometrically, using a NanoDrop instrument (ND-1000, NanoDrop by Thermo Scientific, Wilmington, DE, USA). All RNA used for subsequent manipulations had an $OD_{260}/OD_{280}$ ratio between 1.90 and 2.00 and an $OD_{260}/OD_{230}$ ratio >2.00.

RNA integrity was assessed using denaturing formaldehyde gel electrophoresis. RNA samples (2 μg) in loading buffer (50% formamide [v/v], 17% formaldehyde [v/v], 1X MOPS, 10% [v/v] glycerol, 0.05% bromophenol blue [w/v]) were heated for 10 min at 65 $^{o}$C, cooled on ice for 5 min, then loaded onto a gel containing 1.1% agarose and 18% formaldehyde and 0.7 $mg \cdot mL^{-1}$ ethidium bromide in 1X MOPS. The electrophoresis was carried out for 2 hours at 70 V in 1X MOPS. 1X MOPS contained 0.02 morpholinepropanesulfonic acid (MOPS), 5mM sodium acetate, 1mM EDTA, and pH 7.0. RNA was visualized under UV light and visualized RNA bands were examined for appropriate size and signs of smearing which could indicate degradation.

### 6.3.4 cDNA synthesis and PCR amplification

Following RNA isolation, the synthesis of a single-strand complementary DNA copy (cDNA) of the RNA was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) oligo$(dT)_{18}$ primer using First Strand cDNA Synthesis Kit (Fermentas International Inc., Burlington, ON, Canada) according to manufacturers instructions. Following the RT reaction, the cDNA was diluted 1:10, and a 5 μL aliquot of the diluted RT reaction was amplified by using two arbitrary primers (Table 6.1) and Ready To Go PCR beads (GE Healthcare, Piscataway, NJ, USA). The total reaction volume was 25 μL, 200 μM dNTP, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 units of pure Taq DNA polymerase. The PCR conditions were as follows: 1 cycle of low stringency, 1 min 94 $^{o}$C, 5 min 36 $^{o}$C, 5 72 $^{o}$C; 39 cycles of high stringency, 1 min 94$^{o}$C, 2 min 50 $^{o}$C and 2 min 72 $^{o}$C. All PCR reactions were performed in a PTC-200 thermocycler (MJ Research Products, Waltham, MA, USA).

### 6.3.5 Differential display PCR electrophoresis

The detection and quantification of amplified products was performed by incorporation of a fluorescent Cy 5.0 adapter primer (Sigma-Aldrich, Oakville, ON, Canada) into the PCR products, followed by their separation on a 6% acrylamide (19:1 acrylamide:*bis*-acrylamide; Bioshop, Burlington, ON, Canada), 7 M urea sequencing gel in 1X Tris-Borate-EDTA buffer. The Cy 5.0 adapter primer contains a region that is complementary to a conserved region in the arbitrary primers used to generate the PCR products (Table 6.1). The labeling reaction was set up by adding 1 μL of Cy 5.0 primer (100 μM) to the PCR reaction products and incubating at 95 $^{o}$C for 2 min. Reaction tubes were cooled on ice and 1 μL of dNTPs (25 μM), 2 μL of 10X Klenow Reaction Buffer and 1 μL of Klenow Polymerase (exo$^{-}$, 5U $\mu \cdot L^{-1}$, MBI Fermentas, Burlington, ON) were added. Tubes were gently mixed and incubated at room temperature for 30 min followed by incubation at 37 $^{o}$C for 2 hours. The fluorescently tagged PCR products (7 μL) were mixed with loading dye (80% formamide [v/v], 50 mM Tris-HCl [pH 6.8], 1 mM EDTA, 0.25% bromocresol green [v/v]) and heated to 80 $^{o}$C for 2 min. The mixture was cooled on ice then loaded onto the sequencing gel. Electrophoresis was performed at 1600 V, 55 W for 2 hours. Gels were visualized using a Typhoon 9400 imager (Amersham Biosciences,

Buckinghamshire, UK) in the red fluorescence mode (633 nm). Image manipulations were performed using ImageQuant software v 5.2. Bands appearing to be differentially expressed in the ddPCR gels, were cut from the acrylamide gel using a sterile scalpel.

DNA was extracted from the acrylamide gel using the Qiaex®II Gel Extraction Kit (Qiagen Inc., Mississauga, ON, Canada) according to manufacturers instructions. To further purify these fragments, cDNA was re-amplified using the same primers and conditions as previously stated and the PCR products along with GenerRuler 100bp DNA ladder Plus (MBI Fermentas, Burlington, ON, Canada) were electrophoresed on an agarose gel (gel: 1.2%, 1 XTBE, 0.7 $mg \cdot mL^{-1}$ ethidium bromide; loading dye: 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA supplied with GeneRuler ladder) for one hour at 100 V. DNA in the gels was visualized using a UV transilluminator and the predominant bands that corresponded in size to the original fragments were cut from the agarose gel with a sterile scalpel. Trimmed gel slices were transferred to a microcentrifuge tube and DNA was extracted using the Qiagen gel extraction kit for agarose, according to manufacturer's instructions (Qiagen Inc., Mississauga, ON, Canada). The purified DNA was dissolved in sterile water and the concentration was measured using the NanoDrop.

### 6.3.6 Cloning of cDNA fragments

The purified PCR products were ligated into the pGEM T-Easy (Promega, Fisher Scientific Ltd., Nepean, Ontario, Canada) vector and JM109 high efficiency competent cells (Promega, Fisher Scientific Ltd., Nepean, Ontario, Canada) were transformed with plasmids via heat shock. Manufacturers instructions were followed with these changes: LB medium was used instead of SOC, 1 μL of DNA was added. The transformed JM109 cells were grown for 18 h at 37 $^{o}C$ on AMP/IPTG/X-Gal LB plates (100 $\mu g \cdot mL^{-1}$, 0.5mM and 80 $\mu g \cdot mL^{-1}$ for AMP (MBI Fermentas, Burlington, ON, Canada), IPTG and X-Gal (Promega, Fisher Scientific Ltd., Nepean, Ontario, Canada) respectively). White, fragment containing, colonies were selected and cultured for 16-18 hours in LB broth containing ampicillin (100 $\mu g \cdot mL^{-1}$).

### 6.3.7 Plasmid purification and sequencing

Plasmids were purified by the alkaline lysis protocol originally described by Sambrook et al. (1989). The LB culture was collected twice by centrifugation at 13,000 rpm for 1 min at 4 $^{o}$C. The supernatant was discarded and pellet was resuspended in chilled Solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0) by pipetting up and down or vortexing when necessary. Solution II (0.2 M NaOH, 1% SDS [w/v]) was added to lyse the cells followed by gentle inversion and incubation on ice, which did not exceed 5 min. Lysate was neutralized with Solution III (3M potassium acetate, 5M glacial acetic acid) and allowed to sit on ice for 5 min. Samples were centrifuged at 13,000 rpm for 10 min at 4 $^{o}$C and the supernatant was removed to a new microcentrifuge tube. To remove RNA, RNase A (10 µL of 10 mg·mL$^{-1}$, Bioshop, Burlington, ON, Canada) was added and samples were incubated at 37 $^{o}$C for 25 min. Following digestion, 1:1 phenol:chloroform (vortexed prior to use) was added and samples were vortexed for 30 s, and centrifuged for 2 min at 13,000 rpm. The upper aqueous phase containing the plasmid DNA was transferred to a new microcentrifuge tube, and chilled isopropanol was added. Plasmid DNA was allowed to precipitate at room temperature for 10 min, followed by centrifugation (10 min, 4 $^{o}$C, 13,000 rpm). The supernatant was removed and pellet was washed with 70% ethanol then air-dried. The dry pellet was resuspended in sterile water. Purified plasmids were diluted and sequenced (The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON, Canada). Sequences were compared to those in Genbank using the NCBI network BLAST software (blastn algorithm). Multiple alignments were performed with the CLUSTALW2 program at the EMBL (http://www.ebi.ac.uk/Tools/msa/clustalw2). Sequences with coverage values over 50% were considered for further analysis.

### 6.3.8 Quantitative PCR

Primers were designed using Primer3 version 0.4.0 (Rozen and Skaletsky, 2000) using sequenced cDNA fragments for target genes and sequences available through Genbank for reference genes. To confirm absence of secondary structures that would inhibit primer binding MFOLD software was used (Zuker, 2003) with the theoretical melting temperature of primers. Two micrograms of RNA from the treatments above was reverse

transcribed with oligo(dT)$_{18}$ primer using First Strand cDNA Synthesis Kit (Fermentas International Inc., Burlington, ON, Canada) according to manufacturers instructions. A master mix comprising of SsoFast™EvaGreen® supermix, water and primers was made for each sample. For each reaction (10 μL total) the master mix was loaded into a Hard-Shell® thin-wall, 96 well skirted PCR plate (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and 1 μL of template was added. Each plate also consisted of a NTC (no template control) and "no RT" (no reverse transcriptase at the cDNA synthesis step) control. The plate was then placed in the thermal cycler programmed as follows: initial denaturation and activation of enzyme at 95 $^{o}$C for 30 s; denaturation at 94 $^{o}$C for 5 s, annealing/extension at 64 $^{o}$C for 5 s for 40 cycles. A melt curve was generated after each reaction by heating the products from 64 $^{o}$C to 95 $^{o}$C degrees in 0.5 $^{o}$C increments and taking a fluorescence reading after each temperature increase.

**Table 6.1 Primers used for ddPCR and qPCR amplification.**

Sequences of gene specific primers used to amplify segments from cloned cDNA isolated from ddPCR gels and arbitrary primers and sequencing primers. Primer abbreviations: β-tubulin, TUB; Cytochrome P450, P450; Poly(A)-binding protein, PABP; transcriptional activator containing forkhead-associated domain, FHA1; plasma membrane $H^+$ATPase, PMA; phytoene desaturase, PDS; actin, ACT; knotted-like homoeobox gene, KNOX; elongation factor 1-alpha, EF1A; glyceraldehyde 3-phosphate dehydrogenase, GAPDH. A3/A4 primers selected from Stratagene RAP-PCR kit (Stratagene, Corporation, La Jolla, CA, USA).

| Primer name | Sequence (5' → 3') | Tm ($^{O}$C) |
|---|---|---|
| A3 | AAT CTA GAG CTC CAG CAG | 54.5 |
| A4 | AAT CTA GAG CTC TCC TGG | 53.2 |
| Cy5 Fluorescent | AAT CTA GAG CTC | 26.7 |
| T7 | TAA TAC GAC TCA CTA TAG GG | 47.0 |
| SP6 | TAT TTA GGT GAC ACT ATA G | 42.0 |
| 5' TUB | GCA CCA AGG AGG TTG ATG AG | 64.6 |
| 3' TUB | AGT TGC CAA TGA AGG TGG AC | 63.9 |
| 5' P450 | GCA GCA TCA AAC CCA CAA TA | 63.4 |
| 3' P450 | CTC CTG GCC TCT CAG TTC AG | 64.1 |
| 5' PABP | GGA GCA ACA CCA GGT CTC AT | 64.1 |
| 3' PABP | CAA CCA CCT CCA GCT TTT GT | 64.0 |
| 5' FHA1 | TAT GGT GGG CAC AGA ATC AA | 63.7 |
| 3' FHA1 | CGCAACAGATGACACGACTT | 64.0 |
| 5' PMA | ACC AAG ATT GAT GCC TCC TG | 64.0 |
| 3' PMA | TAA GGT GCT CAG GGA TGG TC | 64.0 |
| 5' PDS | CTG ATG AAA TCG CTG CTG AC | 63.6 |
| 3' PDS | TTC CGG ACG GTC TTG TAA AC | 63.7 |
| 5' KNOX | GGA GAC GGG GTT ACA ACT GA | 63.8 |
| 3' KNOX | CAG TTC CTT TTG CGT TGG TT | 63.8 |
| 5' ACT | CCC ATG CTA TCC TTC GTC TC | 63.5 |
| 3' ACT | ACC ACG CTC AGT CAG GAT CT | 63.9 |
| 5' EF1A | TCA AGT TTG CTG AGC TGG TG | 64.2 |
| 3' EF1A | GAT ACC AGC GTC ACC GTT CT | 64.0 |
| 5' GAPDH | TTC AAC ATG ATT CCA AGC AGC A | 67.6 |
| 3' GAPDH | CGT AAC CCA AAA TGC CCT TG | 66.0 |

All initial manipulations were performed using the Bio-Rad CFX96$^{TM}$ Real-Time PCR System (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and the Ct values were subsequently exported into qBase$^{PLUS}$ (Biogazelle, Ghent, Belgium). Single PCR product was confirmed by running the products out on a 3% agarose gel (1X TBA, 0.7 mg·mL$^{-1}$ ethidium bromide).

### 6.3.9 Reference gene validation for qPCR

The reference genes assessed were: β-tubulin (Genbank accession# FJ032190.1), previously shown to be a good reference gene in cereals such as barley, wheat and oats (Jarosova and Kundu, 2010); actin (Genbank accession# FJ032189.1), which has often been used as a reference gene with mixed results (Migocka and Papierniak, 2010); elongation factor 1-alpha (Genbank accession# FJ032192.1) has previously been shown to be stably expressed in *Triticum aestivum*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (primer for *T. aestivum*, (Crismani et al., 2006)). Primer sets for β-tubulin (*TUB*), β-actin (*ACT*) and elongation factor 1-alpha (*EF1A*) were designed according to published sequences for *S. cereale* while *GAPDH* primer set was previously used for *T. aestivum*, (Crismani et al., 2006). Two stable control genes were chosen using the geNorm algorithm based on their ranking according to their expression stability (Vandesompele et al., 2002).

### 6.3.10 qPCR quantification methodology

Relative transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method (where Ct is the threshold cycle), which compares amplification measurements for gene of interest to that of a reference gene (Livak and Schmittgen, 2001). Additionally, the calculations were corrected for gene-specific amplification efficiency, instead of the assumed efficiency of two copies per cycle of amplification (the 2 in $2^{-\Delta\Delta Ct}$ method, (Pfaffl, 2006)). Incorporating this correction the relative expression ratio of a target gene was calculated as:

$$\text{Ratio} = (E_{TARGET})^{\Delta Ct(TARGET)/} (E_{REF})^{\Delta Ct(REF)}$$

where E is the efficiency of target or reference gene, ΔCt is the difference between the Ct of a control sample and a treated sample ($\Delta Ct_{TARGET} = Ct_{control} - Ct_{treatment}$ and $\Delta Ct_{REF} = Ct_{control} - Ct_{treatment}$).

### 6.3.11 Statistics

To determine differences in gene expression between treatments, data were analyzed using one-way ANOVA and the t-test where appropriate. All statistical analyses were performed using Prism 4 for Macintosh (GraphPad Software, Inc., La Jolla, CA, USA) for the t-tests and SPSS for one way-ANOVA (IBM Corporation, Somers, NY, USA).

## 6.4 Results

### 6.4.1 Plant growth and photosynthetic changes in response to PHC and PGPR

Plant growth was inhibited due to PHC (Figure 6.1). This inhibition was more apparent in the roots than the shoots. Shoots of PHC-grown plants were short and wilted much quicker than plants grown in control soil. When UW4 was used to inoculate seeds of plants grown in PHC impacted soil, some of the growth was restored; specifically root systems were larger (Figure 6.1C). Photosynthetic parameters obtained with PAM fluorometry (Figure 6.1D) revealed that the qN, representing the amount of energy diverted away from photosynthesis to heat dissipation, was raised with PHC exposure relative to control plants. When PGPR were added, qN was lowered and reflected the levels observed in control plants. qP, $F_v/F_m$ and Yield parameters did not change between treatments and were at ~0.95, 0.79 and 0.70 respectively.

### 6.4.2 Qualitative differences between differential display profiles

Differential display profiles were generated using cDNA from both shoots and roots (Figure 6.2) of plants grown in control or PHC-contaminated soils, with or without addition of UW4 as a seed inoculant. Typical ddPCR profiles from shoots and roots did not show acute changes between treatments, but rather subtle changes in band intensity were observed. In the ddPCR profile of both shoots and roots, several bands increased in intensity due to plant growth in PHC-contaminated soil. This was observed in plants with and without UW4 inoculation (Figure 6.2).

### 6.4.3 Identification and sequence analysis of differentially expressed cDNAs

Isolated cDNA were cloned and sequenced. Sequences were compared by BLAST software (blastn algorithm). Table 6.2 lists the putative identification of all isolated cDNA fragment from shoots, while Table 6.3 lists fragments isolated from roots of *S. cereale.* Selected cDNAs will be mentioned below.

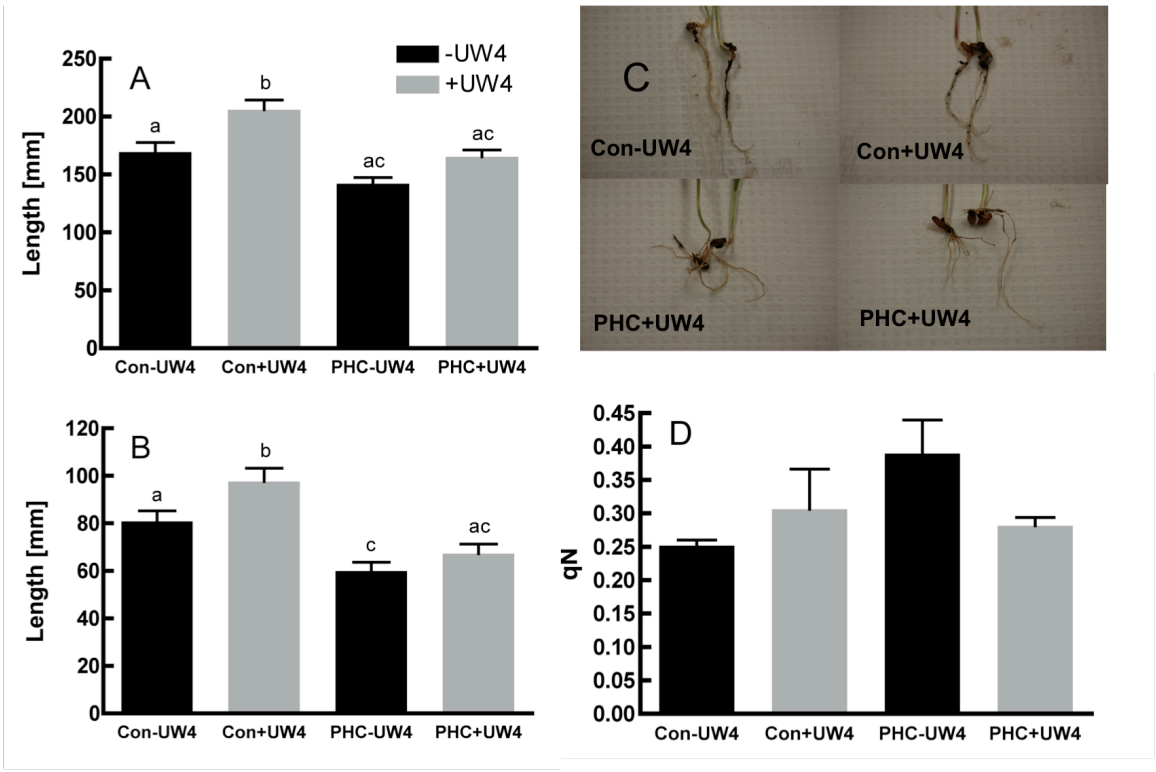



**Figure 6.1 Plant growth and qN PAM parameter in *Secale cereale* plants.**

Shoot and root length was measured on control and PHC contaminated soil. A. *S. cereale* shoot length after 10 days of growth, B. *S. cereale* root length at 10 days after planting, C. Root appearance in control and PHC soil. D. PAM parameter, qN in control and PHC-contaminated soil plants. qP, $F_v/F_m$ and Yield parameters did not change between treatments and were at ~0.95, 0.79 and 0.70 respectively. Results represent three independent trials with n=3, ± SD.

**Figure 6.2 Differential display PCR profiles.**

A. Differential display PCR acrylamide gel with tissue from shoots. B. Differential display PCR acrylamide gel with tissue from roots. C. Close up of a band at ~630-650 bp which was excised from the gel. In all cases from left to right the samples in triplicate were control – UW4, control + UW4, PHC – UW4, PHC + UW4. Three independent replicates were performed for each treatment. Arrows indicate bands that appeared differentially expressed and were sequenced.

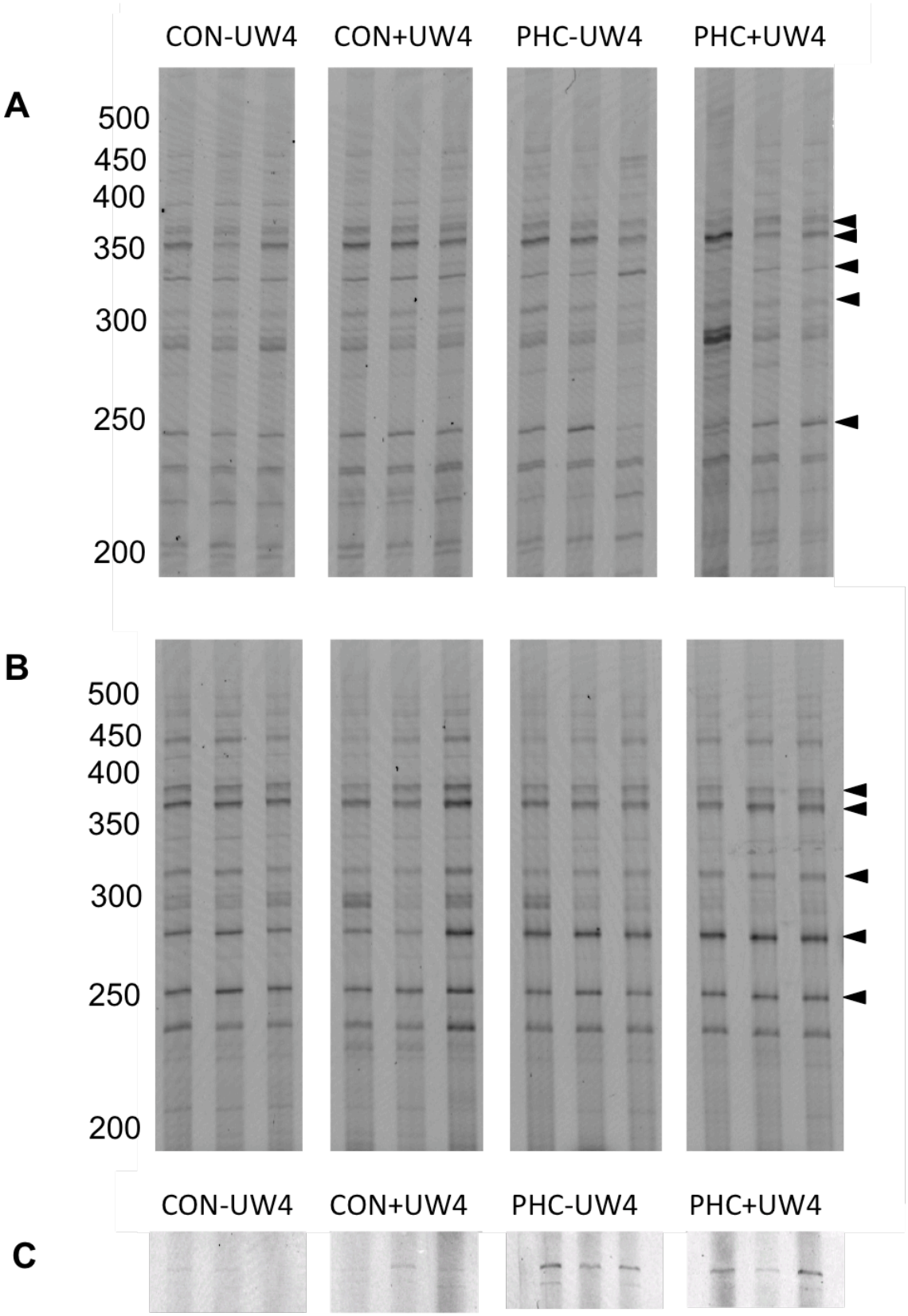

CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
A
500
450
400
350
300
250
200
B
500
450
400
350
300
250
200
CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
C

**Table 6.2 Differentially expressed cDNA in shoots.**

Differentially expressed cDNA fragments identified from shoots of *S. cereale* plants grown in PHC-contaminated soils. Bands isolated from shoots were named S[fragment size][clone #].

| Band | Size (bp) | E-value[a] | Sequence ID[b] | Accession no. |
|---|---|---|---|---|
| **S250#1** | 253 | 2E-78 | *As* Retrotransposon BARBARA truncated | FM242577.1 |
| | | 2E-15 | *Ta* Retrotransposon BARBARA | FN564426.1 |
| **S300#1** | 305 | 1E-08 | No significant match | |
| **S340#1** | 335 | | No significant match | |
| **S365#1** | 364 | 1E-153 | *Ta* phytoene desaturase | FM998042.1 |
| | | 3E-149 | *Ta* phytoene desaturase | FJ517553.1 |
| **S365#2** | 364 | 7E-114 | *Os* phytoene desaturase | AF049356.1 |
| | | 6E-102 | *Zm* phytoene desaturase | L39266.1 |
| **S365#3** | 364 | 9E-155 | *Ta* phytoene desaturase | FM998042.1 |
| **S365#4** | 364 | 2E-153 | *Ta* phytoene desaturase | FM998042.1 |
| **S380#1** | 379 | 4E-173 | *Ta* cDNA clone | AK3344867.1 |
| | | 9E-42 | *Sb* hypothetical protein | XM_002453267.1 |
| **S380#2** | 387 | | No significant match found | |
| **S630#1** | 633 | 1E-36 | *Sc* clone 24_I-C9 microsatellite sequence | EU338559.1 |
| | | | *Ta* Mobile element: retrotransposon | FN564430.1 |
| | | 1E-150 | *As* Inga_3_fragmented | FM242577.1 |
| | | 3E-11 | | |
| **S630#2** | 635 | 6E-122 | *Ta* poly(A)-binding protein (wheatpab)mRNA, complete cds | U81318.1 |
| | | 0 | *Os* similar to Poly(A) binding protein | NM_001068050.1 |
| | | | *Os* polyadenylate-binding protein 2 | AK104792.1 |
| | | 0 | | |
| **S630#3** | 639 | 3E-107 | *Ta* poly(A)-binding protein | E81318.1 |
| **S630#4** | 663 | 1E-36 | *Ta* cDNA clone | AK333885.1 |
| | | 6E-08 | *Os* trehalose-6-phosphate phosphase | AB277360.1 |
| | | | *Zm* trehalose-phosphate phosphatase | |
| | | 1E-04 | | NM_001158750.1 |
| **S700#1** | 697 | 1E-49 | *Ta* cDNA clone | AK333885.1 |
| | | | | NM_001158750.1 |
| | | | | AB277360.1 |

[a] E-values of $10^{-6}$ and lower were considered, values derived from blastn BLAST program (Altschul et al., 1997).

[b] The following abbreviations for plant species were used: *As*, *Aegilops speltoides; Ta, Triticum aestivum*; *Os, Oryza sativa*; *Zm, Zea mays*; *Sb, Sorghum bicolor, Sc, Secale cereale.*

**Table 6.3 Differentially expressed cDNA in roots.**

Differentially expressed cDNA fragments identified from roots of *S. cereale* plants grown in PHC-contaminated soils. Bands isolated from roots were named as R[fragment size][clone#].

| Band | Size (bp) | E-value[a] | Sequence ID[b] | Accession no. |
|---|---|---|---|---|
| **R250#1** | 261 | 2E-73 | *Sb* hypothetical protein | XM_002467128.1 |
| **R250#2** | 253 | | No significant match | |
| **R280#1** | 276 | 4E-44 | *Os* mRNA for KNOX family class 2 homeodomain | AB061817.1 |
| | | 2E-36 | *Zm* homeobox protein knotted-1-like 3 | EU959496.1 |
| **R280#2** | 280 | 9E-53 | *Sb* hypothetical protein | XM_002461074.1 |
| | | 9E-53 | *Zm* Cytochrome P450 CYP727A4 | NM_001159621.1 |
| | | 7E-66 | *Os* Hypothetical CYP P450 family protein | NM_001066984.1 |
| **R300#1** | 350 | | No significant match | |
| **R300#2** | 312 | 8E-125 | *Ta* Plasma membrane $H^+$ATPase | AY543630.1 |
| | | 5E-117 | *Hv* Plasma membrane p-p-type proton pump ATPase | AY136627.1 |
| **R350#1** | 361 | 1E-97 | *Zm* Transcriptional activator FHA1 | EU971449.1 |
| **R350#2** | 361 | 2E-99 | *Zm* Transcriptional activator FHA1 | EU971449.1 |
| **R360** | 379 | 2E-171 | *Os* conserved hypothetical protein | NM_001052454.1 |
| | | 2E-31 | *Sb* hypothetical protein | XM+002453267.1 |
| **R360** | 384 | 9E-42 | *Sb* hypothetical protein | XM_002453267 |
| | | | *Zm* hypothetical protein | EU973587.1 |

[a] E-values of $10^{-5}$ and lower were considered significant (Altschul et al., 1997)

[b] The following abbreviations for plant species were used: *Sb, Sorghum bicolor; Os, Oryza sativa*; *Zm, Zea mays*; *Ta, Triticum aestivum*; *Hv, Hordeum vulgarae*.

Bands were identified based on their size, and further differentiated by R or S to indicate roots and shoots respectively.

#### 6.4.3.1 S630 - PABP and Retrotransposon.

The strong band at approximately 630 bp only appeared in samples where plants were grown in PHC contaminated soil and could represent a particular acclimation to the contaminants (it was located out of the range of the ladder (>500bp)). It was excised from the gel as containing potentially upregulated mRNA (Figure 6.2C). The cDNA, designated S630, was isolated from plants grown in PHC-contaminated soils with PGPR treatment and was 635 bp. The sequence obtained has a very high similarity to a number of plant poly(A)-binding proteins (*PABP*) (Table 6.2). When a band in the same position was cut in the adjacent lane, it yielded a 633 bp fragment. It was homologous to a retrotransposon sequence (Table 6.2).

The shoot cDNA was isolated from band S630 showed significant sequence similarity to poly(A)-binding proteins based on the deduced amino acid sequence from other monocots such as rice (*Oryza sativa*) and common wheat (*Triticum aestivum*). In particular the C-terminus amino acid sequenced matched that of helix 1 – one of the 5 helices that this protein contains at its C-terminus (Figure 6.3A). The C-terminus is critical for proper nuclear shuttling and for PABP dimerization (Kozlov et al., 2001). The *S. cereale* sequence showed at 77% of the amino acid sequence to *T. aestivum PABP* (Genbank: U81318.1) and 80% similarity to *O. sativa PABP* (NM_001068050.1). There was also overlap to the fourth RNA binding domain (Figure 6.3A) of *PABP* (Le and Gallie, 2000).

#### 6.4.3.2 S365 - Phytoene desaturase.

The cDNA isolated from shoots was 365 bp and it came from a band that appeared to be lighter in the control and darker in all the other treatments. Four different clones resulted in the same fragment size and were all identified as similar to phytoene desaturase (*PDS*, Table 6.2). When excised, cloned and sequenced it was determined to be 364 bp.

cDNA from band S365 was identified as phytoene desaturease (*PDS*); the cDNA fragment showed high homology of 99% to *T. aestivum PDS* (FM998042) and 97%

similarity to *O. sativa* hypothetical *PDS* (EEC74643.1, Figure 6.3D). The isolated cDNA fragment was located around a region of high homology between phylogenetically distant organisms located at the C-terminus (Li et al., 1996).

#### 6.4.3.3 R280 Cytochrome P450 and KNOX.

A smaller band at approximately 280 bp was also chosen because it appeared to be upregulated in plants exposed to PHC (Table 6.3). This clone was also identified as cytochrome P450 and knotted-like homoeobox gene (*KNOX*). cDNA from band R280 was identified as a cytochrome P450. The isolated cDNA was most similar to a *Z. mays* cytochrome P450 CYP727A4 (NM_001159621, 90% similarity) and a *O. sativa* cytochrome P450 (NP_001060449.1, 91% similarity). Notably, the conserved Glu-X-X-Arg motif, located in the K helix was located within the found sequence (Figure 6.3E) (Werck-Reichhart and Feyereisen, 2000). Another cDNA product was isolated from band R280, which had 89% similarity to *O. sativa* (KNOTTED1-like homeobox) KNOX familiy class 2 homeodomain protein (AB061817.1) and 91% similarity to *Z. mays* KNOX family class 2 homeodomain protein (NM_001112382). Specifically, helix 2 and helix 3 of the homeodomain were part of the sequence that aligned (Figure 6.3F). Residues that were conserved in class 2 homeodomain proteins were also present in the *S. cereale* fragment (Janssen et al., 1998).

#### 6.4.3.4 R300 Plasma membrane $H^+$ATPase.

In roots, a band at 300 bp appeared to be upregulated due to growth on PHC impacted soil (Table 6.3). cDNA from R300 was identified as plasma membrane $H^+$ATPase (*PMA)*. This cDNA had 91% homology to a *Hordeum vulgare* plasma membrane P-type proton pump ATPase (AY136627.1) and 97% homology to *T. aestivum* (p-type) plasma membrane $H^+$ATPase (AY543630.1). It corresponded to the N-terminus part of the protein and part of the sequence fell on an α-helix 2 (Figure 6.3C).

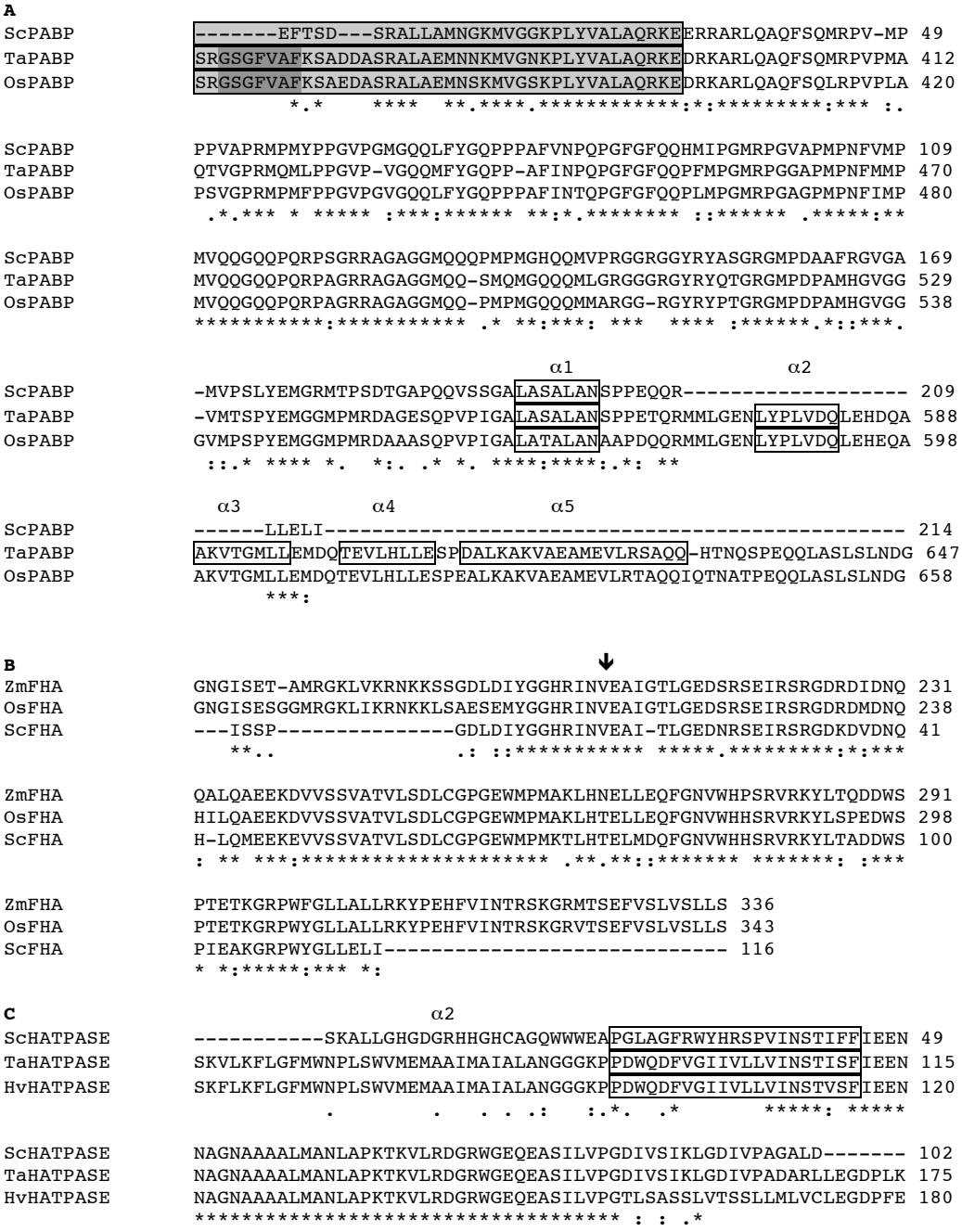
#### 6.4.3.5 R350 FHA1.

A band at 350 bp was visible at uniform intensity in roots and was thus chosen as an experimental control for the differential display method. cDNA was isolated twice from two different replicate lanes (Figure 6.2) and both samples yielded transcriptional

activator *FHA1* (transcriptional activator containing forkhead-associated domain) and exhibited 82% sequence similarity to *Zea mays* transcriptional activator *FHA1* (EU971449) and 30% similarity to *O. sativa* putative transcriptional activator *FHA1* (NM_001070135.2). The lower homology to the *O. sativa* sequence versus the *Z. mays* sequence is likely because the fragment isolated from cDNA matched the amino acid sequence outside its highly conserved FHA residues in *FHA1*. Based on comparison to sequences from other plant species such as *Arabidopsis thaliana* (AAF20224.1), the cDNA obtained in this study aligned towards the C-terminus end of the sequence, following a highly acidic region of the protein (Figure 6.3B). The residues in this acidic region are not highly conserved (Kim et al., 2002).

**Figure 6.3 Analysis of amino acid sequences of isolated fragments.**

Predicted amino acid sequences of the gene fragments isolated from *Secale cereale* and aligned to their homologous sequences. Accession numbers for each predicted protein are listed. (A) *S. cereale* cDNA sequence (S630, ScPABP, poly(A)-binding protein) aligned with that of *Triticum aestivum* (U81318.1) and *Oryza sativa* (NM_001068050.1) PABP. The RNA binding domain (RRM) are outlined in grey boxes; α-helix 1-5 of the C-terminal are indicated by numbers and outlined in blank boxes. (B) R350, (ScFHA1, forkhead associated f) from *S. cereale*, *Zea mays* (EU971449) and *O. sativa* (NM_001070135.2). Arrow indicates possible residues where the acidic region of the protein ends. Conserved α-helix 2 is indicated with a number and outlined in a blank box. (C) $H^+$ATPase fragment (R300) similarity to *Hordeum vulgare* (AY136627.1) and to *T. aestivum* (AY543630.1). Conserved α-helix 2 is indicated with a number and outlined in a blank box. (D) (PDS S365) fragment similarity to *T. aestivum* (FM998042) and *O. sativa* (CM000128.1). Blank box indicates a region of high homology between phylogenetically distant organisms located at the C-terminus. (E) R280 (ScP450) and *Z. mays* P450 CYP727A4. (NM_001159621) and *Oryza sativa* P450 (NP_001060449.1). The Glu-X-X-Arg motif is outlined in a blank box. (F) R280 (KNOX) similarity to *O. sativa* (AB061817.1) and *Z. mays* (NM_001112382). Helix 2 and helix 3 of the homeodomain are outlined are boxed. Homeodomain residues that are conserved in members of Class II are shaded in gray. Identical residues are indicated with (*), conserved substitutions with a colon (:); semi-conservative substitutions are indicated with a period (.). Plant species names are indicated the left of the sequence and amino acid numbers are indicated to the right of the sequence.


A
ScPABP -------EFTSD---SRALLAMNGKMVGGKPLYVALAQRKEERRARLQAQFSQMRPV-MP 49
TaPABP SRGSGFVAFKSADDASRALAEMNNKMVGNKPLYVALAQRKEDRKARLQAQFSQMRPVPMA 412
OsPABP SRGSGFVAFKSAEDASRALAEMNSKMVGSKPLYVALAQRKEDRKARLQAQFSQLRPVPLA 420
ScPABP PPVAPRMPMYPPGVPGMGQQLFYGQPPPAFVNPQPGFGFQQHMIPGMRPGVAPMPNFVMP 109
TaPABP QTVGPRMQMLPPGVP-VGQQMFYGQPP-AFINPQPGFGFQQPFMPGMRPGGAPMPNFMMP 470
OsPABP PSVGPRMPMFPPGVPGVGQQLFYGQPPPAFINTQPGFGFQQPLMPGMRPGAGPMPNFIMP 480
ScPABP MVQQGQQPQRPSGRRAGAGGMQQQPMPMGHQQMVPRGGRGGYRYASGRGMPDAAFRGVGA 169
TaPABP MVQQGQQPQRPAGRRAGAGGMQQ-SMQMGQQQMLGRGGGRGYRYQTGRGMPDPAMHGVGG 529
OsPABP MVQQGQQPQRPAGRRAGAGGMQQ-PMPMGQQQMMARGG-RGYRYPTGRGMPDPAMHGVGG 538
α1
α2
ScPABP -MVPSLYEMGRMTPSDTGAPQQVSSGALASALANSPPEQQR------------------- 209
TaPABP -VMTSPYEMGGMPMRDAGESQPVPIGALASALANSPPETQRMMLGENLYPLVDQLEHDQA 588
OsPABP GVMPSPYEMGGMPMRDAAASQPVPIGALATALANAAPDQQRMMLGENLYPLVDQLEHEQA 598
α3
α4
α5
ScPABP ------LLELI---------- 214
TaPABP AKVTGMLLEMDQTEVLHLLESPDALKAKVAEAMEVLRSAQQ-HTNQSPEQQLASLSLNDG 647
OsPABP AKVTGMLLEMDQTEVLHLLESPEALKAKVAEAMEVLRTAQQIQTNATPEQQLASLSLNDG 658
B
ZmFHA GNGISET-AMRGKLVKRNKKSSGDLDIYGGHRINVEAIGTLGEDSRSEIRSRGDRDIDNQ 231
OsFHA GNGISESGGMRGKLIKRNKKLSAESEMYGGHRINVEAIGTLGEDSRSEIRSRGDRDMDNQ 238
ScFHA ---ISSP----------GDLDIYGGHRINVEAI-TLGEDNRSEIRSRGDKDVDNQ 41
ZmFHA QALQAEEKDVVSSVATVLSDLCGPGEWMPMAKLHNELLEQFGNVWHPSRVRKYLTQDDWS 291
OsFHA HILQAEEKDVVSSVATVLSDLCGPGEWMPMAKLHTELLEQFGNVWHHSRVRKYLSPEDWS 298
ScFHA H-LQMEEKEVVSSVATVLSDLCGPGEWMPMKTLHTELMDQFGNVWHHSRVRKYLTADDWS 100
ZmFHA PTETKGRPWFGLLALLRKYPEHFVINTRSKGRMTSEFVSLVSLLS 336
OsFHA PTETKGRPWYGLLALLRKYPEHFVINTRSKGRVTSEFVSLVSLLS 343
ScFHA PIEAKGRPWYGLLELI---------- 116
C
α2
ScHATPASE -----------SKALLGHGDGRHHGHCAGQWWWEAPGLAGFRWYHRSPVINSTIFFIEEN 49
TaHATPASE SKVLKFLGFMWNPLSWVMEMAAIMAIALANGGGKPPDWQDFVGIIVLLVINSTISFIEEN 115
HvHATPASE SKFLKFLGFMWNPLSWVMEMAAIMAIALANGGGKPPDWQDFVGIIVLLVINSTVSFIEEN 120
ScHATPASE NAGNAAAALMANLAPKTKVLRDGRWGEQEASILVPGDIVSIKLGDIVPAGALD------- 102
TaHATPASE NAGNAAAALMANLAPKTKVLRDGRWGEQEASILVPGDIVSIKLGDIVPADARLLEGDPLK 175
HvHATPASE NAGNAAAALMANLAPKTKVLRDGRWGEQEASILVPGTLSASSLVTSSLLMLVCLEGDPFE 180

```
D
ScPDS           R-----------------------------APAEEWIGRSDTEIIEATMLELAKLFPDEI 32
TaPDs           RSSLLSVYADMSLACKEYYDPNRSMLELVFAPAEEWIGRSDTEIIEATMLELAKLFPDEI 480
OsPDS           RSSLLSVYADMSVTCKEYYDPNRSMLELVFAPAEEWVGRSDTEIIEATMQELAKLFPDEI 456
                *                            ******:************ **********

ScPDS           AADQSKAKILKYHVVKTPRSVYKTVRNCEPCRPLQRSPIEGFYLSGDYTKQKYLASMEGA 92
TaPDs           AADQSKAKILKYHVVKTPRSVYKTVPNCEPCRPLQRSPIEGFYLAGDYTKQKYLASMEGA 540
OsPDS           AADQSKAKILKYHVVKTPRSVYKTIPDCEPCRPLQRSPIEGFYLAGDYTKQKYLASMEGA 516
                ************************: :*****************:***************

ScPDS           VLSGKLCAQSIVQDSKMLSRRSQE--------SSRL 120
TaPDs           VLSGKFCAQSIVQDSKMLSRRSQESLQSEAPVASKL 576
OsPDS           VLSGKLCAQSVVEDYKMLSRRSLKSLQSEVPVAS-- 550
                *****:****:*:* ******* :        :*

E
ScP450          ----------------------------------NLELSWPLSSEEEICGNIVGLMLHGI 26
ZmP450          HHFDQRSCQKSEG--TDPHR--SVFDNMMRNHCLHGAAKGPLNLEEETCGNIMGLMLHGI 356
OsP450          SLIDLSSCQRSERMIKDPCRGFSLLDGVISSRCLNEAAEGPLSSEEEICGNIMGLMLHGI 291
                                           :   . **. *** ****:*******

ScP450          STSANLLCNILTRLILYPKLKDQLYADIVAVHTESSELVMNDVLKMQFVLATVCESARLL 86
ZmP450          STSANLIGNILTRLVLFPELQDQLHEEIVSVCNKSSKVEVDDLLRMQVLLATVCESARLL 416
OsP450          STCANLIGNILTRLALYPNLQCQLHSEIVSGHSESSELKIDDVLRMKFLLATVCESARLL 351
                **.***: ****** *:*:*: **: :**:  .:**:: ::*:*:*:*:.:***********

ScP450          PAGALDS----------------------------------------------------- 93
ZmP450          PAGPLLQRCSLKHDLTLGSGVTVPARSILVVPLHLVQMDASVWGDDADQFNPHRFLKRDI 476
OsP450          PAGPLLQRCSLQQDVNLNSSITIPAGAILVIPLHLVQMEASTWGNDACQFNPNRFLKKEI 411
                ***.* .
F                  α2              α3
OsKN2           AWWQAHSKWPYPTEDDKARLVQETGLQLKQINNWFINQRKRNWHS-NPASSGEKTKKKRN 51
ZmKN2           AWWQAHSKWPYPTEDDKARLVQETGLQLKQINNWFINQRKRNWHS-NPTSSAEKTKKKR- 298
ScKN2           ---------------SRALLVQETGLQLKQINNWFINQRKRNWHSSNTASSSEKTKKKRR 45
                              .:* ************************* *.:**.*******

OsKN2           VTGDGGAEQSW---------------------------- 62
ZmKN2           ---------------------------------------
ScKN2           HGAIVVDWVPDETNMTILLSRLLKYILSLSLFFLSAGALD 85
```

### 6.4.4 Validation of reference genes used for qPCR using the geNorm algorithm

One of the limitations of ddPCR is that many sequences can be obtained from a single ddPCR band (Akhtar et al., 2005). This is due to closely sized cDNA bands migrating together. Because several different genes fragments might be isolated from the same band, a method to confirm differential expression is thus required. The differential expression of selected genes was confirmed with quantitative PCR. To quantify the expression of the gene in question it is further necessary to have a housekeeping gene or any gene assumed to have remained constant under all experimental conditions as a point of reference. Based on previous studies with stable reference genes β-tubulin (*TUB*), β-actin (*ACT*), elongation factor-1 alpha (*EF1A*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were chosen as potential candidates for the normalization (Jian et al., 2008; Jarosova and Kundu, 2010; Migocka and Papierniak, 2010).

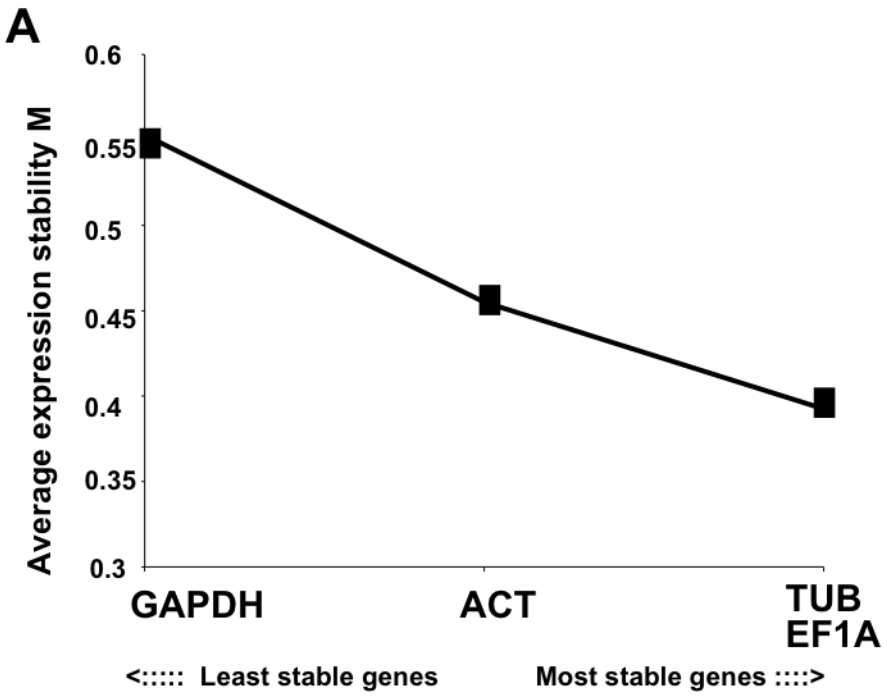
Primers were designed using selected cDNA sequences with Primer3 software, version 0.4.0 (Rozen and Skaletsky, 2000). Sequences of all primers used in this study are listed in Table 6.1. Primers were first tested with a representative cDNA sample (created by combining randomly selected shoot and root cDNA) to ensure they only amplify a single product. The primer sets that resulted in production of only a single product at an optimal temperature (± 4 $^{o}$C around the expected Tm of primer) were selected for further analysis. The selected optimal temperature was then used to test the amplification efficiency of each primer set using a cDNA dilution series. Amplification efficiency may frequently vary; a poorly binding primer set could result in lower than 80% efficiency (Wang and Seed, 2006). Conversely, primer dimers and secondary products may lead to an artificial increase in efficiency, which may obscure the measurement in the qPCR reaction and lead to efficiency values of over 100%. In all qPCR reactions, to check for single product, reaction products were analyzed on a 3% agarose gel as previously described. All primers used for further analysis had 90-105% efficiency; a standard dilution series was run with each set of samples to confirm that amplification efficiency with each plate.

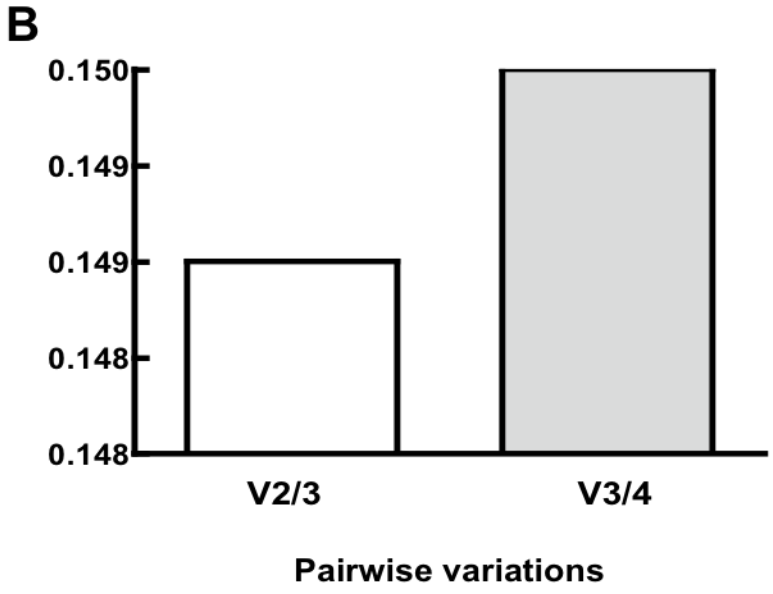
The reference genes were used to normalize the PCR reactions for the amount of RNA added (Livak and Schmittgen, 2001). The current suggested qPCR reaction uses more

than one reference gene (Pfaffl, 2006). Gene expression stability with different treatments was evaluated using the geNorm algorithm (Vandesompele et al., 2002). The geNorm algorithm calculates the gene expression stability measure M. Genes with the lowest M value have the most stable expression, while the highest values indicate the least stable expression (Figure 6.4A). The reference genes in order of stability from least to most stable were *GADPH*, *ACT*, *TUB/EF1A*. The optimal number of reference genes for reliable normalization is determined by pairwise variations $V_n/V_{n+1}$ (Figure 6.4B). The optimal pairwise variation value should not exceed 0.15 (Vandesompele et al., 2002); the value for $V_2/V_3$ was 0.149, and thus it was determined that two genes are sufficient for normalization and addition of the third reference gene for normalization offered no significant improvement (Figure 6.4B). A combination (geometric mean) of *ACT* and *TUB* were chosen as reference for normalization. *ACT* was chosen instead of *EF1A* because of greater amount of *ACT* data available at the time of experiments.

**Figure 6.4 Reference gene stability analysis.**

Stable control genes were chosen using the geNorm algorithm (Vandesompele et al., 2002) based on their ranking according to their expression stability. Stable reference genes were tested using a sample set with four treatments (control – UW4, control + UW4, PHC – UW4, PHC + UW4). A. Average expression stability values of control genes. Highest M value indicates least stable genes. B. Determination of the optimal number of control genes for normalization. A value of less than 0.15 for V2/3 indicates two reference genes are sufficient for reference gene normalization. Figures generated with geNorm software.


A
0.6
0.55
0.5
0.45
0.4
0.35
0.3
Average expression stability M
GAPDH
ACT
TUB
EF1A
<::::: Least stable genes
Most stable genes ::::>


B
0.150
0.149
0.149
0.148
0.148
V2/3
V3/4
Pairwise variations

### 6.4.5 Transcript level changes in response to PHC treatment

To investigate gene expression changes in response to PHC in soil, gene expression was first normalized to control plants without UW4 inoculation (in addition to normalization to reference genes); Overall, transcript levels changed to a greater extent in shoots when plants were exposed to PHC (Figure 6.5) and to a lesser degree in roots of those plants (Figure 6.6). In shoots, the general trend was a transcript increase in all genes when plants were exposed to PHC-contaminated soil; a further increase was observed when PHC-exposed plants that were inoculated with PGPR. Particularly, *PABP*, *FHA1* and *P450* increased in response to PHC (Figure 6.5A, D, F), by 59% and 70% and 65% respectively.

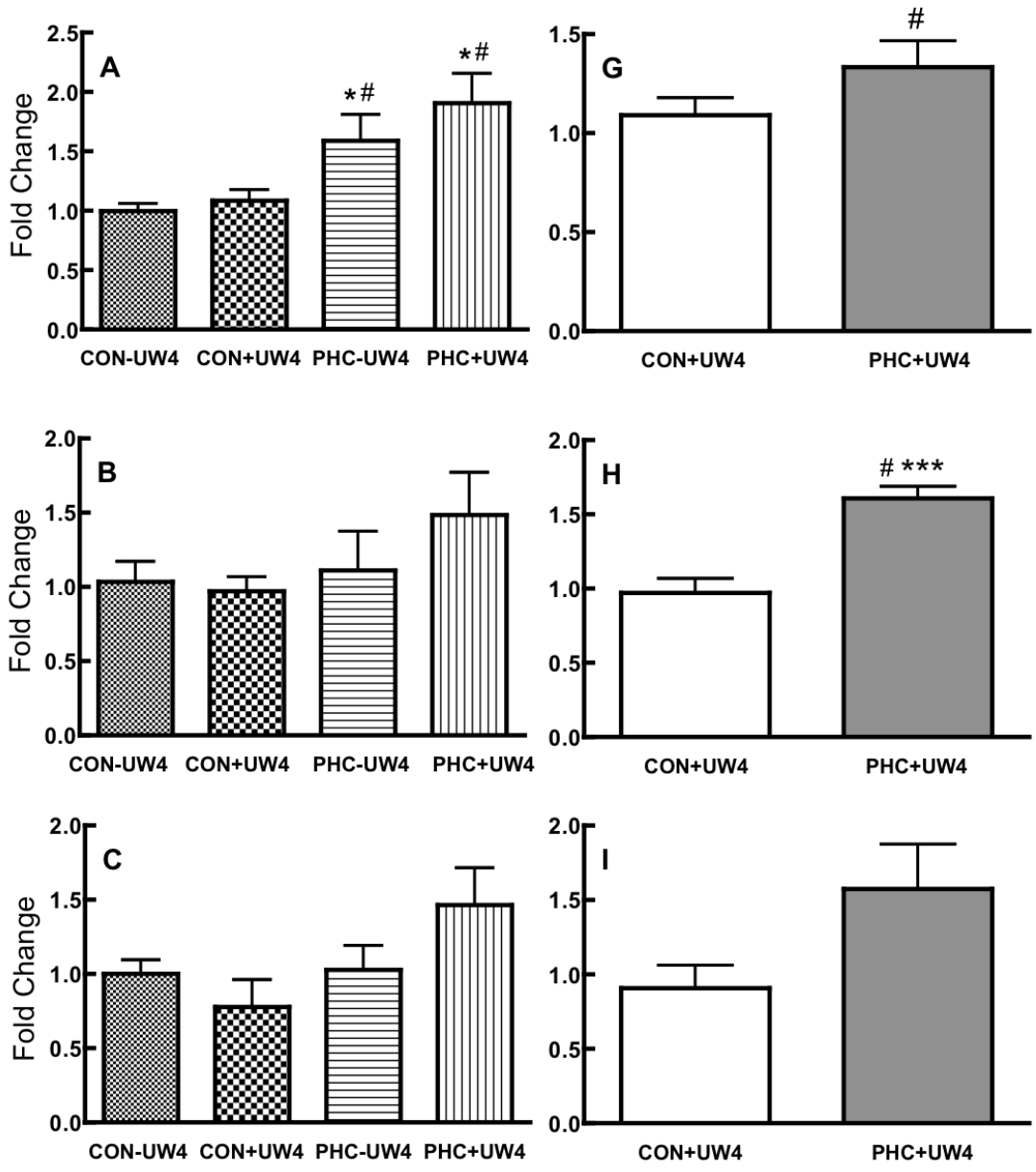
In root tissues, *PDS* showed an increase of 47% in response to PHC. *PABP*, *PMA* and *KNOX* increased by 15%, 27% and 12% respectively in response to PHC in roots (Figure 6.6 D, E, C), albeit not significantly. Notably, *P450* expression decreased by 25% in roots in response to PHC (Figure 6.6). *FHA1* expression in roots did not change.

### 6.4.6 Transcript level changes in response to PGPR treatment

To investigate the influence of PGPR on plants grown in control and PHC-contaminated soil, a second normalization strategy was used: control plants with UW4 were normalized to control plants without UW4, and similarly, PHC plants with UW4 were normalized to PHC treated plants without UW4. In shoots, changes in gene expression in plants grown in PHC-contaminated soil, which received UW4 inoculation were significantly different than plants grown in control soil with UW4 treatment in the case of *FHA1*, *PDS* and *PMA* (Figure 6.5). Further, in control soil, treatment of UW4 modestly lowered the expression of all genes but *FHA1*. However when PGPR inoculated plants were grown on PHC impacted soils, expression of all six genes was increased relative to plants grown on PHC contaminated soils without UW4. Particularly, *PABP* in shoots increased by 28%, *FHA1* increased by 33%, *PMA* increased by 40%, PDS increased by 60%, *KNOX* increased by 58%, and *P450* transcript increased by 24%. In root tissues, PGPR only had an effect on the gene *PMA*. *PMA* significantly increased by 33% when plants were exposed to PHC and UW4.

**Figure 6.5 Shoot gene expression analysis with quantitative PCR.**

Shoot tissue expression of FHA1 (A), PDS (B), KNOX (C), PABP (D), PMA (E) P450 (F), normalized to control plants without PGPR. Where there are four bars, statistical analysis was performed with a one-way ANOVA and LSD post hoc test ($P < 0.05$). # indicates values different than CON-UW4 and * indicates values significantly different than CON+UW4. Shoot tissue expression of FHA1 (G), PDS (H), KNOX (I), PABP (J), PMA (K) P450 (L) gene expression in control +UW4 plants normalized to control –UW4 plants and PHC-exposed +UW4 plants normalized to that of PHC-exposed plants without PGPR. Where there are two bars, statistical analysis was performed with a two-tailed t-test. In all cases * indicates values significantly different than CON+UW4 (*, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$). # indicates values different than 1 as determined by a two-tailed t-test.


A
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
*#
*#
CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
G
1.5
1.0
0.5
0.0
#
CON+UW4
PHC+UW4
B
Fold Change
2.0
1.5
1.0
0.5
0.0
CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
H
2.0
1.5
1.0
0.5
0.0
# ***
CON+UW4
PHC+UW4
C
Fold Change
2.0
1.5
1.0
0.5
0.0
CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
I
2.0
1.5
1.0
0.5
0.0
CON+UW4
PHC+UW4

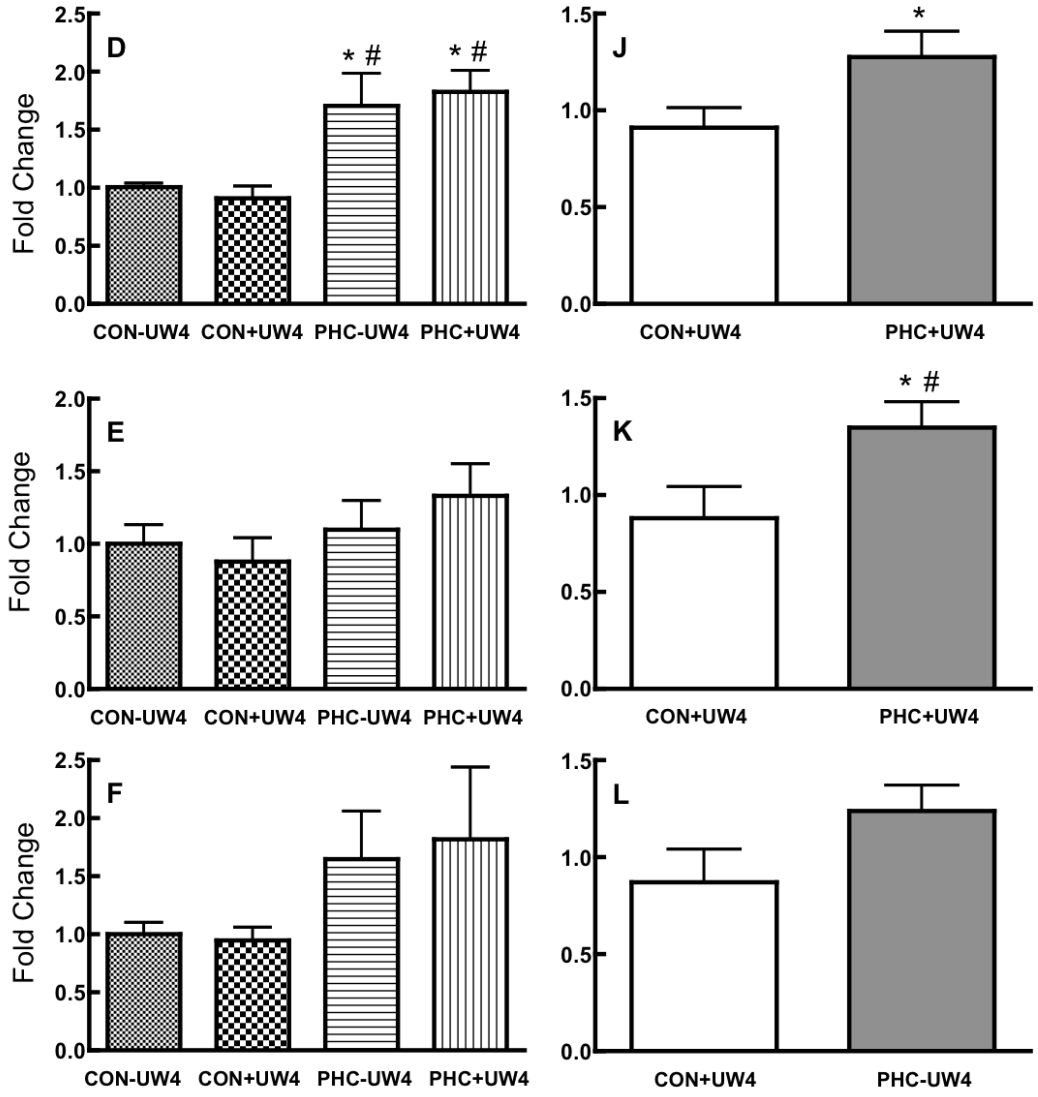
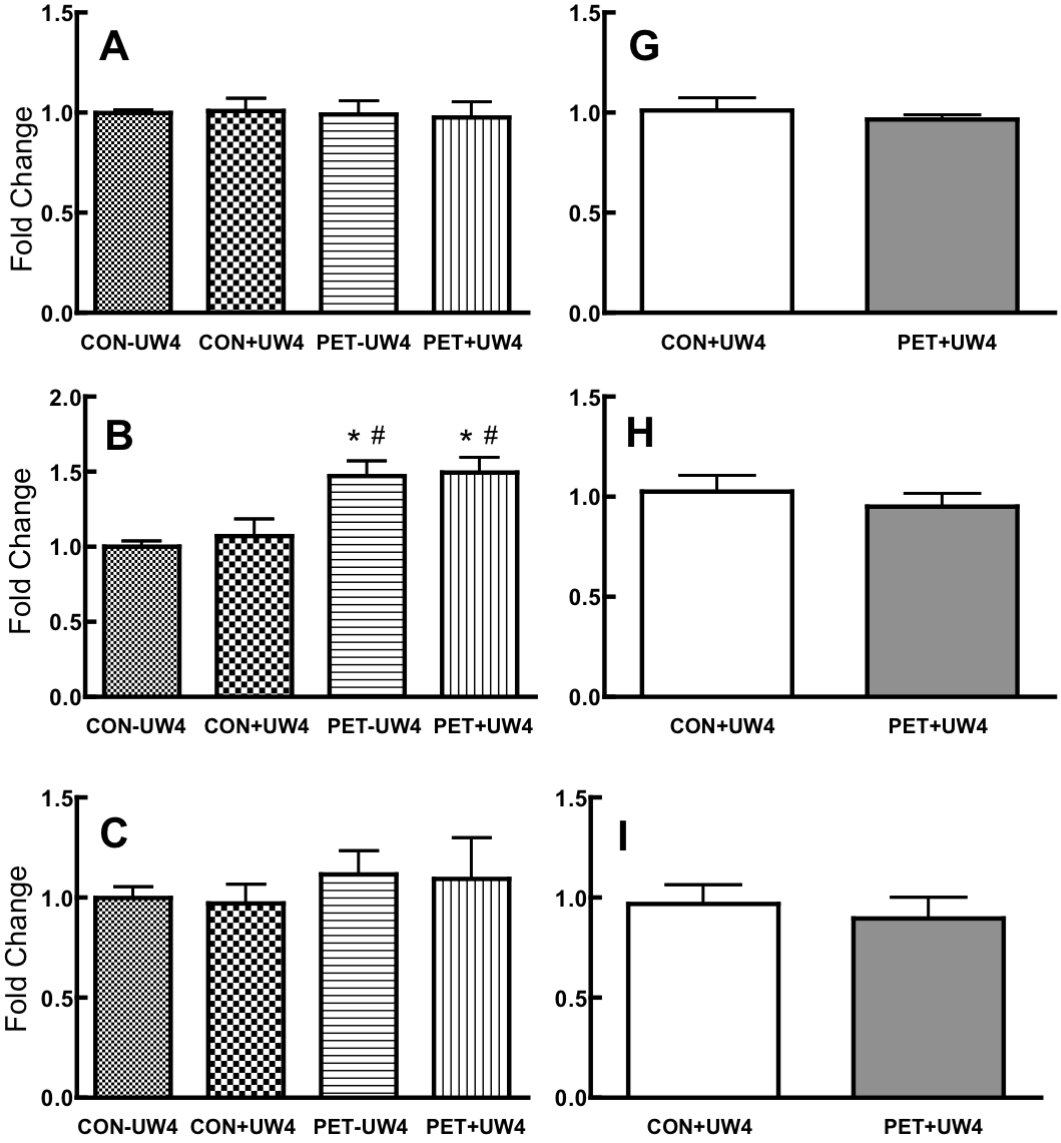

D
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
* #
* #
CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
J
1.5
1.0
0.5
0.0
*
CON+UW4
PHC+UW4
E
Fold Change
2.0
1.5
1.0
0.5
0.0
CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
K
1.5
1.0
0.5
0.0
* #
CON+UW4
PHC+UW4
F
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
CON-UW4
CON+UW4
PHC-UW4
PHC+UW4
L
1.5
1.0
0.5
0.0
CON+UW4
PHC-UW4

**Figure 6.6 Root gene expression analysis with quantitative PCR.**

Root tissue expression of FHA1 (A), PDS (B), KNOX (C), PABP (D), PMA (E) P450 (F), normalized to control plants without PGPR. Where there are four bars, statistical analysis was performed with a one-way ANOVA and LSD post hoc test ($P < 0.05$). # indicates values different than CON-UW4 and * indicates values significantly different than CON+UW4. Root tissue expression of FHA1 (G), PDS (H), KNOX (I), PABP (J), PMA (K) P450 (L) gene expression in control +UW4 plants normalized to control –UW4 plants and PHC-exposed +UW4 plants normalized to that of PHC-exposed plants without PGPR. Where there are two bars, statistical analysis was performed with a two-tailed t-test. In all cases * indicates values significantly different than CON+UW4 (*, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$). # indicates values different than 1 as determined by a two-tailed t-test.


A
Fold Change
1.5
1.0
0.5
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CON-UW4
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G
CON+UW4
PET+UW4
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* #
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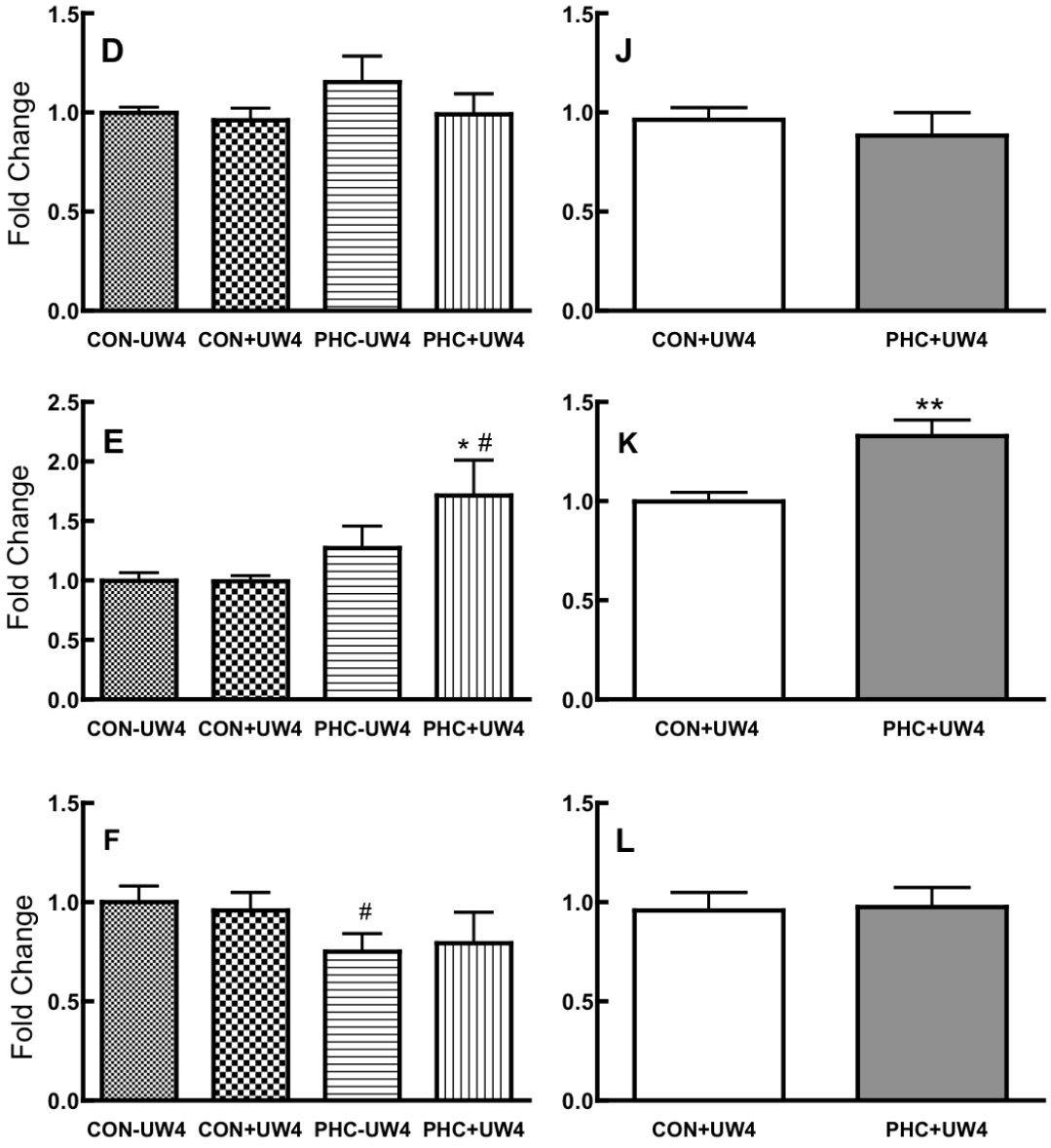
H
C
I


D
Fold Change
1.5
1.0
0.5
0.0
CON-UW4
CON+UW4
PHC-UW4
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E
2.5
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* #
K
**
F
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L

## 6.5 Discussion

Previous work on phytoremediation of PHC-contaminated soil showed that PGPR positively affected growth and remediation (Huang et al., 2005; Gurska et al., 2009). PHC-induced production of plant stress hormone ethylene characteristically resulted in decreased biomass production (Glick et al., 2007). PGPR, including UW4, express ACC deaminase, which hydrolyzes the immediate precursor to ethylene, ACC, to ammonia and α-ketobutyrate (Glick, 2003) (Figure 6.7). This impedes production of ethylene in plants (Glick et al., 1998). Notably, what is affected is the harmful stress ethylene but not the small initial burst of ethylene that is needed to activate plant stress responses(Glick et al., 2007). As a result of this, PGPR enhance germination and plant growth under PHC stress, which promotes phytoremediation (Glick, 2003; Huang et al., 2005; Gurska et al., 2009).

Fall rye plants used in this study showed a decrease in growth in response to PHC as well as increased photosynthetic heat dissipation (qN) due to stress. A PGPR strain, *Pseudomonas putida* (UW4) ameliorated these negative effects in fall rye plants. To better understand the PGPR-plant interaction at the molecular level, ddPCR was employed to identify genes with altered expression under PHC induced stress, with and without UW4 inoculation. Differential expression of genes was examined in both roots and shoots of *S. cereale* plants. Several differentially expressed genes were identified, and their altered expression due to PHC exposure was confirmed with qPCR. It was found that shoots appeared to exhibit the most prominent changes in the expression of the genes examined here. There was some upregulation of all six genes examined, two of which were statistically significant. In roots, two genes were upregulated significantly and one gene appeared to be downregulated.

ddPCR is a useful technique for exploratory examinations of changes in gene expression due to contaminants. Previous studies have been successful at providing insight into contaminant modes of action (Akhtar et al., 2005). The value of this method lies in the fact that it does not require previous DNA sequence knowledge of the organism or the response of the organism to a particular stress. Further, the sequence data obtained from this method may result in finding new genes, e.g. those important for phytoremediation. One of the limitations of ddPCR is the possibility of two similarly

sized cDNA fragments migrating together and being isolated from the same region of the sequencing gel. This was the case with *knotted1*-like homeobox (*KNOX*) and cytochrome P450 (*P450*), which were both isolated from the region of 280 bp from roots. Not only were two cDNAs isolated from the same band, but also their accumulation did not follow the same pattern. In roots, *P450* decreased with PHC treatment while *KNOX* increased. This isolation of false positives can be addressed by confirming the expression of a gene with another technique such as qPCR or northern hybridization (Akhtar et al., 2005). Finally, one of the concerns with ddPCR is that the threshold for detection is quite high, and often changes of twofold or less cannot be detected (Liao and Freedman, 1998). In this study, very few changes in the ddPCR profile were observed and it is likely that many changes were below detection limit of ddPCR gels.

The ddPCR profile of the shoots revealed two strongly upregulated mRNAs in PHC treated plants; cDNAs at 356 bp and 630 bp. The gene found at 365 bp was phytoene desaturase (*PDS*). *PDS* is a key enzyme in the synthesis of carotenoids, integral accessory pigments in photosynthesis, precursors to the plant hormone abscisic acid and photoprotectors (carotenoids protect the photosynthetic apparatus from deleterious oxidative reactions occurring under high light stress) (Sandmann, 2002). Oxidative stress is a driving force behind carotenoid synthesis – they are able to scavenge reactive oxygen species and inhibit membrane lipid peroxidation (Salguero et al., 2003; Khairnar et al., 2003) (Figure 6.7). PDS mRNA has been shown to increase under different stress conditions in peppers and in unicellular green algae (Bouvier et al., 1998; Grünewald et al., 2000). Oxidative stress in plants in current study may come from PAHs, which are present in PHC. PAHs have been shown to induce oxidative stress in *Arabidopsis thaliana*, *Sinapis alba*, *Triticum aestivum and Phaseolus vulgaris* and *Lemna gibba* (Alkio et al., 2005; Babu et al., 2005; Liu et al., 2009a; Paškova et al., 2009). Therefore it is reasonable that this gene is upregulated in the presence of PHC. PHC caused an increase in the PDS transcript in roots but none in shoots. This may be an indication that oxidative stress due to toxicants such as PAHs is more prominent in roots than shoots, which may be a result of higher concentrations of toxic compounds in roots than shoots. Higher accumulation of PAHs in roots than shoot has been observed with some PAHs

(Kacalkova and Tlustos, 2011). PGPR increased *PDS* levels in shoots but did not cause a change in *PDS* expression in roots, possibly because PDS was already induced.

Another mRNA upregulated in shoots was poly(A)-binding protein, *PABP*. *PABP* is highly conserved in eukaryotes and its protein function is at the crux of translation of mRNA. It has critical roles in post-transcriptional events, and in regulation of mRNA stability and turnover. Its activity is regulated by phosphorylation (Le et al., 2000). PABP initiates eukaryotic translation by binding to the poly(A) tails of cytoplasmic mRNA, and recruiting the ribosome to the mRNA at the 5'end (Pierrat et al., 2007); PABP regulates mRNA turnover through protecting mRNA termini from attack by nucleases, and enhances translational activity by through promoting the circularization of the mRNA (Le et al., 1997 ; Preiss and Hentze, 2003 ; Chekanova and Belostotsky, 2003). Finally, PABP also has a role in mRNA degradation, by binding to AU-rich regions of certain mRNAs, and may be involved in recruiting nucleases to mRNA degradation sites in the cytoplasm (Bag and Bhattacharjee, 2010). Furthermore, evidence has amassed suggesting that PABP is involved in mRNA transport from the nucleus to the cytoplasm (Chekanova and Belostotsky, 2003) (Figure 6.7).

With PABP important for mRNA translation and metabolism, it is not surprising that it would be subject to stress-induced changes in expression. Recent literature has shown that mRNA transcript levels do not always correlate to protein production(Kawaguchi and Bailey-Serres, 2002) and because translation requires a great deal of energy, it is a key regulatory step during stress. Indeed, global changes in translation have been shown in plants under drought and sugar starvation conditions (Kawaguchi et al., 2004; Nicolai et al., 2006). Specifically with stress, mRNA may not be exported out of the nucleus or, if exported, mRNA stability and turnover may be altered, ultimately changing final protein levels following transcription (Ali and Reddy, 2008). Thus, *PABP* is a good candidate for this regulated expression.

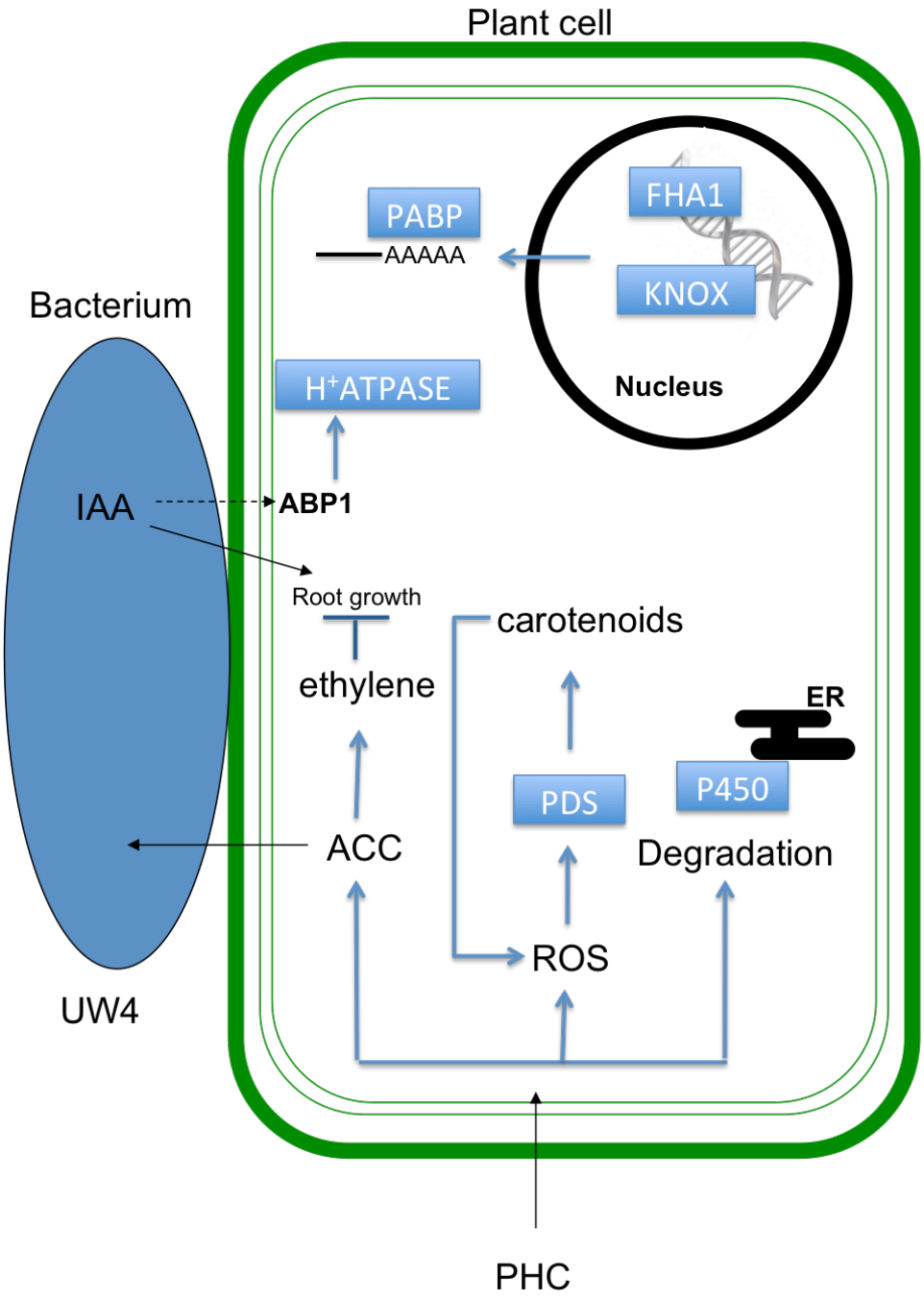


**Figure 6.7 Cellular functions of isolated genes.**

Schematic of putative cellular function with differentially expressed gene products. Cell function with PHC stress and the PGPR, UW4.

Relative transcripts levels of *PABP* in shoots, identified by ddPCR, were confirmed by qPCR (Figure 6.2); both with PHC and PHC with UW4 there was a strong increase in the *PABP* accumulation compared to control plants. In roots, there was only a modest increase in *PABP* transcript in response to PHC, but with PHC combined with UW4, transcript levels did not appear to change compared to control with no PGPR. Studies performed with five-day-old wheat plants following a heat shock and in canola plants with Ni exposure found that *PABP* levels increased in response to these stressors (Gallie et al., 1998; Czarny, 2008). Further, in an experiment by Czarny (2008) on the roots of 6 day-old seedlings treated with UW4, there was a significant 1.2 fold change in putative *PABP*, and this is in line with what was observed here. Interestingly, PABP has been found to accumulate, along with mRNA, into stress granules and processing bodies, acting to sequester and to protect mRNA in the former or degrade it in the latter (Weber et al., 2008). This process may account for additional PABP needed for mRNA storage or degradation under stress. Overall the increase in *PABP* transcript with PHC may be due to its many functions in mRNA regulation and processing during stress. The reason for the significant *PABP* increase in shoots but not in roots in the present study may be that PABP is simply more needed in the shoots. Interestingly in roots, *PABP* levels increased with PHC but decreased in the presence of PHC and PGPR, suggesting some reversal of the stress effect due to PGPR may exist in roots.

Cytochrome P450 monooxygenases (P450s) catalyze a plethora of oxidative reactions for both natural and xenobiotic compounds. Among a host of other functions, they are involved in the biosynthesis of plant compounds that are instrumental in the defense against harmful UV rays, plant defense and as components of structural polymers (Whitbred and Schuler, 2000; Burken, 2003). Notably, they are recognized as the key enzyme in the initial step of metabolism of contaminants in plants as well as other organisms (Schwitzguébel and Vanek, 2003). The oxidation reactions carried out by this enzyme family are part of transformation steps in the biotransformation processes, which increase solubility of the compound (Burken, 2003) (Figure 6.7). cDNA from band R280 was highly similar to *Zea mays* P450 CYP727 A4. This particular P450 does not have a known function however it belongs to the CYP727 clan, which is exclusive to monocots (Nelson et al., 2004; Nelson, 2006). When taking into

consideration that P450s may retain as little as 16 percent of amino acid identity (Schwitzguébel and Vanek, 2003), the resemblance to this particular P450 may be significant. Notably Xie et al., 2010 (Xie et al., 2010) found a potential microRNA sequence that targets Cytochrome P450 CYP727A4, which they have assigned to stress response.

Plant P450s involved in detoxification may be induced by compounds such as PAHs. Plants are able to metabolize a carcinogenic PAH, benzo(*a*)pyrene, and studies with cell cultures of red goosefoot have shown quinone metabolites which point to involvement of P450 enzymes (Harms et al., 2003). In general, metabolism of PAHs has been well characterized (Harms et al., 2003; Olson et al., 2003) and it is likely that plant metabolism of PAH mirrors that of other organisms where P450s are involved.

*S. cereale* cytochrome P450 cDNA was downregulated in roots, but in shoots it was upregulated in response to PGPR and PHC. The fact that this P450 was downregulated in the roots but upregulated in shoots may indicate that detoxification takes place in the shoots more so than in the roots. If, however, the P450 is not directly involved in detoxification but rather in synthesis of plant compounds, it could be a product needed for plant acclimation to PHC stress.

The *knotted1*-like homeobox (*KNOX*) genes encode a protein domain called the homeodomain that has a structural motif of helix-loop-helix-loop-turn-helix. They are common to a group of transcription factors involved in developmental regulation (Janssen et al., 1998). While class I *KNOX* genes have been shown to be involved in maintaining meristem identity in cells, the function of class II genes remains unknown (Janssen et al., 1998). However, recent evidence indicates that these may have a role in nutrient transport through carbohydrate translocation by acting on genes encoding sugar carriers or sugar metabolism (Testone et al., 2009). The *KNOX* cDNA fragment isolated from roots showed a structural similarity to class II *KNOX* genes; the sequence overlapped a segment of the second helix and the loop-turn-helix structure that follows. *KNOX* mRNA increased in shoots when plants were inoculated with UW4. In roots PHC modestly increased transcript levels but the additional presence of UW4 did not further increase transcript levels of *KNOX*. If

KNOX indeed have a role in carbohydrate translocation by acting on genes encoding sugar carriers or sugar metabolism, this may relate well to plant PHC stress response, as plants under PHC stress have been shown to decrease biomass and change biomass allocation from shoots to roots (Nie et al., 2010).

The plasma membrane $H^+$ATPase (PMA) is integral to the plant response to environmental stressors. In plant cells, it establishes a proton electro-chemical gradient across the plasma membrane and the tonoplast, and is functionally connected to stomatal opening, cell elongation and intracellular pH regulation. The expression of *PMA* gene family is regulated at the pre-transcriptional, transcriptional and post-transcriptional levels. The expression of *PMAs* have been shown to be modulated by a number of environmental stressors such as low phosphorous levels, aluminum stress and salt stress (summarized in Liu et al., 2009b). In particular, it has been speculated that enhanced lipid peroxidation could be one of the causes for the *PMA* activation under stress conditions (Veselov et al., 2002). As previously discussed, both IAA and ACC are integral in the association between PGPR and plants. Some PGPR, including UW4, are capable of production of IAA, which is posited to enter the plant cell and ultimately increase plant growth. External application of IAA has been shown to induce *PMA* (Shen et al., 2006). Further, this has been proposed to happen through the auxin receptor, ABP1 (Kawano, 2003; Figure 6.7). In this study, the presence of UW4 increased the production of *PMA* mRNA in shoots when PHC was present in the soil. In roots, on the other hand, it was increased equally by PHC and PHC with UW4 treatments. It appears that *PMA* may be upregulated due to PHC and UW4, likely through a combination of these two mechanisms environmental stressor and IAA signaling.

A gene that was upregulated in shoots by PHC and further with UW4 was *FHA1*, a transcriptional activator containing forkhead-associated domains (FHA). These transcription factors are linked to the RNA polymerase III function and are integral in regulation of rRNA processing (Kim et al., 2002). In yeast, Wade et al. (2004) found FHA1 to be almost exclusively specific to the promoters of ribosomal protein. The *N. thaliana FHA1* FHA domain was found to be similar to that of yeast and functioned to regulate rRNA processing and cell growth, suggesting the two genes are functionally similar (Kim et al., 2002). To date,

relatively little is known about the expression of FHA1 in plants under stress. In a single study, the FHA1 tomato homolog was induced by salt stress (Zhou et al., 2007). The *FHA1* in shoots showed a marked increase in response to PHC, and increased expression when UW4 was present. In roots the transcript was confirmed to be unchanged irrespective of treatment. The increase in *FHA1* mRNA could be indicative of the increased transcriptional load taken on by the plant in shoots but not in roots.

Here, as in previous studies, more changes in gene expression were observed in shoots than in roots. For example, Czarny (2008) found that 965 genes showed differential expression in shoots, but only 223 in roots in response to UW4 with or without active ACC deaminase gene. Czarny (2008) also found that in roots treated with UW4 the biosynthetic processes, response to stress, defense response and response to a biotic stimulus were down-regulated.

Root tissues exhibited less prominent changes in gene expression than shoot tissues. However, root tissue serves primarily as structural support and a passageway for water and nutrients from soil, thus it may not exhibit as many changes in gene expression as shoot tissue. Overall, PGPR elicited transcriptional changes in PHC-treated plants that cannot be observed in control plants; the response to UW4 is distinctly different with and without stress conditions. Our results suggest that UW4 offers further protection by stimulating enzymes that are part of stress defense mechanisms. The genes identified in this study suggest that oxidative stress response might be important in PHC stress, contrary to previous genetic studies that found PHC to induce responses related to mechanic stress and anoxic stress due to poor quality of PHC contaminated soil (Peña-Castro et al., 2006). However, this is in line with previous evidence showing that PAHs cause oxidative stress, cell death, necrosis, upregulation of antioxidant systems and reduced plant growth (Weisman et al., 2010). PAHs may be the cause of oxidative stress directly, as it has been shown with *Lemna gibba* where a PAH derivative 1,2-dihydroxyanthraquionone can, in the presence of excess Cu, lead to generation of reactive oxygen species (ROS) (Babu et al., 2005) Furthermore, the ROS production may result from necrosis. There exist similarities in the genes upregulated by PAH-mediated stress to those upregulated during the pathogenic hypersensitive response in

plants, and, additionally, necrotic lesions characteristic of the hypersensitive response produce ROS (Weisman et al., 2010).

In conclusion, a number of genes that were differentially expressed in response to PHC and UW4 were identified. In many cases only subtle changes in gene expression were detected. This is largely due to the sensitivity of qPCR and could not be detected with ddPCR. The gene changes in roots and shoots of fall rye give a better understanding of how the plant copes with excessive PHC stress and how UW4 modulates this stress. The genes from this study could not only be used as biomarkers of petroleum stress but also could be of potential value in phytoremediation strategies.

## 6.6 Acknowledgements

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# Chapter 7
# Analysis of shoot tissue polyphenols and flavonoids in *Secale cereale* and *Festuca arundinacea* grown in PHC contaminated soils and PGPR inoculation

## 7.1 Overview

Phenolics and flavonoids have been shown to be important in rhizosphere communication between plants and microbes, shaping the rhizosphere microbial community by acting as sources of carbon to soil microbes and are involved in plant stress responses. Thus, phenolics and flavonoid levels in *Secale cereale* and *Festuca arundinacea* plants were measured following ten days of growth in PHC contaminated soil with and without a plant growth promoting rhizobacteria (PGPR), *Pseudomonas putida* UW4. The gene expression of chalcone synthase, the first committed step in flavonoid synthesis, was analyzed. Phenolics and flavonoid levels were analyzed using high performance liquid chromatography (HPLC) and putative phenolic and flavonoid peaks were identified based on their resemblance to previously acquired spectra. The particular protocol used here was effective in separating flavonoids in plant extracts, although other phenolics may be extracted and detected as well. Thus, unless specified this chapter refers to flavonoid analysis with the understanding that other phenols may be detected as well. PHC decreased the levels of chalcone synthase mRNA transcript, and PGPR addition partially alleviated this effect. PGPR had influence on total phenolics accumulation in *S. cereale* and in *F. arundinacea* the effect of PGPR varied with the presence of PHC. Changes to plant phenolics showed a plant species specificity. Two peaks, corresponding to apigenin (flavonoid) derivatives, in *S. cereale* were decreased when PHC was present compared to control soil. In *F. arundinacea* the most prominent peak, corresponding to chlorogenic acid, was found to be increased in PHC contaminated soils.

## 7.2 Introduction

Soil contains a diverse microbial community, and among the most numerous residents are the bacteria (Curl and Truelove, 1986). A group of those are referred to as plant growth-promoting rhizobacteria, or PGPR. These free-living bacteria are capable of acting within the rhizosphere to reduce plant stress, through a variety of mechanisms loosely grouped into either PGPR synthesizing compounds and supplying to the plants or PGPR assisting nutrient uptake by the plants (Glick, 2003). The plant stress hormone, ethylene is thought to mediate the stress response and PGPR are able to decrease levels of ethylene (Glick et al., 1998). Supplementing soil or seed with PGPR prior to plant growth was first proposed in the 1950s as a viable crop supplement, and suggested to improve available nitrogen for plant use (reviewed in Zehnder et al., 2001). The use of these organisms has now been expanded to include use as biological control agents of soil-borne pathogens (Zehnder et al., 2001), and to facilitate plant growth and remediation of contaminated soils (Huang et al., 2005; Gurska et al., 2009).

Remediation of soil contaminants by plants is referred to as phytoremediation. It has been tested on both organic and inorganic contaminants such as petroleum hydrocarbons (PHC) and metals respectively (reviewed in Salt et al., 1998; Chaudhry et al., 2005). Toxicity may be a hindrance to successful phytoremediation applications in the field because contaminants often have negative impacts on plant growth, limiting the effectiveness of phytoremediation (Huang et al., 2005). Microbes that positively influence plant growth, the PGPR, have been used to alleviate toxic effects of contaminants, thus increasing biomass available for remediation (Glick, 2003; Huang et al., 2005). In phytoremediation of organic compounds, the vast majority of degradation takes place in the rhizosphere, where a plethora of microorganisms supported by plant exudates such as amino acids, aromatics, phenols and sugars, are able to degrade contaminants (Hutchinson et al., 2001). Using PGPR may result in growth of larger, healthier roots that, through greater amounts of exudates produced, support increased numbers of degrading microorganisms (see Chapter 3). The large variety of plant-derived compounds shapes the rhizosphere community so that each plant species is able to create a unique ecosystem, some capable of degradation of contaminants.

The evolution of xenobiotic degradation in plants has been suggested to have arisen from pathways originally targeted to the phenolics (Singer et al., 2003; Singer et al., 2004; Shaw et al., 2006). Structural similarities between phenolics and many xenobiotic compounds (e.g. the PAH phenanthrene and flavonoid confusarin) (Shaw et al., 2006) has been noted before. Furthermore, there is also a possibility that plants may increase degradation of certain contaminants by providing the soil microflora with polyphenolic compounds (Donnely et al., 1994). This could create a priming effect where degradative enzymes used for phenolic degradation would also metabolize contaminants. These compounds in turn will induce bacterial enzymes that degrade a variety of pollutants. This raises the possibility that phenolic production may be regulated by the presence of contaminants (Siciliano and Germida, 1998b).

Plants naturally produce more than 8000 different phenolic compounds for varied functions. The abundance and diversity of these compounds make phenolics ideal candidates to mediate plant and soil microorganism interactions. It has long been established that flavonoids, common 15-carbon plant phenolics, are exuded into the rhizosphere (Curl and Truelove, 1986). They are known to serve as nutrient sources for soil microbes, as antimicrobial agents and as inducers of microbial gene expression (Pillai and Swarup, 2002). Following synthesis, flavonoids are glycosylated and stored in vacuoles of the plants (Dardanelli et al., 2009); it has been hypothesized that these will eventually be released to the rhizosphere during root senescence (Shaw et al., 2006). Flavonoid exudation has not been widely investigated, however in a study with *Arabidopsis thaliana* it was found that flavonoids accounted for 37% of all secondary metabolites exuded (Narasimhan et al., 2003), highlighting the importance of these plant derived compounds in rhizosphere ecology.

Fletcher et al. (1995) have reported that flavonoids, along with other phenolics stimulate PAH degrading microorganisms. Flavonoids have been reported to promote root colonization by microbes, and better utilizers based on this criterion were able to increase degradation of PCBs (Narasimhan et al., 2003). Understanding phenolics and flavonoid exudation, as part of the complex plant-microbe-contaminants system may allow for use of

plants that specifically stimulate growth of appropriate microbes that degrade organic contaminants, increasing effectiveness of phytoremediation systems.

Many microorganisms present in the rhizosphere have been confirmed to respond to phenolics and flavonoids in a variety of ways, including degradation of these compounds. Some of these microorganisms include species belonging to *Rhizobia, Agrobacterium, Pseudomonas, Bacillus,* and *Rhodococcus.* Recently, it has been reported that a plant growth-promoting rhizobacterial strain of *Pseudomonas putida* can utilize the flavonoids naringenin, daidzenin, apigenin and naringin (Pillai and Swarup, 2002). Flavonoids released by legume roots activate the genes required for triggering nodulation in *Rhizobium* spp (Pueppke, 1996). Flavonoids also serve as agents in defense against microbial pathogens and in allelopathic interactions (Shaw et al., 2006). Several studies have shown that flavonoids increase colonization of plants by microorganisms, however this is not likely a result of microbe utilization of flavonoids as growth substrates or flavonoid improvement of microbial growth rate, but may be due to induction of microbial colonization genes (reviewed in Shaw et al., 2006). Conversely bacteria can alter plant exudate production or release, by degradation or modification of exudate pattern by inducing changes in the plant phenylpropanoid pathway. Also, a number of studies indicated that the presence of microbes causes changes in gene expression of flavonoid synthesis enzymes (reviewed in Shaw et al., 2006). There is a vast potential for a diverse set of interactions between plants and free-living, non-rhizobial microbes mediated via flavonoids and phenolics, such as those between plants and PGPR and grown in PHC.

Based on the above considerations, production of plant phenolics and flavonoids was examined in fall rye (*Secale cereale*) and tall fescue (*Festuca arundinacea*) with PHC contamination and the presence of PGPR. It is likely that phenolics and flavonoids play an important role in plant stress response to PHC, however the accumulation of specific phenolics and flavonoids under PHC stress in plants has not yet been examined. The objective of this study was to evaluate whether plants under PHC stress modify the pattern of flavonoid production in shoots and further if there are changes in plant flavonoid levels in the presence of a PGPR, *Pseudomonas putida* (UW4). Changes in flavonoids/phenolics were

observed in response to PHC stress and changes in flavonoid/phenolic production in response to PGPR were PHC dependent.

## 7.3 Materials and Methods

### 7.3.1 Bacterial strains preparation and plant growth

Fall rye seed (*S. cereale*, Ontario Seed Ltd., Waterloo, ON, Canada) were inoculated with *P. putida* UW4 (Glick, 1995; Shah et al., 1998) by soaking the seeds in bacterial suspension for 15 min and air drying for >1 hour prior to planting. Water was used for seed coating of control plants. Bacterial inoculum was prepared by growing UW4 (500 μL frozen stock in 100 mL of TSB (50 $g \cdot L^{-1}$, Fisher Scientific, Ottawa, Ontario, Canada)) for 24 h at RT with agitation. The bacterial culture was centrifuged (5000 rpm, 20 min at 4 $^{o}$C) and re-suspended in autoclaved deionized water to obtain a final OD of 1.5-2. The seeds were sown in PHC-contaminated soil, and control soil, in plastic four cell plant plug trays (Jack van Klaveren Co., St. Catherines, ON, Canada). Contaminated soil characteristics were described in Gurska et al. (2009) (Chapter 2). Control soil was obtained from uncontaminated land approximately 20 meters from the site. Plants were grown in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA) with lighting conditions with approximate ratio of PAR:UVA:UVB (~100:10:1), typically found in the mid-latitudes in the USA (Gerhardt et al., 2008) and a 16 h light/8 h dark photoperiod . Plants were harvested 10 days after seeding. Shoots and roots were rinsed in deionized water, separated and immediately placed in 80% methanol for extraction as described in the next section.

### 7.3.2 Quantitative PCR analysis of chalcone synthase transcript

Primers were designed using Primer3 version 0.4.0 (Rozen and Skaletsky, 2000) using sequenced cDNA fragments for target genes and sequences available through Genbank for reference genes. To confirm absence of secondary structures that would inhibit primer binding MFOLD software was used (Zuker, 2003) with the theoretical melting temperature of primers. Two micrograms of RNA from the treatments above was reverse transcribed with oligo$(dT)_{18}$ primer using First Strand cDNA Synthesis Kit (Fermentas International Inc.,

Burlington, ON, Canada) according to manufacturers instructions. A master mix comprising of SsoFast$^{TM}$EvaGreen$^{®}$ supermix, water and primers was made for each sample. For each reaction (10 µL total) the master mix was loaded into a Hard-Shell® thin-wall, 96 well skirted PCR plate (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and 1 µL of template was added. Each plate also consisted of a NTC (no template control) and "no RT" (no reverse transcriptase at the cDNA synthesis step) control. The plate was then placed in the thermal cycler programmed as follows: initial denaturation and activation of enzyme at 95 $^{o}$C for 30 s; denaturation at 94 $^{o}$C for 5 s, annealing/extension at 64 $^{o}$C for 5 s for 40 cycles. A melt curve was generated after each reaction by heating the products from 64 $^{o}$C to 95 $^{o}$C degrees in 0.5 $^{o}$C increments and taking a fluorescence reading after each temperature increase.

The reference genes primers used here were from previously published sequences: β-tubulin (Genbank accession# FJ032190.1), previously shown to be a good reference gene in cereals such as barley, wheat and oats (Jarosova and Kundu, 2010); actin (Genbank accession# FJ032189.1), which has often been used as a reference gene with mixed results (Migocka and Papierniak, 2010) and chalcone synthase in *S. cereale* (Genbank ID, X92547.1). Stability of the reference genes was analyzed using the geNorm algorithm (Vandesompele et al., 2002) based on their ranking according to their expression stability as described in previous chapters. Primers used were as follows: 5'TUB (5'- GCA CCA AGG AGG TTG ATG AG, Tm 64.6 $^{O}$C), 3'TUB (5'- AGT TGC CAA TGA AGG TGG AC, Tm 63.9 $^{O}$C ), 5'ACT (5'- CCC ATG CTA TCC TTC GTC TC, Tm 63.5 $^{O}$C), 5'ACT (ACC ACG CTC AGT CAG GAT CT, Tm 63.9 $^{O}$C), 5' CHS (CAT GTT GGG GTT GTC CTG TA, Tm 63.3 $^{O}$C) and 3' CHS (GTC AAA TCG CAG ATC AGG AA, Tm 62.8 $^{O}$C). All initial manipulations were performed using the Bio-Rad CFX96$^{TM}$ Real-Time PCR System (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and the Ct values were subsequently exported into qBase$^{PLUS}$ (Biogazelle, Ghent, Belgium). Single PCR product was confirmed by running the products out on a 3% agarose gel (1X TBA, 0.7 mg·mL$^{-1}$ ethidium bromide). Relative transcript levels were determined using the $2^{-\Delta\Delta C}{}_{T}$ method, which compares amplification measurements for gene of interest to that of a reference gene (Livak and Schmittgen, 2001). Additionally, the calculations were corrected for gene-specific

amplification efficiency, instead of the assumed efficiency of two copies per cycle of amplification (the 2 in $2^{-\Delta\Delta CT}$ method, (Pfaffl, 2006)). Incorporating this correction the relative expression ratio of a target gene was calculated as:

$$\text{Ratio} = (E_{TARGET})^{\Delta Ct(TARGET)/} (E_{REF})^{\Delta Ct(REF)}$$

where E is the efficiency of target or reference gene, ΔCt is the difference between the Ct of a control sample and a treated sample

$$(\Delta Ct_{TARGET} = Ct_{control} - Ct_{treatment} \text{ and } \Delta Ct_{REF} = Ct_{control} - Ct_{treatment}).$$

### 7.3.3 Phenolic and flavonoid extraction and analysis

Phenolic analysis was performed on the first leaf from fall rye plants and on several leaves of tall fescue plants. For fall rye, discs (0.4 $cm^2$, 20 mg of tissue, fresh weight) were removed from 3 separate plants of each treatment. For tall fescue several blades of grass were cut and combined to make up the appropriate weight (20 mg of tissue). Phenolics were extracted overnight at 4$^o$C in the dark in 1 mL of 80% methanol. Extracts were centrifuged at 13,000 rpm for 10 minutes to remove any particulate matter and supernatant was transferred to amber HPLC vials and stored in the dark at 4$^o$C prior to HPLC analysis.

HPLC separation was done using a Hypersil C18 5U 250x4.6 mm column (Cat# 30105254630; ThermoScientific, Nepean, ON, Canada). The column was washed and equilibrated prior to each analysis by running 80% acetonitrile/20% HPLC water (water with pH 3.0 with phosphoric acid) through the column for 15 minutes followed by 8% acetonitrile for 30 min at a flow rate of 1 mL/min, all at room temperature. 100 μL samples were loaded into the column via an autosampler and eluted from the column by increasing the concentration of acetonitrile over 30 min. Acetonitrile concentration was raised from 2.5%-2% (0-2 min), to 3.5% (2-4 min) to 12% (4-8min) to 15 (8-15min) to 17 (15-20min) to 26 (20-49 min) to 29 (49-50min) to 100 (50-55min).

Following phenolic/flavonoid separation with the HPLC, data were analyzed using the Ezchrome ™ software from Shimadzu. Phenolics/flavonoids between 5-40 minutes retention time were identified by comparison with spectra stored in the HPLC software library. The

overall phenolic/flavonoid content (total area under the flavonoid peaks) of uncontaminated control soil – PGPR samples and treated samples were obtained (Figure 7.3).

The peak of each compound was calculated as % of the total peak height in each sample (i.e. % of total soluble phenolics/flavonoids). These values were scaled to reflect the relative accumulation of each compound by multiplying the % of total soluble phenolic/flavonoids for each peak by the total phenolic/flavonoid accumulation in the particular treatment normalized to control treatment, giving the relative accumulation of a specific phenolic/flavonoid according to method of Gerhardt et al. (2008). Standard deviations (SD) were calculated for means of the independent experiment and scaled in the same way as the % of total soluble phenolics/flavonoids so that error bars remained relative to transformed data. Results are representative of three independent experiments.

### 7.3.4 Statistics

To determine differences in gene expression between treatments, data were analyzed using one-way ANOVA. Phenolic/flavonoid content changes were analyzed using a two-way ANOVA ($P < 0.05$). All statistical analyses were performed using SPSS version 16 (IBM Corporation, Somers, NY, USA).

## 7.4 Results

### 7.4.1 Plant growth

Plants were grown for ten days in PHC-contaminated and control soil. PHC resulted in stunted growth and lower germination rate in both fall rye and tall fescue plants (Figure 7.1). Further, fall rye plants displayed changes in leaf pigments, with an increase in red coloration to leaves when PHC contamination was present. Tall fescue germinated very poorly in PHC-contaminated soil after 10 days, with only a few plants emerging at this time (Figure 7.1). Plant growth was improved with UW4 treatment and fall rye pigment changes were not as pronounced in plants with UW4.

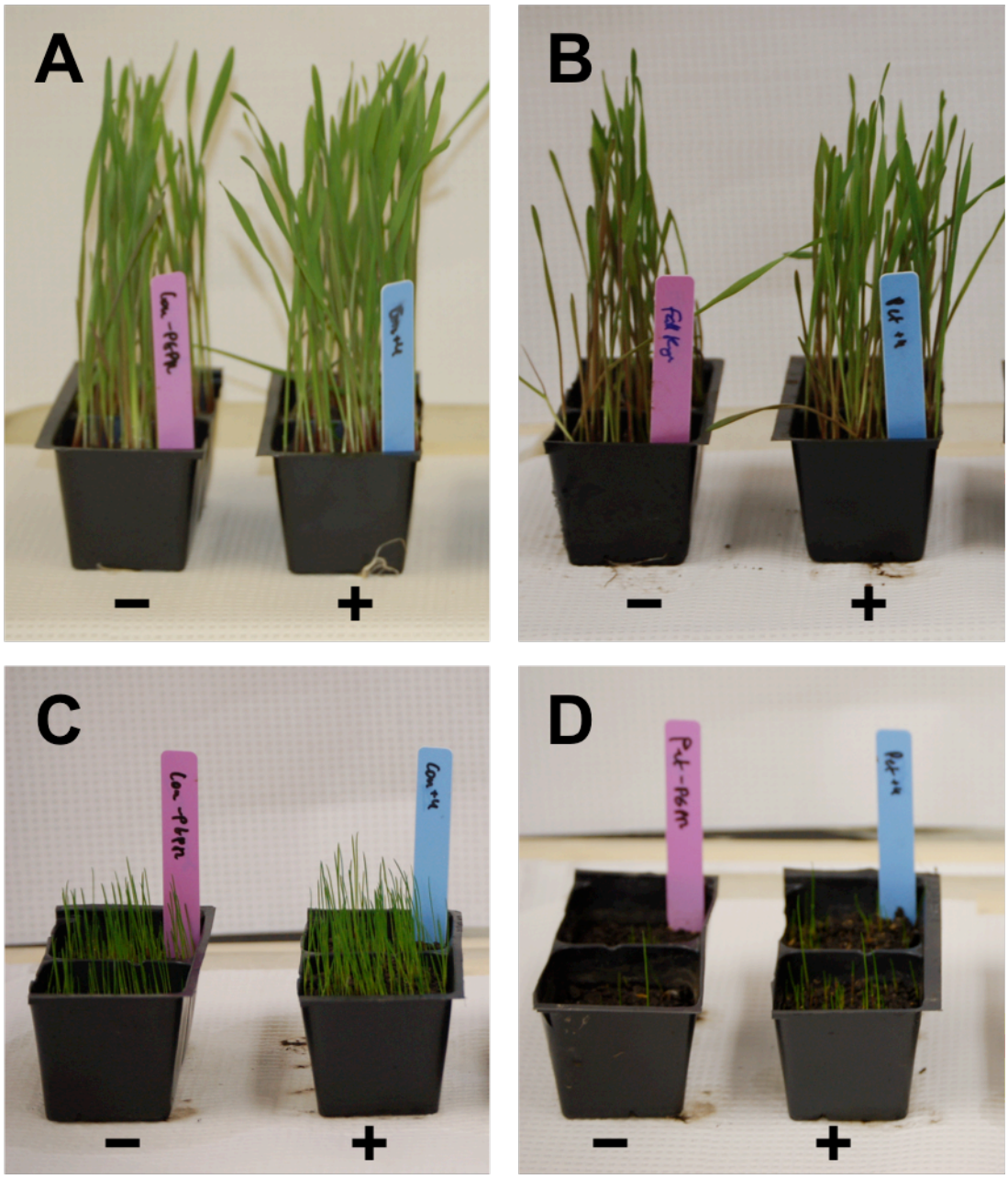


**Figure 7.1 Plant growth of fall rye and tall fescue plants used for phenolic/flavonoid extraction.**

Fall rye plants were grown for 10 days. Fall rye plants grown in uncontaminated soil (A) and PHC-contaminated (B) soil. Tall fescue plants were grown in uncontaminated soil (C) and PHC-contaminated soil (D). PGPR addition indicated by "–" or "+". The size of the bottom of each container is 4.7 cm x 4.7 cm.

### 7.4.2 Chalcone synthase transcript measurements

To quantify the expression of the gene in question using the $2^{-\Delta\Delta C}{}_T$ method, it is necessary to have a gene assumed to have remained constant under all experimental conditions as a point of reference. Based on previous studies with stable reference genes β-tubulin (*TUB*), β-actin (*ACT*) were chosen as potential candidates for this normalization (Jarosova and Kundu, 2010),(Jian et al., 2008; Migocka and Papierniak, 2010). A detailed description of the normalization process is provided in Chapter 6. The reference genes were used to normalize the PCR reactions for the amount of RNA added (Livak and Schmittgen, 2001). A combination (geometric mean) of *ACT* and *TUB* were chosen as reference for normalization (Pfaffl, 2006). To investigate CHS gene expression changes in response to PHC and PGPR in fall rye shoots, CHS gene expression in each treatment was normalized to control plants without UW4 inoculation (in addition to normalization to reference genes); PHC appeared to decrease CHS transcript levels in shoots and PGPR may have restored these levels to some extent although the changes were not significant (Figure 7.2).

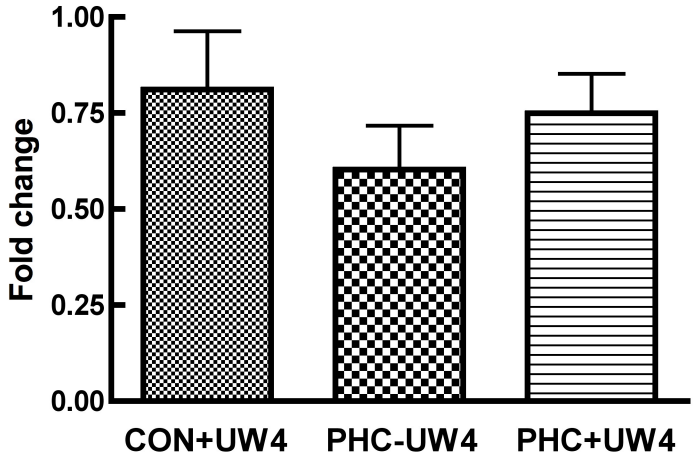


**Figure 7.2 Fall rye shoot CHS expression analysis with quantitative PCR.**

Shoot tissue expression of CHS normalized to control plants without PGPR (following normalization to the reference gene). Statistical analysis was performed with a one-way ANOVA and LSD post hoc test ($P < 0.05$) and was not significant, n=9±SD.

### 7.4.3 Phenolic and Flavonoid Analysis in *S. cereale*

Phenolic/flavonoid content was analyzed in the shoots and roots of plants. Root phenolic/flavonoid levels could not be replicated between experiments, likely due to low levels of these present in roots of plants. Phenolics/flavonoids were extracted in 80% methanol after collecting tissue from 10 day-old plants. Relative levels of phenolic/flavonoid accumulation were analyzed using high performance liquid chromatography in both uncontaminated and PHC-contaminated soil.

For fall rye, two-way ANOVA indicated that there was a significant effect of PGPR on total phenolic/flavonoid accumulation ($P < 0.05$) but no effect of PHC on total phenolic/flavonoid accumulation ($P > 0.05$). Levels of phenolics/flavonoids decreased with PGPR when PHC was present and increased when PHC was not present (Figure 7.3).

PGPR inoculation had an effect on the overall levels of flavonoids in *S. cereale* but this did not reveal whether the total increase in flavonoids resulted from an increase in all of the flavonoids, or if some were preferentially accumulated, lost or remained the same. Therefore the effects of PHC and PGPR treatment were evaluated for individual flavonoids/phenolics. Each major flavonoid peak identified in the HPLC profiles of fall rye (Figure 7.3) was calculated as percentage of total flavonoids/phenolics. Each peak data was normalized to reflect the increase in total flavonoids (peak % of total soluble flavonoids x total soluble flavonoids in treated/total soluble flavonoids in uncontaminated soil without UW4 (giving the relative accumulation of particular flavonoids). This calculation accounted for relative increases in total flavonoids between treatments. Based on these calculations, peak 5 and 6 decreased in fall rye when plants were grown in PHC-contaminated soil both with and without PGPR and peak 2 increased in tall fescue when these plants were grown in PHC contaminated soil with UW4.

In fall rye, peak 5 and 6 were consistently found to be lower in PHC plants, and even more so in plants treated with UW4. Their UV spectra closely resembled that of the apigenin standard and are thus likely two previously identified and characterized fall rye flavonoids: apigenin glycosides, and the C-glucosyl-apigenin-O-glycosides (Dellamonica et al., 1986).

The compound with a larger sugar molecule (galactoside has 6 carbons whereas arabinose has 5 carbons) will have a higher retention time so the two apigenins are isovitexin 2”-O-arabinoside and isovitexin 2”-O-galactoside in increasing order of retention time. Absorbance spectra of peak 5 and 6 were compared to the laboratory standard apigenin and were close in resemblance (Figure 7.4).

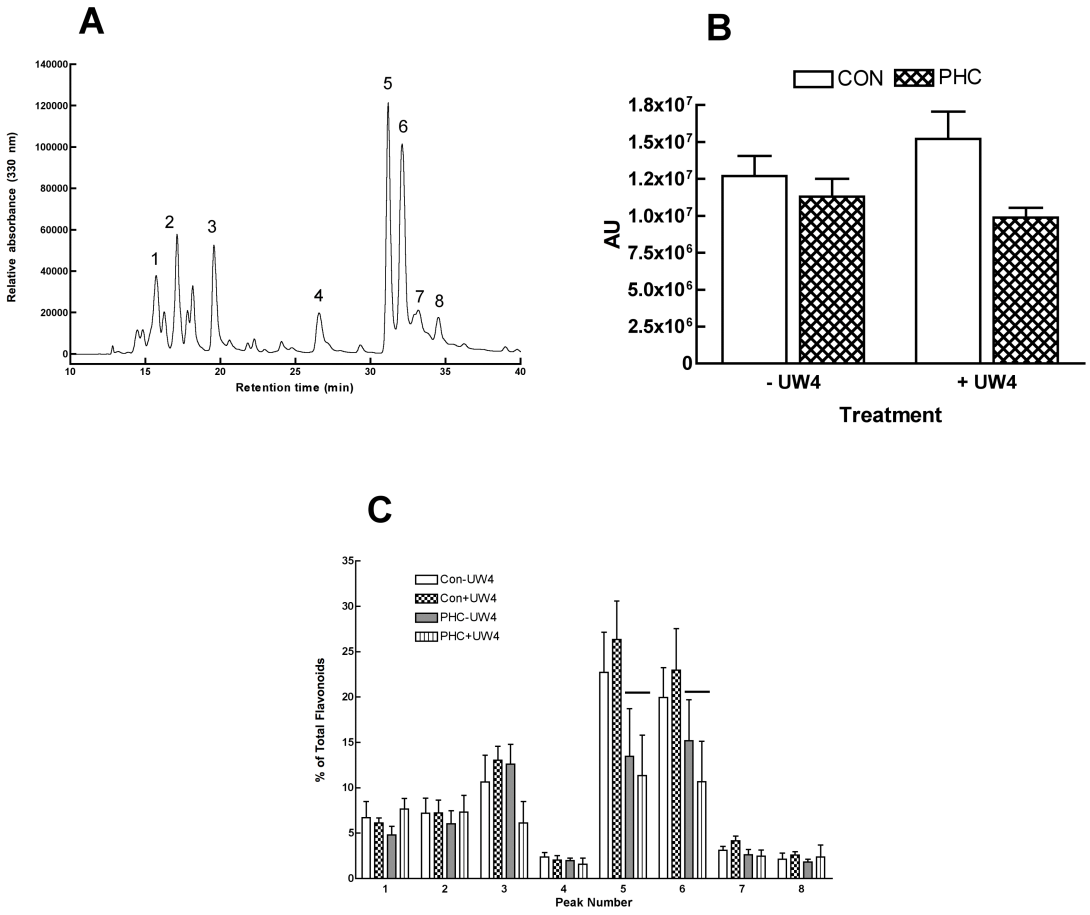


**Figure 7.3 HPLC separation and analysis of flavonoids in *S. cereale*.**

Typical flavonoid/phenolics profile is shown in (A). Each number indicates a peak, which was consistently observed in *S. cereale* chromatograms. Relative levels of flavonoid accumulation in S. cereale (B) exposed to uncontaminated and PHC-contaminated soil for 10 days. Individual peaks were examined in (C) in their respective PHC and UW4 treatments. Each bar represents n = 9 ± SD. Bars indicate peaks which are lower in PHC – UW4 and PHC + UW4 treatments. Flavonoids were extracted in 80% methanol and analyzed by HPLC. Each bar is a mean ± SD of three independent experiments. AU, arbitrary units measured at 330 nm.

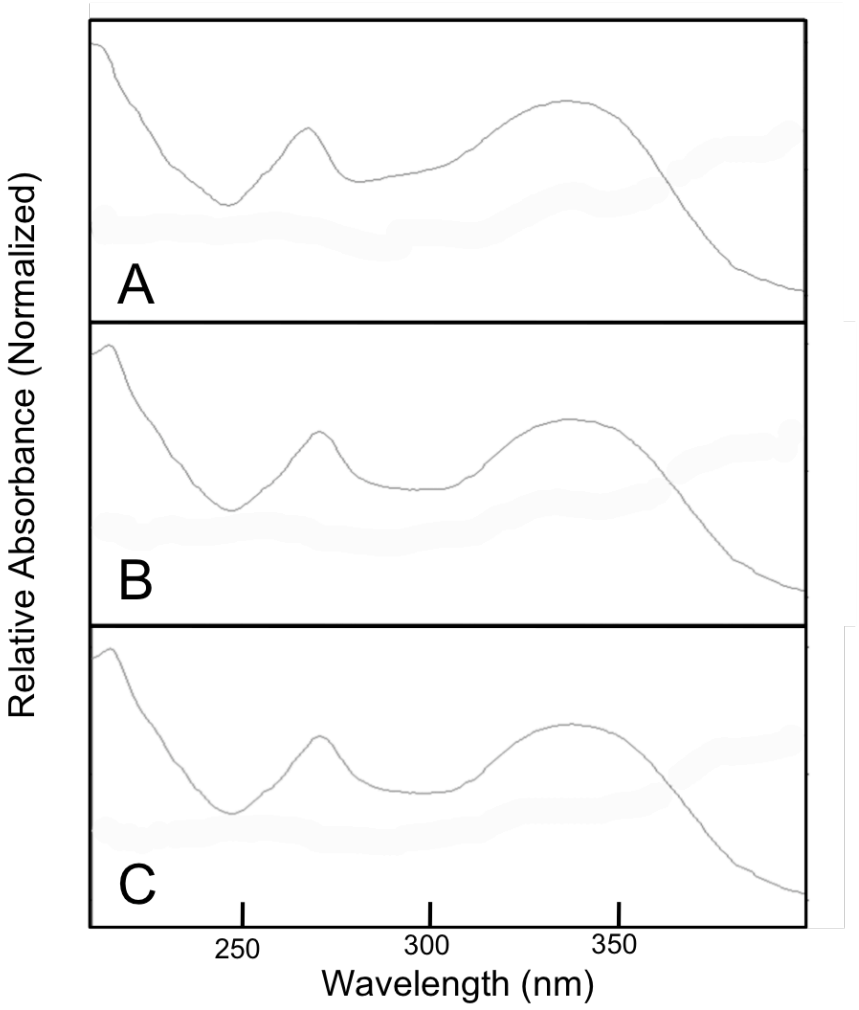


**Figure 7.4 Absorbance spectra of unidentified flavonoid peaks from fall rye and apigenin.**

Absorbance spectra of flavonoid apigening (A) and peaks 5 (B) and 6 (C). Absorbance spectra of two unknown peaks from *S. cereale* leaves were obtained from HPLC elution. Those spectra were compared to spectra of known flavonoids. Both absorbance spectra closely matched that of apigenin.

### 7.4.4 Phenolic and Flavonoid Analysis in *F. arundinacea*

Phenolic/flavonoid content was analyzed in the shoots and roots of plants. As with fall rye plants, root phenolic/flavonoid levels could not be replicated between experiments, due to low levels of phenolics plants. Tall fescue had even less plant root tissue than fall rye at the 10 day time point.

For *F. arundinacea*, there was a significant statistical interaction between PHC and bacteria when two-way ANOVA was conducted ($P < 0.05$), suggesting that PGPR changed the effect of PHC on phenolic/flavonoid production. PGPR inoculation had an effect on the overall levels of flavonoids in *F. arundinacea* however this did not reveal whether the total increase in flavonoids resulted from an increase in all of the flavonoids, or if some were preferentially accumulated, lost or remained the same. Therefore the effects of PHC and PGPR treatment was evaluated for individual flavonoids in both plant species. Each major flavonoid peak identified in the HPLC profile of tall fescue (Figure 7.4) was calculated as percentage of total flavonoids similarly to fall rye. Based on this analysis, peak 2 increased in tall fescue when these plants were grown in PHC contaminated soil with UW4. The UV spectra of peak 2 closely resembled that of chlorogenic acid standard; tall fescue was previously found to have large amounts of chlorogenic acid in its phenolic extracts (Johnson et al., 2002). Chlorogenic acid is not a flavonoid however, it is a phenolic compound belonging to the hydroxycinnamic acid (HCA) derivative group that would be detected in the range analyzed (Naczk and Shahidi, 2006). The smaller peak, peak 1 was compared to the spectra of chlorogenic acid and was closely matched. Again, it is likely chlorogenic acid with a larger sugar molecule which will have a higher retention time so the two chlorogenic acids will have sugars with different molecular masses, with the smaller one appearing on the chromatogram in less time than the larger molecule. Absorbance spectra of peak 1 and 2 were compared to the laboratory standard of chlorogenic acid and resembled it closely (Figure 7.6). There were a number of smaller peaks following peak 2, however due to their small size they could not conclusively be indentified or analyzed. Those would likely have been the flavonoid rutin which has been characterized in tall fescue (Johnson et al., 2002).

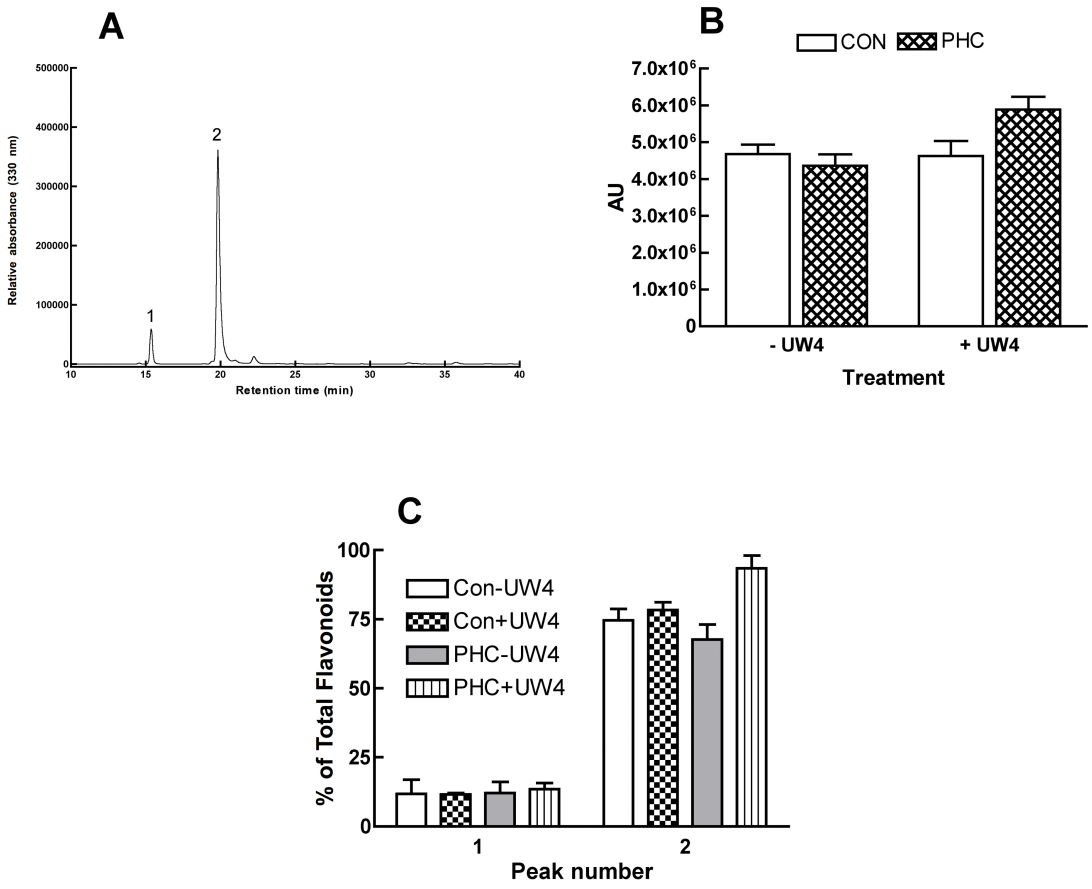


**Figure 7.5 HPLC separation and analysis of flavonoids in *F. arundinacea*.**

Typical profile is shown in (A). Each number indicates a peak, which was consistently observed in *F. arundinacea* plant chromatograms. Relative levels of flavonoid accumulation in *F. arundinacea* (B) exposed to uncontaminated and PHC-contaminated soil for 10 days. Individual peaks were examined in (C) in their respective PHC and UW4 treatments. Each bar represents n = 9 ± SD. Bars indicate peaks which are lower in PHC – UW4 and PHC + UW4 treatments. Flavonoids were extracted in 80% methanol and analyzed by HPLC. Each bar is a mean ± SD of three independent experiments. AU, arbitrary units measured at 330 nm.

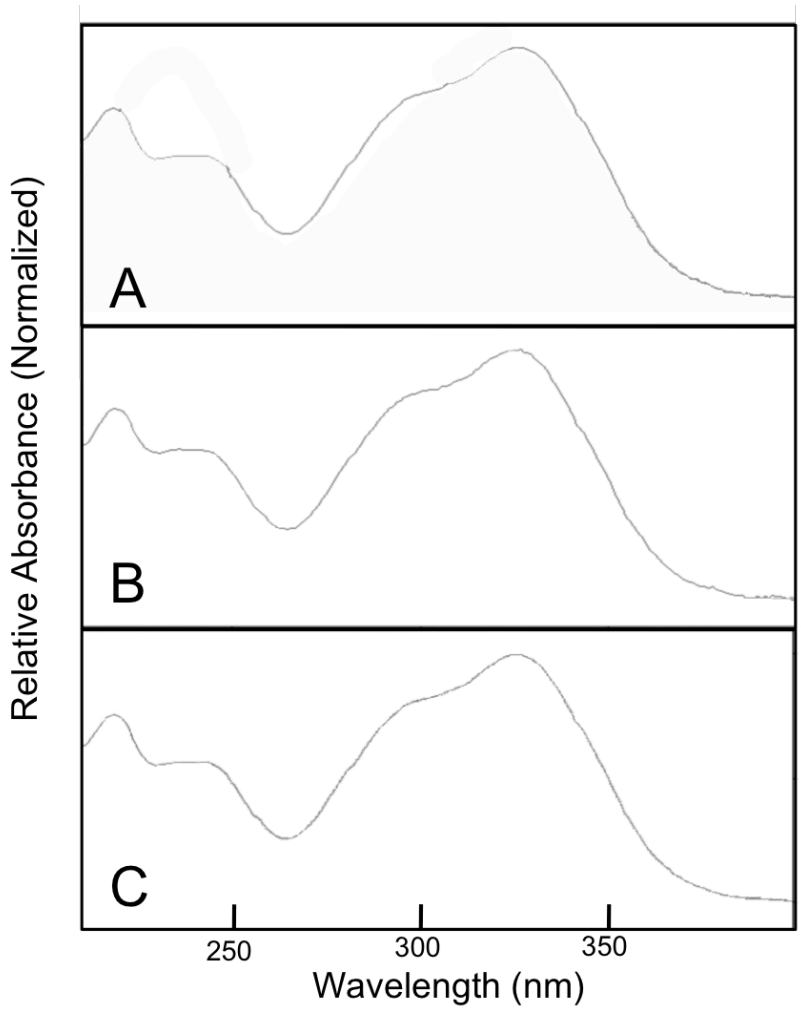


**Figure 7.6 Absorbance spectra of unidentified flavonoid peaks from tall fescue and chlorogenic acid.**

Absorbance spectra of chlorogenic acid (A) compared to flavonoid peaks 1 (B) and 2 (C) from tall fescue leaves. Absorbance spectra of two unknown peaks from tall fescue leaves were obtained from HPLC elution. Those spectra were compared to spectra of known flavonoids. Both absorbance spectra closely matched that of chlorogenic acid.

## 7.5 Discussion

Plant growth was inhibited in both fall rye and tall fescue when grown in PHC-contaminated soil, compared to those in control soil. Notably, when fall rye was grown in PHC-contaminated soils, plants had a distinctive red colour, likely due to accumulation of plant phenolics (anthocyanins), which are a characteristic feature of plant stress (Winkel-Shirley, 2002). This effect was also partially alleviated when PGPR were used with PHC-grown plants. When plant seeds were inoculated with UW4, this partially restored plant growth in contaminated soil. Plant growth was also improved when plants were grown in uncontaminated control soil. Along with increased plant growth, photosynthetic parameters such as non-photosynthetic quenching were improved when plants were grown in PHC (results presented in Chapter 6).

The role of flavonoids in plant stress response is complex. Flavonoids are products of the central phenylpropanoid pathway, where the first committed step is at the enzyme chalcone synthase (CHS). In the current study, CHS levels appeared to decrease when plants were grown in PHC-contaminated soils. However, when UW4 was present there was a modest increase in CHS transcript relative to plants without UW4 grown in uncontaminated soil. This result is not unexpected. In a study by Czarny (2008) where microarray data was examined, CHS gene expression in 6 day-old *Brassica napus* seedlings decreased with application of UW4. A decrease in transcript levels was also observed in 3-week old *B. napus* plants grown in nickel-spiked soil. The correlation between these studies corroborates that CHS may be downregulated either due to UW4 or due to contaminant stress. The lower expression of CHS, is likely a result of a larger stress response where secondary plant metabolite pathways show lower gene expression. Interestingly, previous studies link flavonoids as being involved in controlling polar transport of the plant growth regulator auxin, although more investigation is needed in this area (Winkel-Shirley, 2002). Mutants that were deficient in CHS activity have been shown to have elevated auxin transport, and had elevated levels of an auxin, indole acetic acid (IAA), in upper root tissues (Murphy et al., 2000; Brown et al., 2001). Further, it has been described that rhizobia promote flavonoid

production as a method to interfere with auxin transport (Wasson et al., 2006). CHS transcript levels likely have implications on auxin levels that lead to the stress response observed in PHC contaminated soils. This may be a way PGPR control stress responses in plants through increased auxin levels that may, downstream, lead to lowered stress.

The initial screening of changes in shoot flavonoids/phenols were revealing of PGPR effects on the plants studied. Comparing shoot versus roots allowed for close examination of those changes without the setback of variability due to low phenolics levels in the roots or in the soil matrix. With the presence of bacteria in the soil where flavonoids changes are studied, there is a possibility that rhizoexudates may be degraded prior to detection, or that their synthesis can be modulated by microbes (Dardanelli et al., 2009). This problem was overcome by examining shoot flavonoid levels. It was reasoned that studying PHC effects in soil in such a complex system is challenging and their complete recovery in a non-hydroponic system will be unfeasible due to degradation and loss due to matrix binding or background extraction. Thus, flavonoid production in the shoots was examined.

One assumption made in the current study was that the effects of PHC and PGPR would be observed in plant shoots and would approximate what is happening in the roots, in respect to both gene expression and flavonoid production. Measured CHS and flavonoid content in the shoot tissue may not depict what is happening in the roots accurately however. Roots possess their own CHS, localized to the cortex cells and the epidermal cells of the elongation zone of the root tip, where flavonoids accumulate (Saslowsky and Winkel-Shirley, 2001). Changes in flavonoid levels may be more visible in root tissue than in shoot tissue because roots are in closer proximity to contaminants. Further, exudate-bound flavonoids may be derived from the roots, so measured changes in shoot flavonoids does not imply change to exudation to the rhizosphere. Nonetheless, physiological effects in shoot flavonoids were observed in response to PGPR and were further modulated in the presence of PHC, demonstrating that PHC and PGPR affect the plant shoot physiology.

The most important finding in this study was the determination that UW4 modulates total flavonoid levels in both fall rye and tall fescue and this effect changes with the presence of

PHC. The decrease in two peaks of fall rye and the overall decrease in total flavonoid accumulation are somewhat counterintuitive when one considers the hypothesis that plants grown in PHC contaminated soil would increase exudation to stimulate microbial growth. If PHC has an effect on plants it would be expected that flavonoid levels would increase, to then increase exudation. This can be explained in the following ways. To reiterate, the flavonoid levels in shoots were examined, and what is happening in the shoots may not reflect what is happening in the roots. It is possible that while some phenolics are decreased (flavonoids), other phenolics that were not detected by this method were increased. In a recent study investigating salt stress and PGPR influence on flavonoid patterns exuded by soy bean roots, it was discovered that only four flavonoids were exuded under all conditions, some were not detected when salt was present while others where not detected when PGPR were present (Dardanelli et al., 2009). This confirms that plant contaminant stress and PGPR, result in a complex flavonoid response where individual flavonoids may play a variety of complex roles that remain to be elucidated.

Fall rye has been shown to have two epidermal flavonoids, which decreased with PHC and with UW4. Those have previously been characterized in fall rye (Dellamonica et al., 1986). The predominant phenolic compound detected in tall fescue belonged to the hydroxycinnamic acid (HCA) derivative group (Naczk and Shahidi, 2006). As it is a phenolic compound similar to flavonoids it was detected through the same analysis as flavonoids because it gave a UV spectra. Chlorogenic acid has previously been reported in root exudates (Campbell et al., 1997) and is a potential allolochemical (Khanh et al., 2005). It appeared that chlorogenic acid increased when UW4 was added to seeds grown in PHC-contaminated soils. This shows potential for further investigation as tall fescue has been documented to be a good PHC phytoremediator in numerous studies (Epuri and Sorensen, 1997; Kulakow et al., 2000; Huang et al., 2004a, 2004b), and the increase in cholorogenic acid in the shoots, if mirrored in the rhizosphere may be a factor that stimulates the PHC degrading community.

## 7.6 Acknowledgements

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# Chapter 8
# General Conclusions

Phytoremediation of petroleum hydrocarbons (PHC) promises to be a viable remediation strategy if toxicity of PHC can be overcome and plant growth increased to facilitate remediation. Current research has focused on optimizing amendments to spur phytoremediation and one avenue that has potential is the use of plant growth promoting rhizobacteria (PGPR) to facilitate plant growth and remediation. The proceeding chapters examined the application of PGPR to alleviate PHC toxicity to plants and increase remediation. The effects of PGPR on plant performance were assessed as well as remediation over the course of three years at each field site. It was shown that performance of PGPR-enhanced phytoremediation is better than phytoremediation without PGPR amendments at PHC-contaminated sites.

Despite these growth improvements remediation over time appeared to plateau. This was particularly evident at the site with low PHC contamination. One potential cause for this may be the increase of biogenic compounds that are released during the lifetime of plants on site and their improper removal from the soil samples during analysis. These compounds can artificially increase PHC concentrations if not properly removed from sample extracts, as there is currently no way to differentiate between biogenic and petrogenic compounds during standard sample analysis. When it is considered that recalcitrant PHC components, such as PAHs, continue to be remediated this raises concerns when overall PHC content is not decreasing. Thus, biogenics accumulation in soils undergoing phytoremediation must be investigated further.

Bacterial populations in the soil were monitored temporally to evaluate the effects of PGPR on degradation and on native bacterial community in the soil and it was found that PGPR-enhanced phytoremediation had higher number of microbes in the rhizosphere but addition of PGPR did not drastically change the microbial community. In a separate study,

newly isolated PGPR were investigated and their influence on plant growth was examined with the possibility of site-specific PGPR use that would comply with regulatory demands. This evaluation found that combinations of PGPR often perform better than individual species. If possible, these PGPR should be tested in the field, where the mixtures are expected to promote plant growth and increase remediation.

In the greenhouse, using field soils, the effects of high levels of PHC on plant physiology and plant gene expression were studied. The gene changes in roots and shoots of fall rye provided a better understanding of how the plant copes with PHC stress and how UW4 modulates this stress. It is hoped that further investigation, particularly of the cytochrome P450 gene will provide more information about its function in plant stress. Cytochrome P450 gene found may be implicated in detoxification mechanisms, and is likely the first time this sequence was examined in *Secale cereale*. The gene expression study combined with plant physiology data indicated that plants were only moderately stressed from the high levels of PHC and that PGPR alleviated this stress. Phytoene desaturase indicated oxidative stress response upon PHC exposure that was enhanced with UW4. Plasma membrane $H^+$TPase mediates stress responses in the cell and may be upregulated in response to the PGPR production of plant hormones, which is an established plant growth promotion mechanism. The established primers for these genes can be used to evaluate other stressors in *S. cereale*.

Data from analysis of phenolics and flavonoids showed that PGPR alter production of these compounds in shoots when grown under PHC stress. Of particular interest, is the chlorogenic acid that may play a role in remediation of organics in the rhizosphere. It would be useful to determine if chlorogenic acid is exuded when plants are experiencing PHC stress, and to further elucidate its activity on rhizosphere bacteria, providing more insight into plant-microbe rhizosphere interactions.

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# Appendix A

# Supplementary Data for Chapter 1

**Table A1. Average nutrient and petroleum contaminant levels in soil from the experimental sites.**

| Soil Parameter | Percentage or Concentration |
|---|---|
| Sand | 46.3% |
| Silt | 38.5% |
| Clay | 15.2% |
| Phosphorous | 66 mg $kg^{-1}$ |
| Potassium | 394.4 mg $kg^{-1}$ |
| Magnesium | 179.3 mg $kg^{-1}$ |
| Organic matter | 20.1% |
| Total phosphorous | 111.7 mg $kg^{-1}$ |
| pH | 7.1 |
| Fraction 2 | 2.9 g $kg^{-1}$ (±0.71; n≥3) |
| Fraction 3 | 26.5 g $kg^{-1}$ (±6.36; n≥3) |
| Fraction 4 | 11.3 g $kg^{-1}$ (±2.47; n≥3) |
| Total PHC | 129.3 g $kg^{-1}$ (±7.78; n≥5) |

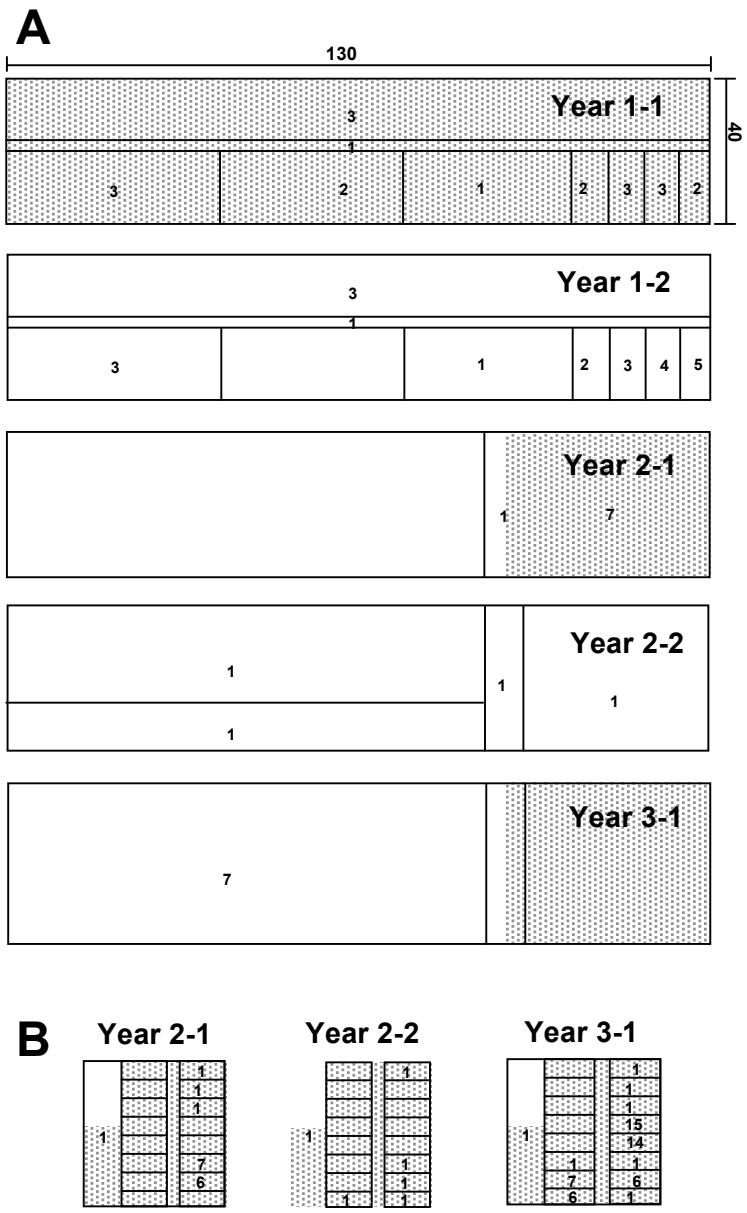


**Figure A1. Planting schematic for Sarnia landfarm.**

A. Planting schematic for site 1: year 1 first planting (year 1-1), year 1 second planting (year 1-2), year 2 (year 2-1), year 2 second planting (year 2-2), year 3 first planting (year 3-1). B. Planting schematic for site 2, from left to right: year 2 first planting (year 2-1), year 2 second planting (year 2-2), year 3 first planting (year 3-1). Numbers indicate: 1, unplanted soil; 2, annual ryegrass – PGPR; 3, annual ryegrass + PGPR; 4, tall fescue – PGPR, 5, tall fescue + PGPR; 6, annual ryegrass/tall fescue mixture – PGPR; 7, annual ryegrass/tall fescue mixture +PGPR; 8, triticale – PGPR; 9, triticale + PGPR; 10, barley/fall rye – PGPR; 11, barley/fall rye +PGPR; 12, fall rye – PGPR; 13, fall rye + PGPR; 14 barley/annual ryegrass/tall fescue – PGPR; 15, barley/annual ryegrass/tall fescue + PGPR. Empty blocks indicate no over-seeding if block previously planted. One planting only if only first planting indicated. Shaded areas indicate tilling prior to planting.

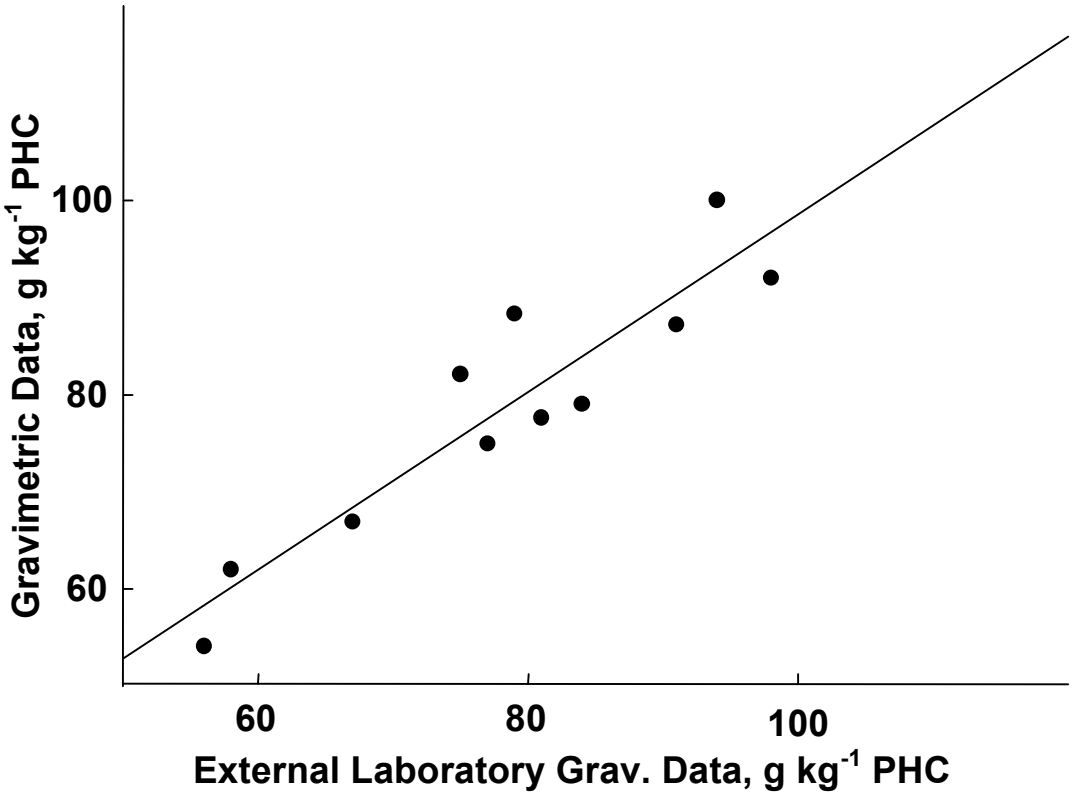


**Figure A.2 Gravimetric data comparison.**

Standard curve comparison of gravimetric data obtained in our laboratory and external laboratory gravimetric data for a representative sampling performed at the land farm in September 2006 ($r^2 = 0.85$, equation of the line: $y = 0.93x + 0.48$).

**Table A.2 Depletion of PHC in test plots at site 2.**

Measurements performed in year 2 (2005) and year 3 (2006). Average PHC levels in g $kg^{-1}$ of soil, where error bars represent ±SE (n≥2). AR - Annual ryegrass, TF – tall fescue, B – barley, FR – fall rye. Asterisks indicate numbers that are significantly different at the end of the growth period from beginning of growth period for the given treatment ($p > 0.05$). Symbol $^{\#}$ indicates numbers where remediation with PGPR differed significantly from that without PGPR ($p > 0.05$)

| Year | Plants | PGPR | Sample collection time | PHC | % Remediation |
|---|---|---|---|---|---|
| 2005 | AR | - | Spring 05 | 120.8±9.6 | |
| | | | Spring 06 | 106.7±1.1 | 11 |
| | | + | Spring 05 | 112.2±1.3 | |
| | | | Spring 06 | 94.5±3.5* | 15 |
| | TF/AR | - | Spring 05 | 113.5±7.8 | |
| | | | Spring 06 | 91.9±8.7 | 19 |
| | | + | Spring 05 | 131.2±9.6 | |
| | | | Spring 06 | 102.4±6.3* | 21 |
| | B/FR | - | Spring 05 | 87.1±5.9 | |
| | | | Spring 06 | 62.4±0.8$^{\#}$ | 28 |
| | | + | Spring 05 | 63.9±2.5 | |
| | | | Spring 06 | 52.7±0.06*$^{\#}$ | 17.5 |
| | Blank | - | Spring 05 | 89.0±32.8 | |
| | | | Spring 06 | 88.5±38.0 | 0.6 |
| 2006 | TF/AR | - | Spring 06 | 92.6±6.1 | |
| | | | Autumn 06 | 80.5±8.0 | 13 |
| | | + | Spring 06 | 99.0±7.6 | |
| | | | Autumn 06 | 84.0±1.3 | 15 |
| | B/TF/A | - | Spring 06 | 65.3±2.9 | |
| | | | Autumn 06 | 64.2±6.2 | 1 |
| | | + | Spring 06 | 73.6±7.9 | |
| | | | Autumn 06 | 60.3±6.0 | 18 |
| | Blank | - | Spring 06 | 100.8±22.4 | |
| | | | Autumn 06 | 92.1±24.5 | 8.6 |
| | | | **+ PGPR** | **- PGPR** | |
| Remediation Ave. (%) | | | 18 | 15 | |
| Significance ($P < 0.05$) | | | Yes | No | |

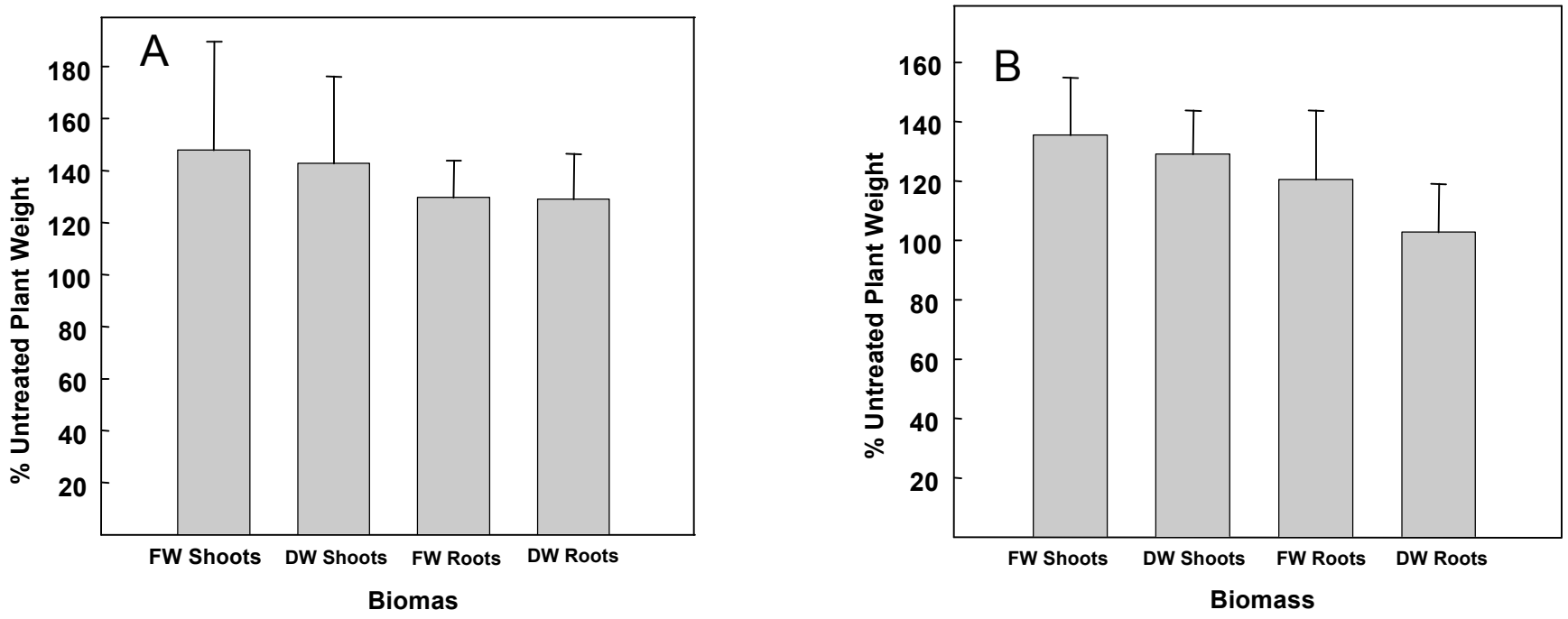


**Figure A3. Plant growth at Sarnia landfarm.**

Average dry weight (DW) and fresh weight (FW) measurements for 2006 growth season at site 2 using a mixture of annual ryegrass and tall fescue (A), or a mixture of annual ryegrass, tall fescue and barley (B). In all cases data was normalized to plants grown – PGPR (100%) and represent increase of + PGPR relative to – PGPR. Bars represent the mean ± SE (n≥24) measurements of different time points (May 29, July 6, August 13, and September 2). Statistical analysis using one-way ANOVA between – PGPR and + PGPR was performed and no significant differences were detected (P>0.05).

# Appendix B

## Supplementary Biolog data

| Microbiological Variables | | 2006 | | | 2007 | | |
|---|---|---|---|---|---|---|---|
| | | **Unvegetated** | **- PGPR** | **+PGPR** | **Unvegetated** | **-PGPR** | **+PGPR** |
| **Carbohydrates** | D-cellobiose | 1.31±0.08 | 1.52±0.08 | 1.45±0.06 | 1.96±0.76 | 1.10±0.21 | 1.54±0.15 |
| | α-D-lactose | 0.35±0.13 | 1.25±0.16** | 1.37±0.15** | 0.49±0.19 | 1.17±0.22 | 1.35±0.19* |
| | β-methyl-D-glucoside | 0.83±0.21 | 0.96±0.08 | 0.86±0.05 | 0.86±0.63 | 0.40±0.08 | 0.90±0.30 |
| | D-xylose | 0.94±0.32 | 1.40±0.11* | 1.48±0.06** | 1.71±0.23 | 1.42±0.15 | 0.36±0.10 |
| | i-erytritol | 0.48±0.19 | 1.25±0.08** | 1.12±0.12** | 0.09±0.08 | 0.86±0.11** | 0.99±0.12** |
| | D-mannitol | 1.88±0.36 | 1.87±0.10 | 1.69±0.09 | 2.48±0.31 | 2.00±0.36 | 2.14 ±0.17 |
| | *N*-acetyl-D glucosamine | 1.54±0.19 | 1.23±0.05** | 1.15±0.04** | 1.27±0.46 | 1.10±0.21 | 1.60±0.21 |
| | Glucose-1-phosphate | 0.20±0.09 | 0.71±0.14** | 0.59±0.09** | 0.13±0.08 | 0.38±0.12 | 0.69±0.13** |
| | Glycerol-1-phosphate | 0.14±0.02 | 0.19±0.02 | 0.21±0.02 | 0.42±0.10 | 0.42±0.26 | 0.19±0.01 |
| | Galactonic lactone acid | 0.74±0.11 | 0.58±0.05a | 0.73±0.04a | 0.43±0.13 | 0.82±0.11* | 0.75±0.09 |
| | **Average** | 0.84±0.08 | 1.07±0.01** | 1.00±0.01** | 0.98±0.13 | 0.97±0.06 | 1.15±0.07 |
| **Carboxyl** | Pyruvic acid methyl ester | 1.41±0.31 | 0.91±0.05** | 1.03±0.06* | 0.89±0.27 | 0.71±0.16 | 0.93±0.13 |
| | D-glucosaminic acid | 1.13±0.21 | 0.84±0.04 | 0.84±0.04 | 1.11±0.33 | 0.86±0.16 | 0.70±0.07 |
| | Galacturonic acid | 1.39±0.25 | 1.18±0.09 | 1.03±0.05* | 1.51±0.47 | 1.75±0.36 | 1.95±0.29 |
| | γ-hydroxybutiric acid | 0.72±0.49 | 0.65±0.21 | 1.04±0.13 | 0.79±0.32 | 1.12±0.25 | 0.97±0.20 |
| | Itaconic acid | 1.12±0.20 | 1.11±0.11 | 0.97±0.06 | 0.19±0.07 | 0.81±0.22* | 0.51±0.18 |
| | α-ketobutiric acid | 0.10±0.04 | 0.46±0.15** | 0.35±0.06* | 0.09±0.06 | 0.09±0.04 | 0.09±0.08 |
| | D-malic acid | 0.81±0.19 | 0.80±0.07 | 0.78±0.05 | 0.58±0.12 | 0.74±0.28 | 0.68±0.16 |
| | **Average** | 0.94±0.06 | 0.88±0.03 | 0.88±0.02 | 0.74±0.06 | 0.87±0.09 | 0.83±0.05 |
| **Polymer** | Tween 40 | 0.64±0.09 | 0.59±0.05** | 0.50± 0.04** | 3.42±0.62 | 3.40±0.43 | 2.46±0.21 |
| | Tween 80 | 2.01±0.20 | 1.47±0.13 | 1.29±0.05 | 2.63±0.66 | 2.68±0.56 | 1.98±0.10 |
| | α-cyclodextrine | 1.18±0.40 | 1.30±0.15 | 1.58±0.04* | 0.13±0.06 | 0.45±0.22 | 0.43±0.17 |
| | Glycogen | 0.68±0.18 | 1.13±0.06** | 1.27± 0.03** | 1.04±0.32 | 0.20±0.09 | 0.52±0.18 |
| | **Average** | 1.47±0.14 | 1.35±0.06 | 1.37±0.03 | 1.81±0.24 | 1.68±0.29 | 1.61±0.08 |

**Table continued on next page**



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| Microbiological Variables | | 2006 | | | 2007 | | |
|---|---|---|---|---|---|---|---|
| | | Unvegetated | - PGPR | +PGPR | Unvegetated | -PGPR | +PGPR |
| **Shannon's S*** | **7 day of incubation** | 3.15±0.05 | 3.34±0.02 | 3.34±0.02 | 2.74±0.14 | 3.17±0.16 | 3.17±0.05 |
| | **AWCD*** | 3.09±0.06 | 3.19±0.06 | 3.19±0.09 | 2.65±0.33 | 3.06±0.08 | 3.11±0.11 |
| **Amine** | L-arginine | 1.29±0.34 | 1.13±0.07 | 1.12±0.04 | 1.17±0.49 | 1.30±0.17 | 1.17±0.19 |
| | L-asparagine | 1.82±0.11 | 1.49±0.07* | 0.13±0.06** | 1.65±0.65 | 2.41±0.20 | 2.45±0.13 |
| | L-serine | 1.54±0.18 | 1.09±0.04** | 1.15±0.06** | 0.57±0.30 | 1.03±0.23 | 1.23±0.19 |
| | L-phenylalanie | 1.18±0.12 | 0.75±0.07** | 0.90±0.06** | 0.40±0.14 | 0.48±0.11 | 0.44±0.08 |
| | L-threonine | 0.95±0.41 | 0.86±0.17 | 0.66±0.10 | 0.07±0.03 | 0.31±0.28 | 0.20±0.12 |
| | Glycyl-L-glutamic acid | 0.71±0.13 | 0.56±0.11 | 0.61±0.05 | 0.13±0.04 | 0.39±0.07 | 0.60±0.16** |
| | **Average** | 1.25±0.06 | 0.98±0.06** | 0.97±0.03** | 0.67±0.20 | 1.00±0.05 | 1.02±0.05 |
| **Amino** | Phenylethylamine | 0.64±0.28 | 0.82±0.17 | 1.06±0.09* | 0.44±0.23 | 0.55±0.14 | 0.55±0.13 |
| | Putrescine | 0.64±0.09 | 0.59±0.05 | 0.49 ±0.04* | 0.64±0.19 | 0.79±0.12 | 0.64±0.12 |
| | **Average** | 0.64±0.17 | 0.71±0.06 | 0.78±0.05 | 0.54±0.15 | 0.67±0.02 | 0.59±0.10 |
| **Phenol** | 2-hydroxybenzoic acid | 0.11±0.08 | 0.53±0.16** | 0.54±0.10** | 0.10±0.05 | 0.22±0.11 | 0.07±0.04 |
| | 4-hydroxybenzoic | 1.14±0.18 | 0.93±0.04* | 0.93±0.05* | 0.52±0.22 | 0.83±0.19 | 1.00±0.13 |
| | **Average** | 0.63±0.10 | 0.73±0.08 | 0.74±0.05 | 0.31±0.10 | 0.53±0.12 | 0.54±0.07 |

* Shannon's S was determined using data collected at day 7 of incubation and using the appropriate AWCD values of 0.5 and 0.7 as described in section 3.4.7.