Field Application of Plant Growth Promoting Rhizobacteria Enhanced Phytoremediation on an Urban Brownfield and the Derivation of Direct Soil Contact Values for Weathered Petroleum Hydrocarbons (Fraction 3)

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

There are over 30,000 contaminated brownfield sites in Canada, many of which are located in desirable urban areas; and therefore have a high value for potential redevelopment. The remediation of urban brownfield sites presents a challenge as they frequently contain very complex mixtures of contaminants. Phytoremediation is a cost-effective technology that uses plants to remediate a range of contaminants from the soil. However, the capabilities of currently used phytoremediation systems are often limited due to poor bioavailability of the contaminants, growth inhibition caused by poor soil quality, and the presence of contaminant mixtures. One way to enhance phytoremediation systems is the use of plant growth promoting rhizobacteria (PGPR), naturally present soil bacteria that are able to directly and indirectly influence plant growth and reduce the production of stress ethylene in plants. PGPR enhanced phytoremediation systems (PEPS) have been successfully used to remediate rural sites; however, they have not yet been applied to urban brownfields.

In this study a two year field trial was conducted on an urban brownfield site in Toronto, Ontario, Canada, to evaluate the ability of PEPS to enhance plant growth and remediate petroleum hydrocarbons (PHCs) on a mixed contaminant site. The effectiveness of PEPS was evaluated through plant biomass production and PHC remediation. Based on germination rates and biomass production in the field, sunflowers (*Helianthus annuus* var Mammoth Russian) were recommended for PEPS on urban brownfields. PGPR did not have a consistent effect on plant biomass production during the field trials. The impact of PGPR on the native microbial community was evaluated to ensure there were no adverse effects. Bacterial numbers in the rhizosphere of all planted treatments increased throughout the second field season. The plant species utilized, rather than the PGPR treatment, had the largest impact on the microbial community. A significant decrease in the PHC concentrations was observed during the second field season in planted plots treated with PGPR, with an average percent remediation of 25 percent (%). Therefore, based on the results of the two year field trial, PEPS shows promise as an effective remediation technique for urban brownfield remediation.

Concerns were raised that the developed fine- and coarse-grained Ecological Tier 1 Canada-wide standards (CWS) for PHC fraction 3 (F3) based on freshly spiked soils are overly conservative and may result in unnecessary and costly remediation. Although the PHC CWS were revised using more recent toxicological data from field studies, the applicability of the current guidelines to sites with historical PHC contamination warrants further investigation as studies with a limited number of PHC concentrations and a ranked response approach were relied upon to derive the current PHC CWS for F3.

In this study, plant toxicity tests were conducted to examine the toxicity of weathered PHC (mostly F3) in a coarse-grained soil to derive direct soil contact values for ecological receptors. Coarse grained field and reference soils were obtained from a landfarm site where PHC sludge had been spread for approximately 35 years. Toxicity tests using plants were conducted following standardized test protocols developed by Environment Canada. Endpoint effective concentrations (EC)/and inhibitory concentrations (IC) 25% were calculated to derive soil standards for F3 in coarse-grained soil protective of plants exposed through direct contact with soil. The proposed guideline values derived for the weathered F3 of 659 and 1,961 milligrams per kilogram (mg/kg), respectively, for agricultural/residential and industrial land use are higher than the current ecological Tier 1 CWS for F3 in coarse-grained soil (300 mg/kg for agricultural/residential land use and 1,700 mg/kg for commercial/industrial land use) and support the derivation of remediation targets higher than the current guideline. Additional studies with a more sensitive test species (i.e., earthworms) and a wider range of PHC concentrations are recommended to confirm this conclusion.

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Here's to all the wrong reasons!

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List of Abbreviations

° C	Degrees centigrade
%	Percent
μL	Microlitre
μmoL	Micromole
A-KB	α-Ketobutyrate
ACC	1-Aminocyclopropane-1-carboxylate
AdoMet	S-Adenosyl-methionine
ANOVA	Analysis of variance
AWCD	Average well colour development
bp	Base pair
BTEX	Benzene, toluene, ethylbenzene, and xylene
CCME	Canadian Council of Ministers of the Environment
CFU	Colony forming unit
CLPP	Community level physiological profiling
cm	Centimetre
ddH$_2$0	Double distilled water
DDT	Dichlorodiphenyltrichloroethane
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
d.w.	Dry weight
EC	Effective concentrations
EC	Environment Canada
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FID	Flame ionization detector
FCSI	Federal Contaminated Sites Inventory
g	Grams
hrs	Hours
IAA	Indole acetic acid
IC	Inhibitory concentrations

ICPIN	ICPIN computer program
km	Kilometres
L	Litre
lbs	Pounds
LC	Lethal concentration
LCL	Lower 95% confidence limit
m	Metre
M	Moles
m^2	Square metre
MEQ	Milliequivalents
mg/kg	Milligrams per kilogram
min	Minute
mL	Millilitre
mm	Millimetres
MOE	Ontario Ministry of the Environment
NA	Not applicable
NaCl	Sodium chloride
NC	Not calculated
nm	Nanometres
OD	Optical density
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PEPS	PGPR enhanced phytoremediation systems
PGPR	Plant growth promoting rhizobacteria
PHCs	Petroleum hydrocarbons
PHC CWS	Canada-wide standard for petroleum hydrocarbons in soil
PLFA	Phospholipid fatty acid
ppm	Parts per million
qPCR	Quantitative polymerase chain reaction
r^2	Coefficient of determination for regression analysis
rpm	Revolutions per minute

RRA	Ranked response approach
RRD	Ranked response distribution
RT	Room temperature
SD	Standard deviation
SE	Standard error
Triton X-100	Polyethylene glycol *tert*-octylphenyl ether
TSA	Tryptic soy agar
UCL	Upper 95% confidence limit
UCM	Unresolved complex mixture
UPGMA	Unweighted pair group method using arithmetic averages
USEPA	United States Environmental Protection Agency
UW3	*Pseudomonas* sp.
UW4	*Pseudomonas putida*
v/w	Weight by volume

Chapter 1

Introduction

Industrial and commercial development, urbanization, energy production, and mining have resulted in long-term detrimental effects to the environment. In Canada, it is estimated that there are over 30,000 contaminated brownfield sites (Ontario Ministry of the Environment [MOE], 2007). Brownfield sites are classified as unutilized plots of land where industrial or commercial activity historically took place (MOE, 2007). Examples include old landfills, abandoned factories, dry cleaners, former oil well sites, print shops and gas stations. Most brownfield sites are located in desirable urban areas, and therefore have a high value and potential for re-development. However, in some cases remediation may be required prior to re-development for the protection of human health or ecological receptors. The remediation of urban brownfield sites, in particular, presents a challenge as they frequently contain very complex mixtures of contaminants. For example metals, polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons (PHCs) and/or polychlorinated biphenyls (PCBs) may be present together.

Although many strategies have been developed to remediate urban brownfield sites, the most commonly used remediation technology is still excavation and disposal *via* incineration or as landfill. Other *ex-situ* techniques such as chemical soil washing or soil vapour extraction offer a rapid clean-up solution; however, they are costly, invasive and often have mixed results, particularly on brownfield sites. *In situ* treatments, such as bioremediation, use microbial organisms found in or added to the soil to remediate organic contaminants (Gerhardt *et al.*, 2006). Landfarming enhances the microbial breakdown of contaminants; however, microbial populations are often unable to reach biomass levels required for effective remediation due to the poor soil quality of brownfield sites. Usually, bioremediation is not practical for brownfield remediation due to the limited number of contaminants it can be applied to, as well as time constraints.

The discovery of metal hyperaccumulator plants prompted the use of phytoremediation to remediate contaminated sites as an alternative *in situ* treatment (Gerhardt *et al.*, 2006). Since then, phytoremediation has been used to effectively remediate a variety of contaminants such as herbicides, PAHs, PCBs, explosives, salt and metals mainly in rural locations (Gerhardt *et al.*, 2006). Therefore, phytoremediation, which is a cost-effective *in situ* technology, has the ability to remediate complex contaminant mixtures in soils on urban brownfield sites.

1.1 Phytoremediation

Phytoremediation is defined as the use of plants to remove, destroy or sequester undesirable substances from environmental media (*e.g.*, soil, water, air). Compared to invasive off-site treatments, phytoremediation has many advantages. These include: 1) the preservation and enhancement of soil structure and texture; 2) promotion of microbial biomass; 3) low cost; and, 4) potential for rapid clean-up (Gerhardt *et al.*, 2006; Greenberg *et al.*, 2006; Yang, 2008). Depending on the contaminant of concern, there are various ways to utilize phytoremediation to clean up a hazardous site including rhizodegradation, phytodegradation, phytoextraction and phytostabilization. Phytodegradation and rhizodegradation result in the breakdown of organic contaminants into smaller compounds or mineralization to carbon dioxide. While phytodegradation involves uptake and degradation of the contaminants within plants, rhizodegradation occurs in the rhizosphere (root zone) as a result of microbial activity supported by plant roots and plant exudates (Arthur *et al.*, 2005). Phytodegradation targets munitions, herbicides (e.g., atrazine), chlorinated solvents, dichlorodiphenyltrichloroethane (DDT) and PCBs, while rhizodegradation can be used for PAHs and PHCs (Susarla *et al.*, 2002).

Unlike their organic counterparts, metals cannot be degraded but only phytoextracted or phytostabilized. Phytostabilization reduces the spread of soil contaminants *via* absorption or precipitation within the rhizosphere, and/or root absorption (Mendez and Maier, 2008; Susarla *et al.*, 2002). Phytoextraction involves the uptake of soil contaminants into plant root tissue, which in some cases is followed by translocation to aerial tissue (*e.g.*, stem, leaves, fruit) (Susarla *et al.*, 2002). Although this method has been repeatedly used for metal extraction, it was believed that hydrophobic organic compounds (*e.g.*, PCBs) would be unable to cross the plant plasma membranes preventing uptake and translocation (White *et al.*, 2005). However, the uptake of hydrophobic and persistent organic compounds (*e.g.*, PCBs) has been demonstrated, particularly in some cucurbit species (White *et al.*, 2005; White, 2002; Lunney *et al.*, 2004; Zeeb *et al.*, 2006; Whitfield-Aslund *et al.*, 2007); therefore, phytoextraction may also be applied to some organic contaminants. Phytoextraction can be used to target metals, salt, benzene, toluene, ethylbenzene, and xylenes (BTEX), short-chained aliphatics, pentachlorophenol, and radionuclides (Susarala *et al.*, 2002).

Although a number of phytoremediation field trials on brownfield sites have been conducted within the past two decades (Whitfield-Aslund *et al.*, 2007; Agnello *et al.*, 2016), a need exists to optimize these phytoremediation systems before they can be applied to brownfield sites on a wide-scale. As well, commercial acceptance and development is required. The success of a phytoextraction system depends on the bioavailability of the contaminants, and the ability of the plants to accumulate large amounts of the

contaminants in aboveground tissue. A key component is thus to produce as much biomass as possible. However, based on the current literature, the capabilities of currently used phytoremediation systems are often limited due to poor bioavailability of the contaminants, growth inhibition caused by plant stress due to poor soil quality, and the presence of complex contaminant mixtures in soil. For instance, in-situ phytoremediation attempts on various brownfield sites containing weathered PCBs were only able to remove 0.08 to 1.3 percent (%) of the PCBs during one growth season due to low bioavailability and poor plant up-take (Zeeb *et al.*, 2006; Whitfield Aslund *et al.*, 2007; White *et al.*, 2005).

Brownfield sites commonly contain a complex mixture of soil contaminants, including multiple metals; however, polymetallic soils have been found to have a severe impact on the phytoextraction capabilities and growth of plant species (Marchiol *et al.*, 2004; 2007; Madejon *et al.*, 2003; Kayser *et al.*, 2000). Furthermore, the presence of metals has been shown to inhibit the degradation of organic contaminants by microorganisms (Sandrin and Maier, 2003). It is clear that innovative solutions are required to increase plant growth and contaminant availability under these circumstances to maximize contaminant removal.

1.2 Effect of Plant Growth Promoting Rhizobacteria (PGPR) on Phytoremediation

Based on the results of previous phytoremediation trials, improving plant growth in stressful conditions, such as those found on urban brownfield sites, is critical for successful phytoremediation. One way to overcome this obstacle is the utilization of plant growth promoting rhizobacteria (PGPR), which are free-living soil bacteria that are beneficial to plants especially under stress conditions (Kloepper *et al.*, 1989). High concentrations of bacteria are commonly found in close association with plant root systems within the rhizosphere. These bacteria may affect plant growth directly by secreting compounds that assist plant uptake of various nutrients. Specifically, bacteria may fix atmospheric nitrogen, synthesize siderophores to solubilize iron and other metals, secrete enzymes which modulate plant growth, synthesize phytohormones (*e.g.*, auxins, cytokinins), or possess mechanisms which solubilize minerals (Glick, 1995; 2003; Belimov *et al*, 2005).

In addition to the above, many PGPR contain 1-aminocyclopropane-1-carboxylate (ACC) deaminase which can lower the level of stress ethylene in plants (Glick *et al.*, 1998; Glick, 1995; 2003). Ethylene is a plant hormone which plays an important role in both plant development and stress responses. For example, a high concentration of ethylene can inhibit root elongation (Glick, 2003). ACC-deaminase is proposed to mitigate growth inhibition due to high ethylene concentrations (Glick *et al.*, 1998) (Figure 1-1). PGPR that possess ACC deaminase activity can bind to the root surface or seed of a developing plant. Once bound, the PGPR are in close enough proximity to take up small molecules from the seeds and/or
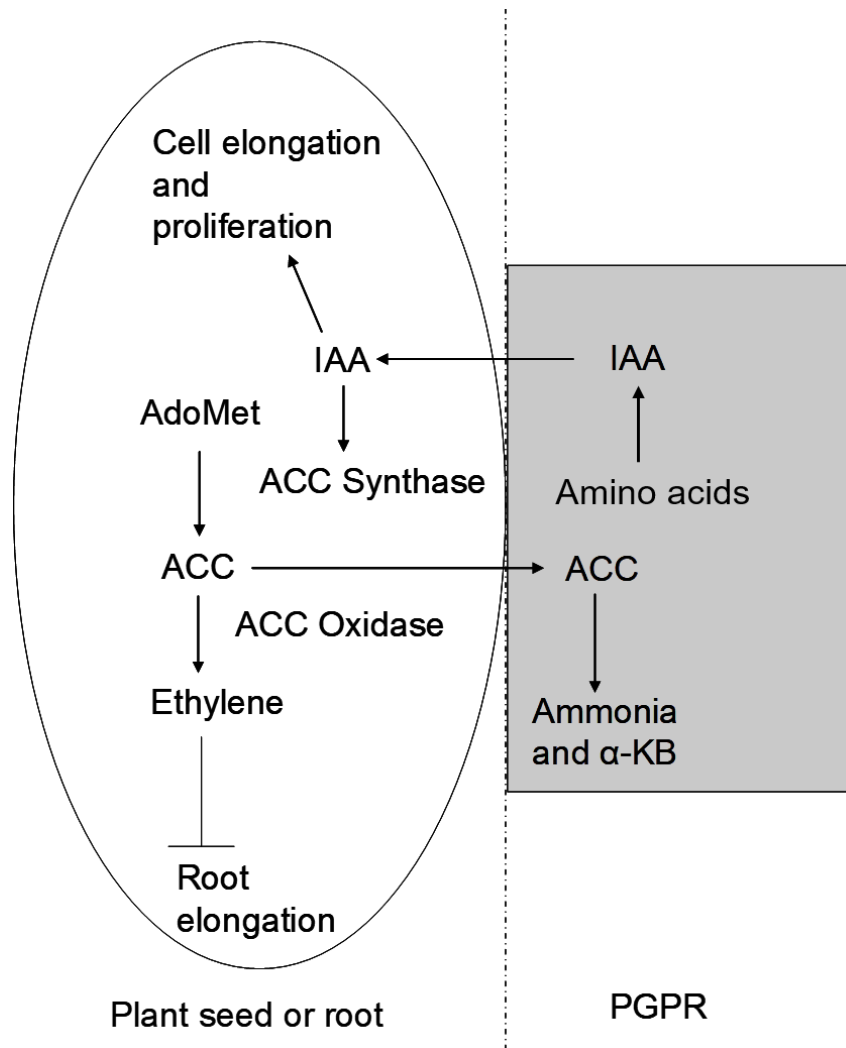
Figure 1-1 A schematic model of how plant growth promoting rhizobacteria (PGPR), bound to a seed or plant root, lower the concentration of stress ethylene and facilitate plant growth. Arrows represent a chemical or physical step, whereas ____|__ indicates inhibition. Key: IAA: indoleacetic acid; ACC: 1 aminocyclopropane-1-carboxylic acid; AdoMet: S-adenosyl-methionine; α-KB: α-ketobutyrate. (Glick *et al.*, 1998; adapted from Figure 1).

root exudates which can stimulate the PGPR to synthesize and excrete indole acetic acid (IAA). The bacterial IAA, in addition to naturally produced plant IAA, induces cell proliferation, cell elongation and/or the production of ACC synthase, which is responsible for the production of ACC. ACC is the immediate precursor to plant ethylene. The ACC produced by the plant is exuded by the roots or seeds, and subsequently taken up by the PGPR. Within the bacterial cell, ACC deaminase is able to convert ACC into ammonia and α-ketobutyrate (providing the bacteria with reduced nitrogen and a four carbon sugar). To maintain the equilibrium between the internal and external ACC concentrations, the plant must exude an increasing amount of ACC. Lowered ACC concentrations in the plant lowers the amount of ethylene that can be produced, alleviating stress induced growth inhibition (Glick *et al.*, 1998).

1.3 PGPR Enhanced Phytoremediation Systems

PGPR Enhanced Phytoremediation Systems (PEPS) have been developed to remediate persistent PHCs, salt, DDT and PAHs from soils (Chang, 2007; Gurska *et al.*, 2009; Huang *et al.*, 2004a;b; 2005; Wang, 2008a). Such systems combine PGPR with plant species, such as grasses, which can proliferate together under high contaminant concentrations to remediate contaminated sites located across Canada. Typical plant species used include: barley (*Hordeum vulgare*), oats (*Avena sativa*), tall fescue (*Festuca arundinace*), fall rye (*Secale cereale*), pumpkins (*Cucurbita pepo* cv. *Howden*), and sugar beets (*Beta vulgaris*). In these studies, the application of PGPR was found to enhance phytoremediation through accelerated plant growth, specifically in the roots, and increased biomass production (Chang, 2007; Gurska *et al.*, 2009; Huang *et al.*, 2004a;b; 2005; Wang, 2008a). This approach has also been used by others to enhance metal phytoextraction systems (Burd *et al.*, 1998; 2000; Kumar *et al.*, 2008; Rajkumar *et al.*, 2006; Rajkumar and Freitas, 2008; Wu *et al.*, 2006; Di Gregorio *et al.*, 2006) and degradation of organic contaminants (Agnello *et al.*, 2016; Phillips *et al.*, 2009; Asghar *et al.*, 2017; Plociniczak *et al.*, 2017; Hou *et al.*, 2015; Cowie *et al.*, 2010). Typically, treatment with PGPR did not greatly alter the metal or salt concentration found in plant tissue (Burd *et al.*, 1998; 2000, Wu *et al.*, 2006; Rakumar *et al.*, 2006; Chang, 2007); however, PGPR protected the plants from growth inhibition caused by high contaminant concentrations resulting in increased plant growth and biomass production leading to greater remediation (Burd *et al.*, 1998; 2000; Wu *et al.*, 2006; Kumar *et al.*, 2008; Belimov *et al.*, 2005; Rakumar *et al.*, 2006; 2008; Chang, 2007; Jiang *et al.*, 2008).

To apply the PEPS to brownfield sites in urban areas (e.g., greater Toronto area) modifications will be required as the majority of previously published work has been done on rural sites. For instance, the currently applied PEPS rely heavily on various grass species (*e.g.*, tall fescue, perennial rye grass, *etc.*) and PGPR species isolated from a natural site in Waterloo, Ontario. However, brownfield sites may

require different plant species, which address each of the contaminants of concern. For example, some plant species such as zucchini (*Curcurbita pepo*) can bioaccumulate organics and metals simultaneously (Mattina *et al.*, 2003). Sunflower (*Helianthus annuus*), corn (*Zea mays*) and soybean (*Glycine max*) have been demonstrated to have fairly high phytoextraction potential on brownfield soils contaminated with multiple metals (Madejon *et al.*, 2003; Lombi *et al.*, 2001; Fellet *et al.*, 2007). Switchgrass is another species of interest, as it has shown potential for PCB remediation (Chekol *et al.*, 2004) and is relatively salt tolerant. In a greenhouse study in which plants were grown for 13 months on contaminated sediments, switchgrass produced more root and shoot biomass than willow, poplar, eastern gamagrass, arrowhead, and sedge (Euliss *et al.*, 2008).

1.4 Impacts of Phytoremediation on Microbial Communities

Although the main goal associated with phytoremediation is the removal of contaminants from the soil, the restoration of soil quality is also an important endpoint (Epelde *et al.*, 2008). An increased awareness of the importance of microbial diversity as an indicator of soil quality has emerged (Li *et al.*, 2006; Chen *et al.*, 2006). Soil microorganisms are important as they are responsible for the decomposition of organic matter, recycling of plant nutrients, maintenance of the soil structure, rhizodegradation of certain contaminants, and the control of plant pests and plant growth (Chen *et al.*, 2006). Microbial communities respond sensitively to changes in environmental conditions in their external environment as bacteria have a high surface area to volume ratio. Therefore, changes in microbial communities due to anthropogenic contamination may occur prior to detectable changes in soil properties or in plant and animal communities (Winding *et al.*, 2005). As a result the composition of microbial communities can be used as a simple and sensitive biological indicator of anthropogenic effects on soil ecology (Chen *et al.*, 2006) and should be considered as an endpoint for the assessment of soil remediation (Chen and Banks, 2004).

Previous methods of examining soil microbial communities focused on techniques which required the bacteria to be cultured prior to analysis (Li *et al.*, 2006). One example is community level physiological profiling (CLPP) which employs substrate utilization patterns to classify microbial communities. Although this method is fast, highly reproducible, inexpensive, and differentiates between microbial communities, it does not account for unculturable microorganisms (Kirk *et al.*, 2004). Therefore, this method is not ideal for soil microbial community analysis as only 1 to 10% of these soil microorganisms are culturable (Wayne *et al.*, 1987). Polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE) allows for the study of microbial community diversity at the molecular level (Li *et al.*, 2006). As PCR-DGGE is not dependent on the physiological state of the cells or the ability to

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culture them, it includes all soil microorganisms (Gremion *et al.*, 2004; Li *et al.*, 2006). PCR-DGGE has been applied to monitor microbial communities in soil (Winding *et al.*, 2005). DGGE is based on variations within the base composition and secondary structure of a fragment of the rRNA gene between microbial species (Winding *et al.*, 2005). Once a fragment of known size has been amplified, the products are separated based on their melting behaviour. The number and position of the fragments reflects the dominating microbial species within the community (Winding *et al.*, 2005). PCR-DGGE is a reliable, reproducible, and rapid method, and it allows for a large number of samples to be analyzed simultaneously (Kirk *et al.*, 2004). Both PCR-DGGE and CLPP have been used to evaluate the impact of phytoremediation and anthropogenic contamination on microbial communities (Epelde *et al.*, 2008; Palmroth *et al*; 2007; DiGregorio *et al.*, 2006; Li *et al.*, 2006; Gremion *et al.*, 2004; Chen *et al.*, 2006; Chen and Banks, 2004; Kirk *et al.*, 2005).

Soil contaminants, such as metals and organics, can have a detrimental effect on the diversity of native soil microbial communities. Exposure to high metal concentrations, in weathered and artificially spiked soils, shifted the composition of the native microbial communities present in the soil (Muller *et al.*, 2001; Li *et al.*, 2006; Gremion *et al.*, 2004; Chen *et al.*, 2006). Chen *et al.* (2006) observed a negative correlation between microbial diversity and the concentration of available copper in the soil. Furthermore, soils with mixed contaminants, such as urban brownfields, may make it extremely challenging to maintain a diverse microbial community as elevated metal concentrations may inhibit organic-contaminant degrading microorganisms (Sandrin and Maier, 2003).

The growth of plants on a contaminated site has a significant effect on the microbial communities (Gremion *et al.*, 2004; Kirk *et al.*, 2005; DiGregorio *et al.*, 2006). Gremion *et al.* (2004) observed plant induced changes in microbial diversity. This was accompanied by a positive effect on substrate utilization using CLPP. Furthermore, Di Gregorio *et al.* (2006) found that the most significant modification of the soil microbial community, in a phytoremediation system using Indian mustard (*Brassica juncea*), ethylenediaminetetraacetic acid (EDTA), and polyethylene glycol *tert*-octylphenyl ether (Triton X-100™), was due to the presence of Indian mustard. Although the effect of phytoremediation on soil microbial communities has been examined in several studies, limited research has been conducted on urban brownfield sites (Agnello *et al.*, 2016).

PEPS introduces non-native PGPR to contaminated sites during phytoremediation, along with the plant species. Wang (2008b) investigated the effect of non-native PGPR amendment during phytoremediation of a landfarm by quantifying total bacteria (culturing method and quantitative polymerase chain reaction [qPCR]) and DGGE. Although the introduced PGPR expressed a modest effect on the native microbial

community during the early stages of seed development, this effect was masked by enhanced indigenous microbial populations as plant growth continued throughout the season (Wang, 2008b). Further research in this area is required to investigate the effects of plant species and PGPR on native microbial communities from urban brownfield soil.

1.5 Petroleum Hydrocarbon Direct Soil Contact Values for Ecological Receptors

Remediation of urban brownfield sites is often required prior to re-development for the protection of ecological and human health. Potential risks to human health and ecological receptors are determined based on the comparison of site soil concentrations to soil quality guidelines derived for PHCs. In Canada, suggested PHC soil criteria are regulated by provinces and are based on the Canadian Council of Ministers of the Environment (CCME) guidelines. The CCME guidelines were established for separate PHC fractions, which were determined based on petroleum product composition, fate, and human toxicity thresholds initially developed by the Total Petroleum Hydrocarbon Criterion Working Group (Potter and Simmons, 1998; Gustafson *et al.*, 1997). The four fractions are as follows: PHC fraction 1 (F1; >nC6-nC10), fraction 2 (F2; >nC10-nC16), fraction 3 (F3; >nC16-nC34) and fraction 4 (F4; nC35+). The initial soil guidelines established in 2001 for PHCs were derived based on the available ecotoxicological data for plants and soil invertebrates using freshly spiked field and artificial test soils. The adverse effects of PHCs has been attributed to changes in the physical and chemical properties of the soil caused by the PHC in addition to direct PHC toxicity (Roberston *et al.*, 2007; Mikkonnen *et al.*, 2012). For instance, PHC can decrease water availability, and limit water and gas exchange impacting plant growth (Roberston *et al.*, 2007; Adam and Duncan, 2002) by forming hydrophobic films on the exterior surfaces of soil aggregates (McGill *et al.*, 1981; Certini, 2005).

The effect of PHC on soil quality is dependent on the duration of PHC exposure (Wang *et al.*, 2010). As a result, the use of freshly spiked field and artificial soils in plant toxicity tests may not be reflective of the adverse effects on plants and soil invertebrates caused by PHC induced changes to the physical and chemical properties of the soil. This is a particular concern on sites with PHC contaminated soils that experienced prolonged exposure to high PHC concentrations. Conversely, the use of freshly spiked test soils may over-estimate the environmental risk posed by well-weathered PHCs. Weathered PHC can become sequestered in the soil by partitioning into organic matter and diffusion into the soil micropores (Northcott and Jones, 2001; Reeves *et al.*, 2001; Husemann *et al.*, 2004; Tang *et al.*, 2012). It is also degraded over time. As a result, the PHC becomes less accessible, which lowers the available composition of the PHC and diminishes toxicity. Therefore, a soil guideline based on freshly spiked test

soils may result in an over-estimation of risk and unnecessary remediation. Angell *et al.*, (2012) derived soil standards for weathered F2 in fine-grained soils based on plant and invertebrate toxicity testing. The proposed standards for weathered F2 were higher than the current Canada-Wide Standard for Petroleum Hydrocarbons in Soil (PHC CWS) derived using ecotoxicological data for receptors exposed to fresh F2 in soil (Table 1-1). This indicates that the current criteria are overly protective for weathered PHC. Therefore, further investigations on the impact of weathered F2, F3, and F4 on soil receptors are recommended. Additional concerns were raised that the F3 ecological standards may be overly conservative as a lack of toxicity to plants and other organisms was observed at several bioremediated sites with concentrations of F3 above the PHC CWS (CCME, 2008) (Table 1-2). Given that the remediation and management of PHC impacted sites is a multibillion dollar issue in Canada (CCME, 2008), it is important that remediation is driven by accurate effects based on the true risks.

The most recent CCME PHC CWS for F3, established in 2008, was derived based on field trials and ranked response distributions. A long-term field study conducted by Visser (2005) was heavily relied upon to derive the current PHC CWS. This study used a large number of species for the toxicity testing, relevant endpoints, chronic duration, and sound analytical methods. However, a limited number of PHC soil concentrations were used in the field trial; therefore, a ranked response approach (RRA) was applied to derive the current guideline. The RRA involves presenting data for all non-redundant endpoints (e.g., germination rate, root weight or length, and shoot weight or length) for various species (plants and soil invertebrates) from single concentration field plant toxicity studies as a response relative to the control (CCME, 2006). A given field soil was not considered to pose a risk to ecological receptors if the 25th percentile of the combined responses for all non-redundant endpoints and species expressed relative to the control was equal to or greater than 50% for industrial/commercial or 75% for agricultural/residential land use (CCME, 2006). Resulting guidelines using the RRA may provide a concentration range, if several tests concentrations were available, or indicate if the guideline is less than or greater than a single test concentration. Therefore, although the most recent soil contact values for F3 are based on field soils using relevant endpoints, a limited number of PHC soil concentrations were used limiting the accuracy of the resulting guideline. Furthermore, since the completion of the plant toxicity bioassays by Visser (2005), Environment Canada (EC) has established standardized test protocols for measuring emergence and growth of plants exposed to contaminants in soil (EC, 2007). Therefore, the suitability of the current guideline for weathered F3 using standardized test protocols and a dose response curve needs further investigation.

Table 1-1 Summary of the current and proposed ecological Tier 1 Canada-wide standards (CWS) for petroleum hydrocarbons (PHCs) fraction 2 in surface soil (milligrams per kilogram [mg/kg])

	Agricultural/Residential Land Use	Commercial/Industrial Land Use
Current Guidelines Based on Fresh Product	150	260
Proposed Guidelines Based on Weathered Product	262	338

Table 1-2 Current ecological tier 1 Canada-wide standards (CWS) for petroleum hydrocarbons (PHCs) fraction 3 (F3) in fine- and coarse grained surface soil (milligrams per kilogram [mg/kg]).

PHC Fraction	Fine grained soil		Coarse-grained Soil	
	Agricultural/Residential Land Use	Commercial/Industrial Land Use	Agricultural/Residential Land Use	Commercial/Industrial Land Use
F3	1,300	2,500	300	1,700

1.6 Objectives

With over 30,000 brownfield sites in Canada it is evident that there is a need for a remediation technology which can effectively remediate urban brownfield sites in a timely manner. Currently, the success of phytoremediation systems applied to brownfield sites is often limited by the bioavailability of contaminants and growth inhibition due to poor soil quality and complex contaminant mixtures. The application of PGPR to these sites may decrease plant growth inhibition by lowering stress ethylene concentrations; however, this has not been extensively tested on urban brownfield soils. Despite the successful application of the PEPS to PHCs and salt on several rural sites (Chang, 2007; Gurska *et al.*, 2009; Huang *et al.*, 2004a;b; 2005; Wang, 2008a), it is assumed that optimization will be required to apply this system to brownfield sites with mixed contaminants and poor soil quality. Therefore, the first objective of this thesis was to optimize the currently used PEPS by investigating different plant species (e.g., sunflower, switch grass) that are amenable to PEPS and have ideal characteristics for urban brownfield sites. The PGPR effect will also be investigated on an urban brownfield site.

Recently, microbial diversity has emerged as an important indicator of soil health. Although studies have begun to examine the effect of phytoremediation and the introduction of non-indigenous PGPR on native microbial communities, little work has been done on urban brownfield sites with mixed contaminants. Therefore, the second objective of this thesis was to investigate the impact of phytoremediation using PGPR on native microbial communities in urban brownfield soils.

Remediation on urban brownfield sites is driven by soil quality guidelines derived to be protective of human and ecological receptors; therefore, it is important that the soil standards are accurate and effects based to prevent unnecessary remediation. The suitability of the current PHC CWS for weathered F3 has not yet been investigated using standardized protocols and a dose response approach. Therefore, the third objective was to examine the toxicity of weathered F3 to plant species using a well aged soil with high historical PHC concentrations.

Chapter 2

Field Application of PGPR Enhanced Phytoremediation for In Situ Remediation of an Urban Brownfield Site

2.1 Overview

Plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation systems (PEPS) have been successfully used to remediate rural sites contaminated with salt and petroleum hydrocarbons (PHCs). They have not yet been applied to urban brownfields. A two year field study (2008/2009) was conducted on an urban brownfield site in Toronto, Ontario, to evaluate the ability of PEPS to enhance plant growth and remediate PHCs on a mixed contaminant site. Mixed grasses (tall fescue [(*Festuca arundinacea]*, perennial rye grass [*Lolium perenne*], annual rye grass [*Lolium multiflorum]*) and sunflowers (*Helianthus annuus* var Mammoth Russian) were deemed suitable for phytoremediation based on germination rates and biomass production. Consistent PGPR effects on biomass production were not strongly apparent. The impact of the growth of PGPR (*Pseudomonas sp.)* treated plants on the native microbial community was evaluated by community level physiological profiling (CLPP), denaturing gradient gel electrophoresis (DGGE) of bacterial 16S ribosomal deoxyribonucleic acid (16S rDNA) genes, and microbial counts. Soil heterotrophic and PGPR bacterial numbers in the rhizosphere of all planted treatments increased throughout the 2009 field season. The plant species utilized, rather than the bacterial PGPR treatment, had the largest impact on the microbial community based on CLPP and DGGE analysis. A significant decrease in PHCs was observed during the 2009 field season in sunflower and mixed grass plots treated with PGPR (paired t-test, $p<0.05$). An average percent remediation of 25% was observed in the mixed grass and sunflower plots treated with PGPR. The average percent remediation ranged from 2 to 13% in planted plots treated without PGPR and an average percent remediation of 8% in the unplanted controls. PEPS shows promise as an effective remediation technique for urban brownfield remediation.

2.2 Introduction

There are estimated to be over 30,000 brownfield sites in Canada, including gas stations, old landfills, and abandoned industrial sites (Ontario Ministry of the Environment [MOE], 2007). Many brownfield sites are located in desirable urban areas, and have a high value and potential for redevelopment. Remediation of these sites presents a challenge as they frequently contain complex mixtures of contaminants. Although many strategies have been developed to remediate urban brownfield sites, the most commonly used

remediation technology is still excavation and disposal *via* incineration or as landfill materials. Other *ex-situ* techniques offer rapid clean-up solutions; however, they are costly, invasive and have had mixed results, particularly on brownfield sites.

In situ treatments, such as bioremediation, use microbial organisms found in or added to the soil to remediate organic contaminants (Gerhardt *et al.*, 2006). Usually, bioremediation is not practical for brownfield remediation due to the limited number of contaminants it can be applied to, as well as time constraints. The discovery of metal hyperaccumulator plants prompted research on plants to remediate contaminated sites as an alternative *in situ* treatment (phytoremediation) (Gerhardt *et al.*, 2006). Since then, phytoremediation has been used to effectively remediate contaminants such as herbicides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), explosives, salt and metals mainly in rural locations (Gerhardt *et al.*, 2006). Phytoremediation is a cost-effective *in situ* technology, which may be applied to remediate complex contaminant mixtures in soils on urban brownfield sites.

Phytoremediation is the use of plants to remove, degrade or sequester undesirable substances from environmental media (*e.g.*, soil, water, air). Currently used phytoremediation systems often express diminished remediation capabilities on urban brownfields. For instance, soils impacted with metal mixtures have been found to have a severe impact on the phytoextraction capabilities and growth of a variety of plant species (Marchiol *et al.*, 2004; 2007), while elevated metal concentrations may inhibit organic-contaminant degrading microorganisms (Sandrin and Maier, 2003). Innovative solutions are required to increase plant growth under these circumstances to maximize contaminant removal. One method is the use of PGPR, which are a group of free-living soil bacteria that are beneficial to plants (Kloepper *et al.*, 1989). Many PGPR contain 1-aminocyclopropane-1-carboxylate (ACC) deaminase which modulates the level of ethylene in plants (Glick *et al.*, 1998). ACC-deaminase is proposed to mitigate growth inhibition due to high ethylene concentrations (stress ethylene) produced by plants in response to environmental or contaminant stressors (Glick *et al.*, 1998). These PGPR can alleviate plant stress, resulting from high contaminant concentrations, allowing sufficient biomass production for remediation to occur (Glick, 2003; Huang *et al.*, 2005; 2005).

A PEPS has been developed to remediate persistent PHCs, salt, and PAHs from soils (Gurska *et al.*, 2009; Huang *et al.*, 2004a;b; 2005). This approach has also been used by others to enhance metal phytoextraction systems (Burd *et al.*, 1998; Di Gregorio *et al.*, 2006; Roman-Ponce *et al.*, 2017). This system combines PGPR with plant species, such as grasses, which can then grow more aggressively in soils with high contaminant concentrations. PEPS has been used to remediate contaminated rural sites across Canada. In several studies, the application of PGPR was found to enhance phytoremediation

through accelerated plant growth, specifically root growth, and increased biomass production (Gurska *et al.*, 2009; Huang *et al.*, 2004a;b; 2005).

The currently used PEPS relies heavily on mixed grasses (*e.g.,* perennial tall fescue, annual and perennial ryegrass) and cereals (*e.g.*, barley, oats) for PHC and salt phytoremediation. However, these plant species may not be ideal for urban brownfield sites, which commonly contain complex mixtures of contaminants and poor soil quality. These sites may require different plant species that address each of the contaminants of concern. For example, species which are capable of bioaccumulating organics and metals simultaneously, such as zucchini (*Curcurbita pepo*) (Mattina *et al.*, 2003) may be better suited to address the complex nature of urban brownfield sites. Furthermore, sunflower, corn (*Zea mays*) and soybean (*Glycine max*) have demonstrated fairly high phytoextraction potential on brownfield soils contaminated with multiple metals (Madejon *et al.*, 2003; Lombi *et al.*, 2001; Fellet *et al.*, 2007). Switchgrass is another species of interest, as it has shown potential for PCB remediation (Chekol *et al.*, 2004), is relatively salt tolerant, and has the capacity to produce high quantities of root and shoot biomass (Euliss *et al.*, 2008).

Few studies have examined the effect of agronomic practices to enhance phytoextraction in field studies (Ji *et al.*, 2011). Direct seeding is an easy and economical planting method; however, it requires over-seeding and subsequent thinning for certain plant species and may result in non-uniform plots (Li *et al.*, 2003). In addition, slow-growing seedlings planted via direct seeding may be susceptible to weed competition in the field (Li *et al.*, 2003). Transplanting seedlings is an alternative approach to direct seeding. Although transplanting requires additional cost and labour to grow the seedlings in a greenhouse prior to transplanting to the field, it provides uniform plant density, reduces labour for weeding and thinning, and allows the plants an earlier start in the growing season (Li *et al.*, 2003). Therefore, transplanting seedlings may enhance phytoextraction on urban brownfield soils.

Although the main goal associated with phytoremediation is the removal of contaminants from the soil, the restoration of soil quality is also an important consideration (Epelde *et al.*, 2008). Microbial communities respond sensitively to changes in environmental conditions due to the high surface area to volume ratio of bacteria. Therefore, the composition of microbial communities can be used as a simple and sensitive indicator of anthropogenic effects on soil ecology (Chen *et al.*, 2006) and should be considered as an endpoint for the assessment of soil remediation (Chen and Banks, 2004). Microbial diversity in soil can be assessed using biochemical-based techniques (e.g., plate counts and CLPP) and molecular-based techniques (e.g., DGGE) (Kirk *et al.*, 2004). Techniques such as plate counting and CLPP are fast, inexpensive, and can generate large amounts of data; however, these methods only

represent the culturable portion of the microbial community and are biased towards fast growing organisms (Kirk *et al.*, 2004). DGGE is a microbial based approach, in which DNA is extracted from a soil sample and target DNA is amplified using universal primers. The resulting products are separated based on the melting temperatures (denaturing) of the double-stranded DNA to identify differences in microbial communities (Kirk *et al.*, 2004). Soil contaminants, such as metals and organics, can have a detrimental effect on the diversity of native soil microbial communities by shifting their composition (Muller *et al.*, 2001; Li *et al*, 2006) or decreasing microbial diversity (Chen *et al.*, 2006). The effect of phytoremediation on soil microbial communities was examined (Gremion *et al.*, 2004; Kirk *et al.*, 2005); however, limited research has been conducted on urban brownfield sites. Even fewer studies have investigated the impact of PGPR application on native microbial communities (Wang, 2008b; Piromyou *et al.*, 2011).

While PEPS have been used to remediate numerous contaminants on several rural sites, few studies have examined if it is applicable to urban brownfield species with mixed contaminants. Traditional plant species used in PEPS may not be ideal for urban brownfield sites with poor soil quality and complex contaminant mixtures. Few studies have considered different agronomic practices to enhance phytoremediation in field studies. Limited research on the effect of phytoremediation and the use of non-indigenous PGPR via PEPS on soil microbial communities has been conducted on urban brownfields sites. Therefore, the objectives of this study were to: 1) determine whether the PEPS can be successfully applied to remediate petroleum hydrocarbons on an urban brownfield site in the presence of multiple metals; 2) optimize PEPS for remediation of an urban brownfield site by investigating different plant species and two planting techniques (direct seeding and transplanting seedlings); and, 3) determine the impact of phytoremediation using PGPR on the native soil microbial community.

2.3 Materials and Methods

2.3.1 Site Description

Field tests were performed during two consecutive years beginning May 2008 at an urban brownfield site in Toronto, Ontario, Canada. The site was originally created by land filling and was historically used as an industrial waste oil refinery tank farm. In 2007, sub-surface soils from within and surrounding the site were piled and smoothed to form a raised (approximately 1.5 metre [m]) planting site (3,388 square metres [m^2]). Subsequently, the soil was covered with a layer of woodchips approximately 0.5 m thick (Figure 2-1). The site was divided into experimental plots using a paired plot design. Plots were

Figure 2-1 Urban brownfield site in Toronto, Ontario where field tests were completed for two consecutive years, beginning in May 2008. A) Field site prior to planting in 2008; B) Field site prior to planting in 2009; C) Field site following tillage to a depth of approximately 30 centimetres in July 2009; D) Experimental test plots ready for planting in July 2009.

heterogeneous due to the high volume of debris and unconventional tilling techniques. Between the two field seasons the site was tilled to a depth of approximately 30 centimetres [cm] with an excavator, and levelled with a Bobcat MT55 Mini Track Loader (Figure 2-1).

The main physical and chemical characteristics of the soil, as determined by SGS Agrifood Laboratories (Guelph, ON, Canada), were: pH, 7.6, organic matter, 4.8 percent [%], cation exchange capacity, 27 milliequivalents [MEQ]/100 gram [g], sand fraction, 64%, silt fraction, 25%, and clay fraction, 11%. The soil was classified as a sandy loam and considered a coarse soil type by Canadian Council of Ministers of the Environment [CCME] (2008). Soil contaminants included PHCs, metals, and PCBs (Table 2-1). At the beginning of the trial the average total PHC (including fraction 2 [F2] to fraction 4 [F4]) concentration was 3,900 milligrams per kilogram [mg/kg], ranging from 1,688 mg/kg to 9,640 mg/kg.

2.3.2 Field Application of PEPS

In 2008, the site was planted with a combination of mixed grasses (Tall fescue (*Festuca arundinacea*); annual rye grass (*Lolium multiflorum*); and, perennial ryegrass (*Lolium perenne)*), pumpkin (*Cucurbita pepo* var Howden), sunflowers (*Helianthus annuus* var Mammoth Russian), and sugar beets (*Beta vulgaris*). The experimental test plots were seeded or planted, as applicable, between June 2 and June 20, 2009. In 2009, mixed grasses, sunflower, and switchgrass (*Panicum virgatum*) were planted on the site. The experimental test plots were seeded or planted between June 24 and July 10, 2009. Sugar beet seeds were purchased from Agriculture Environmental Renewal Canada Inc., Ottawa, Ontario, Canada, while the other seeds were purchased from Ontario Seed Co., Waterloo, Ontario, Canada. Planting density for the mixed grasses in 2008 ranged between 600 to 900 pounds (lbs)/acre, while in 2009 a seeding rate of 300 lbs/acre was used. A seeding density of 20 lbs/acre was used for switchgrass. The pumpkins and sugar beets were planted at densities of 10,000 and 20,000 plants/acre, respectively. The pumpkins were planted in clusters of 4 seeds. In 2008, the sunflowers were planted at densities ranging between 50,000 to 80,000 plants/acre. In 2009 this density was increased to 320,000 plants/acre; however, plants were thinned a month after planting resulting in a planting density of 160,000 plants/acre. The plants were allowed to grow for the entirety of the growing season (~120 days). They were irrigated and fertilized as needed. The control area consisted of soil that was not vegetated. Minimal weed growth occurred in the control area over the course of the field trial.

Two planting techniques (direct seeding and transplanting) were tested during the 2008 and 2009 field season. During the 2008 field season, one test plot for pumpkins and one for sunflowers applied both of

Table 2-1 Average petroleum hydrocarbon (PHC), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbon (PAH), and metal concentrations in soils from an urban brownfield site in Toronto, Ontario, Canada. The average concentration (± standard error) was presented when multiple data points were available. Chemical concentrations greater than the Table 3: Full-Depth Generic Site Condition Standards in a Non-Potable Ground Water Condition for industrial/commercial land use with coarse-grained soil are presented in bold.

Chemical	Field Soil Concentration (mg/kg)
Petroleum Hydrocarbons (PHCs)	
Fraction 1	< 5
Fraction 2	53 ± 4
Fraction 3	**2782 ± 236**
Fraction 4	1060 ± 106
Polychlorinated Biphenyls (PCBs)	
Total PCBs	**2.6 ± 0.4**
Polycyclic Aromatic Hydrocarbons (PAHs)	
Acenaphthene	0.09
Acenaphthylene	0.13
Anthracene	0.24
Benzo(a)anthracene	0.62
Benzo(a)pyrene	**0.59**
Benzo(b/j)fluoranthene	0.54
Benzo(g,h,i)perylene	0.52
Benzo(k)fluoranthene	0.49
Chrysene	0.66
Dibenz(a,h)anthracene	0.1
Fluoranthene	1.01
Fluorene	0.15
Indeno(1,2,3-cd)pyrene	0.48
1-Methylnaphthalene	0.24
2-Methylnaphthalene	0.39
Naphthalene	0.28
Phenanthrene	0.74
Pyrene	1.14
Metals	
Antimony	5
Arsenic	11
Barium	333 ± 18
Beryllium	<0.5
Cadmium	**2**
Chromium	25
Cobalt	7
Copper	172 ± 40
Lead	**739 ± 40**
Molybdenum	3
Nickel	28
Selenium	<1
Silver	1.6
Thallium	<1
Vanadium	19
Zinc	**368 ± 20**

the planting techniques. Following seed treatment, pumpkin and sunflower seeds were planted in ProMix® and grown in the greenhouse for three weeks to generate the seedlings to transplant to the field The sunflowers were planted 30 cm apart with a row spacing of 1 m, while the pumpkins were planted 50 cm apart, with a row spacing of 85 cm. In 2009, three smaller test plots (2 m^2) paired sunflower seedlings and seeds. The sunflower seedlings were planted 10 cm apart with a row spacing of 25 cm.

2.3.3 Application of PGPR to the Seeds

Two PGPR *Pseudomonas* strains, UW3 (*Pseudomonas* sp.) and UW4 (*Pseudomonas putida*) were used by applying them to the seeds prior to planting (Glick *et al.*, 2005; Hontzeas, 2004, Gurska *et al.*, 2009). Bacterial strains were grown in tryptic soy broth (30 grams per litre (g/L), Fisher Scientific, Ottawa, Ontario, Canada) at room temperature (RT) for 24 hours (hr) until an absorbance of 2 at 600 nanometre (nm) was reached. The bacterial cultures were centrifuged and resuspended in deionized water. A methyl-cellulose polymer (Sigma-Aldrich, Oakville, Ontario, Canada) coating was used to adhere the PGPR to the seeds. A colorant (Color Coat Blue, Becker Underwood, Saskatoon, Saskatchewan, Canada) was added to the bacterial slurry containing PGPR to distinguish the PGPR treated seeds. A Hege 11 liquid seed treater (Wintersteiger, Saskatoon, Saskatchewan, Canada) was used to coat the prepared bacterial slurry onto the seed.

2.3.4 Soil Sampling for PHC and Microbial Analysis

Soil samples (750 g) were collected from unplanted and planted areas at the beginning and end of the field season to a depth of 30 cm using an Edelman auger (Eijelkamp Agrisearch Equipment, Giesbeek, The Netherlands). Three composite soil samples per plot were created by combining 2 (2008) or 4 (2009) grab samples from pre-determined sampling locations. This sampling method was used to minimize the impact of the heterogeneous nature of the site. Samples were collected in plastic bags and stored at 4 degrees centigrade (°C) until further analysis. PHC levels in the soil were determined gravimetrically as Fraction 3 (F3) was the predominant form of the PHCs (72% of total PHCs) at the site. The soil samples (4 g) were air-dried and extracted three times by ultrasonication for 50 minutes (min) into a total of 20 millilitres (mL) of 1:1 hexane/acetone mixture in duplicate (United States Environmental Protection Agency [USEPA], 1998). The extracts were dried completely and weighed to determine the PHC content. Replicate variation was typically less than 10%.

For microbial analysis, soils in the rhizosphere and non-rhizosphere were sampled at monthly intervals in 2009. The initial soil samples were collected in July at the time of planting/seeding. Subsequent samples for microbial analysis were collected on August 11th, September 11th, and October 11th. To obtain

rhizosphere soil, four plant samples were randomly obtained within a 2 m^2 area per plot using a small hand trowel and placed into a pre-labelled Ziploc bag. This was repeated at three locations within each plot. The plant samples were stored at 4°C until further analysis. Non-rhizosphere soil samples were obtained from the same sampling location in areas with no plant growth using a hand held auger. Occasionally, these soil samples were taken slightly outside the 2 m^2 area due to maximal plant coverage. Microbial samples on the blank plots were obtained in the same manner as the non-rhizosphere soil samples.

Rhizosphere soils were removed from the plants by first shaking the plant roots by hand to remove loosely attached soils, and then scraping the remaining soil off the roots with a sterile scapula. The rhizosphere soil was screened through a sterile 2 millimetre (mm) sieve to remove any debris from the sample (*e.g.*, glass, rocks, metal). Composite soil samples were generated for each plot, for microbial counts and CLPP, and each treatment for DGGE analysis.

2.3.5 Plant Biomass Production

Plant samples were collected at mid-season and the end of the field season each year. Mid-season biomass sampling occurred between August 13th and 26th in 2008 and between September 3rd and 4th in 2009. End of season biomass sampling occurred between October 14th and 28th in 2008 and between October 14th and 21st in 2009. Plant growth was measured as root and shoot biomass for pumpkins and sunflowers, and as total biomass for the mixed grasses. For the pumpkins and sunflowers plant biomass was measured by randomly sampling approximately 10 to 30 plants from each plot. Initially, during the 2008 mid-season sampling, the mixed grasses were sampled by isolating a 0.01 m^2 area; however, due to variability in plot coverage this was increased to a 50 cm x 50 cm (0.25 m^2) square of soil, approximately 30 cm in depth. The plants were isolated from the soil and washed with water to remove soil particulates adhered to the roots. The dry weight was measured after the plants were dried in an oven at 60°C for 48 hrs. Measurements were normalized to those of the untreated plants and averaged to obtain annual plant performance in terms of root and shoot, or total biomass (dry weight).

2.3.6 Microbial Plate Counts

To quantify the number of microbes, ~1 g soil samples were aseptically placed in 45 mL of sterilized 0.1% weight by volume (w/v) sodium pyrophosphate solution (pH adjusted to 7.0) in 250 mL sterile Erlenmeyer flasks with 4 g of sterile 2 mm diameter glass beads (Kirk *et al.*, 2005). This was performed in triplicate. The soil solutions were shaken for one hour on a Multi-Mixer at 160 revolutions per minute

(rpm). Each sample was poured into a sterile 50 mL Falcon tube and centrifuged at 1,800 rpm for 2 min to remove the soil from the sample. The supernatant from the soil extract suspension was diluted, between 0 and 200 fold, for the microorganism quantification assays described below. A 5-6 g soil sample from each composite sample was dried at 60°C for 48 hrs to determine the soil moisture content.

2.3.6.1 Total bacterial Number

Culturable aerobic heterotrophic bacterial cells were enumerated in triplicate using the spread plate count method. Diluted soil extract suspensions of 100 microlitre (µL) were spread on tryptic soy agar (TSA) plates supplemented with 75 parts per million (ppm) cycloheximide to inhibit fungal growth (Kirk *et al.*, 2005). Plates were incubated at RT for 48 hrs and enumerated using a colony counter.

2.3.6.2 PGPR

PGPR were enumerated in triplicate using the plate count method. The DF salt medium was prepared following the method described by Penrose and Glick (2003). The component of DF salt per litre was: 4 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, and 5 µL of trace metal solution. The DF salt medium was autoclaved for 30 min and then cooled to 60°C prior to adding filter sterilized glucose (1 g into 10 mL of water). Prior to plating 60 uL of ACC (0.5 moles [M]) was spread onto the plate. The diluted soil extracts (100 µL) were spread onto DF salt plates containing ACC as the sole nitrogen source. Plates were incubated at RT for 48 hrs prior to plate counting. For the microbial plate counts, PGPR were defined as any bacteria with ACC deaminase activity or the ability to utilize ACC as the sole nitrogen source.

2.3.7 Eco-BIOLOG Plates to Assess Substrate Utilization Patterns for Metabolic Diversity

The remaining soil solution suspensions used for microbial analysis were poured into a second sterile centrifuge tube and centrifuged for 10 min at (8,000 x gravity) to pellet the microbial cells. The sterile sodium phyrophosphate medium was removed and replaced with the same volume of sterile 0.85% (w/v) sodium chloride (NaCl). The tubes were shaken and vortexed for 2 min to resuspend the cells (Kirk *et al.*, 2005). All samples were diluted two-fold and inoculated onto an Eco-Biolog (EcoPlate™, Biolog Inc., Hayward, CA, USA) plate (150 µL/well) to determine the metabolic potential of the microbial community over the course of the field season. The Eco-Biolog plate contains 31 different carbon sources, replicated three times. Samples were incubated in the dark at RT and analyzed at 595 nm using a multi-skan plate reader (Multiskan Spectrum v1.0, Thermo Scientific) at 24, 36, 48, 60, 72, 96, 120, 144 and 168 hrs after inoculation.

The Shannon diversity (H') index of catabolic diversity was used to measure species metabolic diversity calculated as $H' = -\sum p_i \ln p_i$, where p_i = (optical density [OD] reading of well i)/(sum of all wells). The time point which had an average well colour development (AWCD) closest to 0.75 was selected for the H' calculation. An AWCD of 0.75 was selected as, at this value the response of the microbial community is visible in most wells and the most active microbial communities have reached maximum colour development (Garland *et al.*, 2001). The value pi was calculated by subtracting the average blank absorbance from each substrate's average absorbance at 595 nm and then dividing this value by the total colour development for all selected substrates. Only substrates which had an average absorbance value greater than 0.25 after blank subtraction were used for the analysis to reduce the number of false positives.

2.3.8 Denaturing gradient gel electrophoresis (DGGE)

DNA was extracted directly from field soils from the three treatments (unseeded soils, soil seeded [sunflowers and mixed grasses] with and without PGPR) using the Ultra Clean Soil DNA Kit (MoBio Laboratories Inc., Carlbad, CA) as per the manufacturer's instructions. Oligonucleotide primers able to amplify a fragment spanning nucleotide position 341 to 518 of *Escherichia coli* (*E. coli*) 16S rDNA were used. The nucleotide sequences were: 341F, 5'- GC clamp-CCTACG GGAGGCAGCAG-3' and 518R, 5'- ATTACCGCGGCT GCTGG-3'. These primers have been used previously to analyze bacterial communities with DGGE (Muyzer *et al.*, 1993). The 40 base pair (bp) GC clamp was attached to the forward primer (341F) to ensure that the DNA fragment was not completely separated into single strands. This enabled strand separation in the polyacrylamide gel to be based on melting behavior of the DNA fragment.

Extracted DNA was subjected to polymerase chain reaction (PCR) amplification. PCR was conducted in a PTC-200 DNA Engine Cycler (MJ Research) with the following program: 5 min at 95°C, and 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 10 min at 72°C and then held at 10°C. The extracted DNA was visualized on agarose gel to assess the quality (Figure 2-2). The PCR products were a mixture of 16s rDNA of various soil bacteria with almost identical sizes, but with different nucleotide sequences.

DGGE was performed on a Bio-Rad DeCode system (Bio-Rad, Mississauga, Ontario, Canada). DGGE used a 30% to 70% denaturing gradient in 10% polyacrylamide gels, which is suitable to DNA fragments ranging from 100 to 300 bp. Gels were run for 14 hours at 85+V according to a previously published
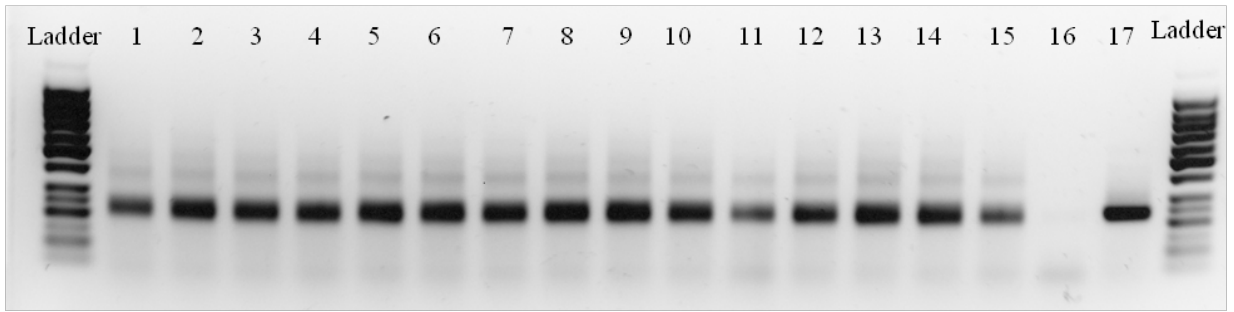
Figure 2-2 Soil deoxyribonucleic acid (DNA) extracted from an urban brownfield site in Toronto, Ontario. DNA was extracted from soils of blank plots and seeded plots (sunflowers and mixed grasses) with and without plant growth rhizobacteria (PGPR) treatment. Polymerase chain reaction (PCR) product (2 micrograms per litre [µg/L]) was loaded into each well of the 2 percent (%) agarose gel. Electrophoresis was performed for 1 hour. The resulting fragments were approximately 220 base pairs in length. Lanes 1, 11, 15: Blank untreated plots; Lanes 2, 5, 13: Sunflower with PGPR; Lanes 3, 9, 14: Sunflower without PGPR; Lanes: 4, 8, 12: Mixed grasses with PGPR treated seeds; Lanes: 6, 7, 10: Mixed grasses without PGPR; Lane 16: Control (ddH$_2$0); Lane 17: Control (UW3 [*Pseudomonas* sp.] and UW4 [*Pseudomonas putida*]).

protocol (Green *et al.*, 2010). Gels were stained using SYBR Green. The gels were visualized on a Tyhpoon Scanner.

Gel images were analyzed using GelCompar II version 5.0 package (Applied Maths, Kortrijk, Belgium). GelCompar II software was used to normalize and compare all of the DGGE profiles. Images of the DGGE gels were matched using the internal control samples and the banks were quantified after a local background subtraction. A tolerance in the band position of 1% was applied. The similarity between two treatments was determined by comparing the intensity pattern of the curve instead of individual bands. Clustering was done with the unweighted pair group method using arithmetic averages (UPGMA) based on the similarities between treatments.

2.3.9 Statistical Analysis

To determine significance between plant biomass production and microbial endpoints (total microbial counts, Shannon diversity index) an analysis of variance (ANOVA) was used with a Tukey-Kramer post-test along with paired t-tests, as applicable. Due to the heterogeneity of the PHC concentrations in the field plots, paired t-tests were used to determine if significant remediation had occurred during the course of the field season.

2.4 Results

During the course of the field seasons (2008 and 2009) the weather was favourable for plant growth in Toronto, Ontario. Weather data was obtained from the Toronto City Centre weather station located approximately 5 kilometres (km) west of the site (Government of Canada, 2019).The average mean temperature ranged from 9°C in October (2008) to 21°C in July (2008) and August (2009). Adequate rainfall occurred throughout the growing season (June to October) with a total precipitation of 450 mm in both 2008 and 2009. The highest precipitation occurred in July (143 mm) and August (145 mm) during the 2008 and 2009 growing season, respectively.

2.4.1 Suitability of Plant Species

Plants were selected based on their suitability to the Southern Ontario climate, previous success with PEPS, or previously demonstrated ability to remediate the contaminants of concern. In 2008, very low germination rates (<5%) were observed for switchgrass and sugar beets (Table 2-2). A low average above ground biomass production (48 grams dry weight per square metre [g d.w./m²]) was observed for pumpkins, which were also plagued with pest problems such as powdery mildew and cucumber bugs. The above ground biomass production varied greatly between the individual plots (Table 2-2). The average

Table 2-2 Germination rate and average and plot specific above ground biomass production for various plant species during two field seasons of plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation system (PEPS) at an urban brownfield site in Toronto, Ontario.

Plant Species	Year	Germination Rate		Plot Specific Above Ground Biomass Production [1]					Average Above Ground Biomass Production (g/m^2) [3]
				Plot	Mid-Season		End of Season [2]		
		- PGPR	+ PGPR		- PGPR	+ PGPR	- PGPR	+ PGPR	
Seeds									
Switchgrass	2009	<5	<5		NC	NC	NC	NC	NC
Sugar beets	2008	<5	<5		NC	NC	NC	NC	NC
Pumpkins	2008	81	84	21/22	0.82 ± 0.22	1.1 ± 0.29	1.8 ± 0.2	1.7 ± 0.3	48 ± 18
		88	88	31/32	1.1 ± 0.28	1.3 ± 0.31	4.7 ± 1.0	2.0 ± 0.4	
		90	89	34/33	3.8 ± 1.1	7.2 ± 3.6	9.2 ± 2.2	7.5 ± 2.0	
Sunflower	2008	87	89	1/2	1.6 ± 0.4	1.4 ± 0.4	3.9 ± 0.6	2.9 ± 0.5	77 ± 16
		78	82	15/16	5.6 ± 2.8	2.0 ± 0.7	7.9 ± 1.0	8.2 ± 1.3	
		69	75	27/28	NC	NC	2.1 ± 0.3	2.3 ± 0.3	
	2009	61	62	7/8	11 ± 2.2	7.5 ± 0.8	20 ± 2.8	18 ± 2.7	462 ± 56
		51	51	21/22	6.7 ± 1.3	6.3 ± 0.5	12 ± 1.6	12 ± 1.2	
		47	46	27/28	7.1 ± 1.1	8.8 ± 2.0	14 ± 1.7	15 ± 2.2	
Mixed grasses[3]	2008	NA	NA	3/4	0.40 ± 0.12	0.43 ± 0.12	246 ± 71	277 ± 56	564 ± 132
		NA	NA	19/20	0.40 ± 0.19	0.47 ± 0.13	171 ± 34	174 ± 9.4	
	2009	NA	NA	1/2	12 ± 2.8	16 ± 3.2	95 ± 20	54 ± 6.4	273 ± 33
		NA	NA	5/6	9.5 ± 3.9	20 ± 9.4	70 ± 17	37 ± 7.4	
		NA	NA	15/16	14 ± 4.9	24 ± 7.0	74 ± 6.7	78 ± 9.2	

[1] Sunflower and pumpkin biomass presented as average dry weight per plant. Mixed grasses biomass is presented as a total biomass (roots and shoots) per 0.25 m^2 quadrant, except for the mid-season 2008 sampling (0.01 m^2).

[2] Above ground biomass was limited to stalk weights only for the sunflowers as a large proportion of sunflowers had lost their flowers prior to end of season sample collection.

[3] Total biomass (root and shoots) is presented

NA – Not applicable; NC – Not calculated

above ground biomass production of the sunflowers in 2008 was 77 g/m^2; however, increasing the planting density in 2009 increased biomass production to 462 g/m^2 (Table 2-2). The above and below.ground biomass production of the mixed grass plots was 564 and 273 g/m^2 in 2008 and 2009, respectively. The decrease in seeding rate from 600 to 900 lbs/acre in 2008 to 300 lbs/acre in 2009 most likely contributed to the decrease in total biomass production of the mixed grasses in 2009

2.4.1 Agronomic Practices

Two seeding techniques were compared at the site to enhance the phytoextraction potential of PEPS. In 2008, the transplanted seedlings produced significantly more above ground biomass (ANOVA Tukey, p<0.05) than those plants that were directly seeded; however, there was only one test plot per species (Table 2-3). In 2009, there was no consistent significant difference between above ground biomass production for the transplanted seedlings versus the plants that were direct seeded (Table 2-3).

2.4.2 PGPR Treatment

PGPR treatment at this site did not significantly increase the biomass production (root, shoot or total biomass) of any of the plant species (paired t-test, p>0.05) (Table 2-2). Although PGPR appeared to enhance the above ground biomass of sunflower and pumpkin seedlings (ANOVA Tukey, p<0.05) in 2008, this effect was not reproducible during the 2009 field season with triplicate test plots (ANOVA Tukey, p>0.05).

2.4.3 PHC Remediation

In 2008, no petroleum hydrocarbon remediation was observed; however, PHC concentrations varied greatly over the field site and plant growth for some species was lower than in 2009. In 2009, the petroleum hydrocarbon concentration significantly decreased over the field season in sunflower and mixed grass plots for seeds treated with PGPR (paired t-test, p<0.05), with an average percent remediation of ~25% (Figure 2-3). The average percent of remediation for the sunflower and mixed grass plots for seeds without the PGPR treatment was 13% and 2%, respectively. An average percent remediation of 8% was observed in the unplanted controls.

2.4.4 Impact of PEPS on the Microbial Community

2.4.4.1 Microbial Plate Counts

After three months of growth in the urban brownfield soil, total heterotrophic and PGPR bacterial numbers in the rhizosphere of all planted treatments were significantly higher than the bulk soil

Table 2-3 Average dry weight above ground biomass production for seeds and seedlings of pumpkins and sunflowers during two field seasons of plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation system (PEPS) at an urban brownfield site in Toronto, Ontario, Canada. End of season biomass for the sunflowers was limited to stalk weights only as a large proportion had lost their flowers prior to the end of season sample location. Sample sizes in 2008 were 10 for mid-season and 20 (pumpkin) and 35 (sunflower) for end of season. Sample sizes in 2009 were 15 (mid-season) and 20 (end of season). * Indicates a significant difference between +PGPR and –PGPR treatment (ANOVA Tukey, <0.05). # Indicates significant differences between seedlings and seed treatments (ANOVA Tukey, <0.05).

Plant Species	Year	Plot	Plant Type	Above Ground Biomass Production (g/plant)			
				Mid-Season		End of Season	
				- PGPR	+ PGPR	- PGPR	+ PGPR
Pumpkins	2008	9/10	Seeds	5.2 ± 2.8	3.2 ± 0.55	3.7 ± 0.7	4.8 ± 0.9
			Seedlings	11 ± 3.5	17 ± 5.7	12 ± 2.2#	26 ± 6.4*#
Sunflower	2008	25/56	Seeds	1.4 ± 0.26	2.4 ± 0.94	1.5 ± 0.19	1.6 ± 0.24
			Seedlings	3.6 ± 0.59	6.2 ± 1.3#	3.3 ± 0.33#	10 ± 2.4*#
	2009	9/10	Seeds	24 ± 3.2	25 ± 2.9	21 ± 6.8	20 ± 3.7
			Seedlings	25 ± 4.4	26 ± 3.3	16 ± 1.9	15 ± 1.6
		11/12	Seeds	17 ± 3.2	18 ± 2.9	16 ± 3.1	17 ± 2.8
			Seedlings	28 ± 5.9	29 ± 5.6	24 ± 5.2	26 ± 5.3#
		25/26	Seeds	8.3 ± 1.2	6.4 ± 0.9	7.9 ± 1.1	6.1 ± 0.89
			Seedlings	11 ± 1.0	12 ± 1.2#	9.7 ± 0.86	11 ± 1.1
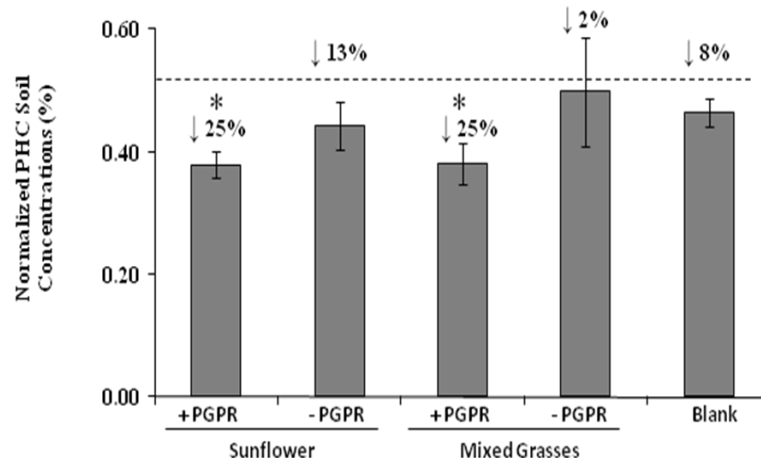
Figure 2-3 Normalized petroleum hydrocarbon (PHC) concentrations at the end of the 2009 field season along with the calculated average percent remediation. Significant decreases in PHC concentrations are indicated with an asterisk (*) for sunflower and mixed grass plots treated with plant growth promoting rhizobacteria (PGPR) (paired t-test, $p < 0.05$) compared to the initial concentration (dashed line).

(ANOVA, p<0.05) (Figure 2-4). Although highly variable, the number of heterotrophic bacteria in the bulk soil decreased over the course of the field season. All of the planted treatments led to increased heterotrophic bacteria numbers in the rhizosphere after the first month of growth (ANOVA, p<0.05). The heterotrophic counts in the mixed grass plots tended to increase over the course of the field season, whereas the sunflower associated counts remained relatively constant. A decrease in the microbial counts was observed in September, which may be due to low soil moisture contents and/or end of season dormancy. No significant differences were observed between plots with and without PGPR treatment (ANOVA Tukey, p>0.05) (Figure 2-4). At the end of the 2009 field season, total heterotrophic and PGPR bacterial numbers in the non-rhizosphere soil of the planted treatments did not significantly differ from the bulk soil (ANOVA Tukey, p<0.05) (Figure 2-5). The number of heterotrophic bacteria in the non-rhizosphere soil was highly variable, but stayed relatively constant over the duration of the field season. PGPR treatment had no significant impact on total heterotrophic or PGPR microbial biomass in the non-rhizosphere soil (ANOVA Tukey, p>0.05). The heterotrophic and PGPR bacterial numbers were significantly higher in the rhizosphere soil (Figure 2-4) compared to the non-rhizosphere (paired t-test, p<0.05) over the course of the field season (Figure 2-5).

2.4.4.2 Community Level Physiological Profiling

The Shannon's diversity index, which is based on the number and evenness of the carbon substrates used by the microbial community on the Eco-BIOLOG plates, decreased over the course of the field season in the blank and sunflower plots, and increased in the mixed grass plots; however, no significant differences between treatments was noted (ANOVA Tukey, p>0.05) (Figure 2-6). Neither PGPR application nor plot treatment had a significant impact on the carbon substrate utilization (ANOVA Tukey, p>0.05). (Figure 2-6)

2.4.4.3 Denaturing Gradient Gel Electrophoresis

The microbial community was monitored using DGGE at the urban brownfield site during the 2009 field season. Generally, soil DNA extracts from the site were similar in terms of bacterial diversity and community structure. The banding patterns of the DGGE profiles were highly conserved between treatments and over the course of the field season (Figure 2-7). The average similarity between the planted treatments and the unplanted blank field plots decreased by approximately 20% as the 2009 field season progressed, with an average similarity of 41% at the end of the field season. A change in the microbial community was noted over the course of the field season based on the cluster analysis presented as an ordination analysis in two dimensional space (Figure 2-8). The microbial community shifted over

30

A)

B)
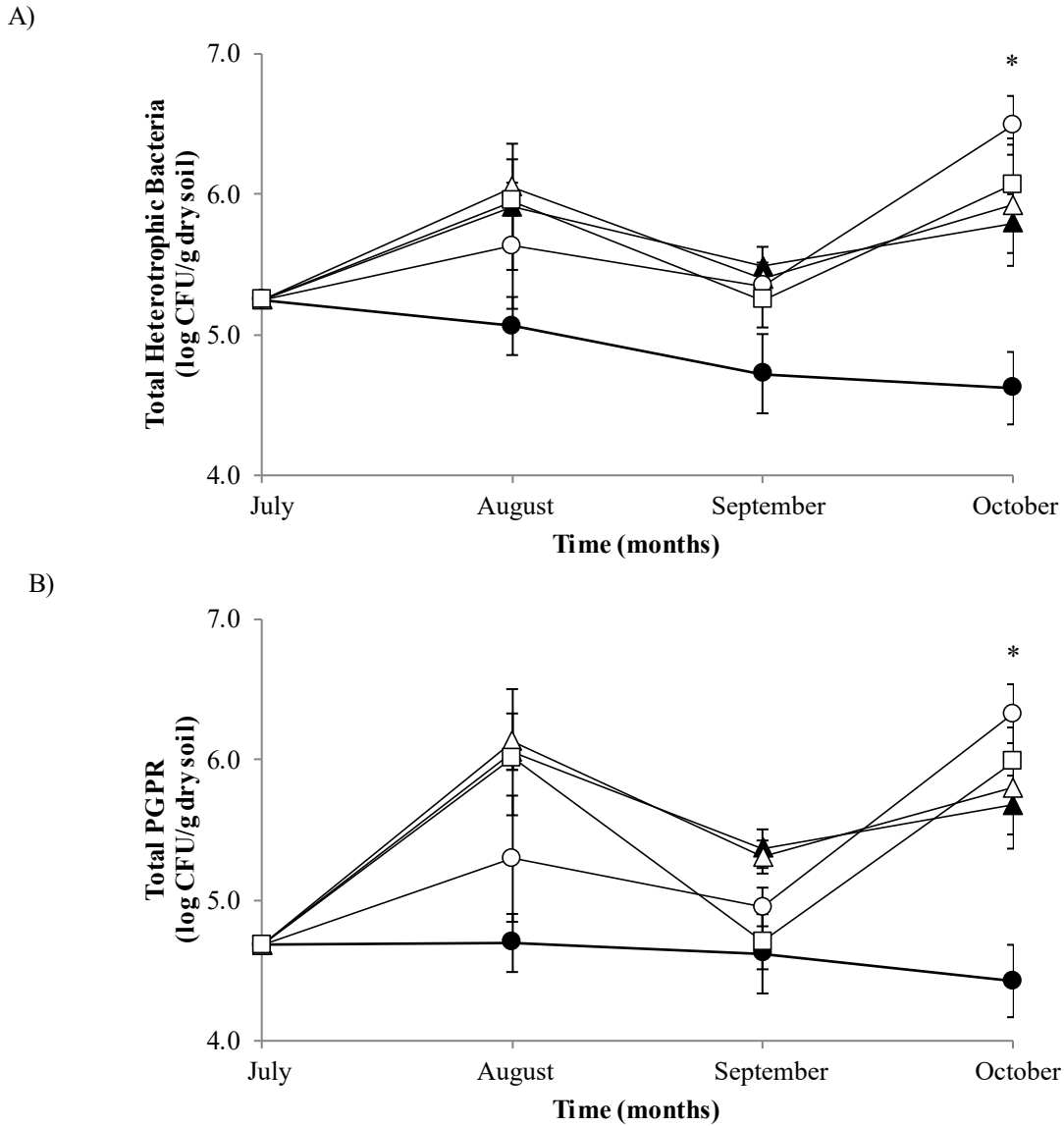
Figure 2-4 Total (A) culturable heterotrophic bacteria; and (B) plant growth promoting rhizobacteria (PGPR) from the rhizospheres of four planted treatments and bulk urban brownfield soil, contaminated with petroleum hydrocarbons (PHCs) and various metals, as determined by plate counts on (A) tryptic soy; and (B) DF agar with 1-aminocyclopropane-1-carboxylate (ACC) as the sole nitrogen source. Symbols: ▲ = Sunflower +PGPR; Δ = Sunflower –PGPR; ○ = Mixed grasses +PGPR; □ = Mixed grasses – PGPR; ● = Bulk soil; * = significant difference (ANOVA Tukey, p<0.05). Data are the means ± standard deviation (n = 3).

A)
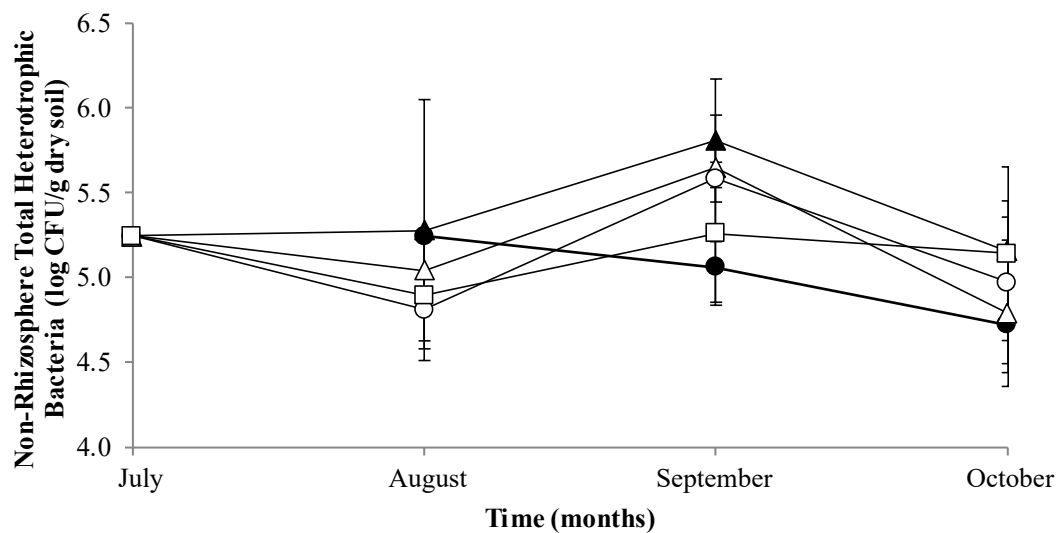
B)
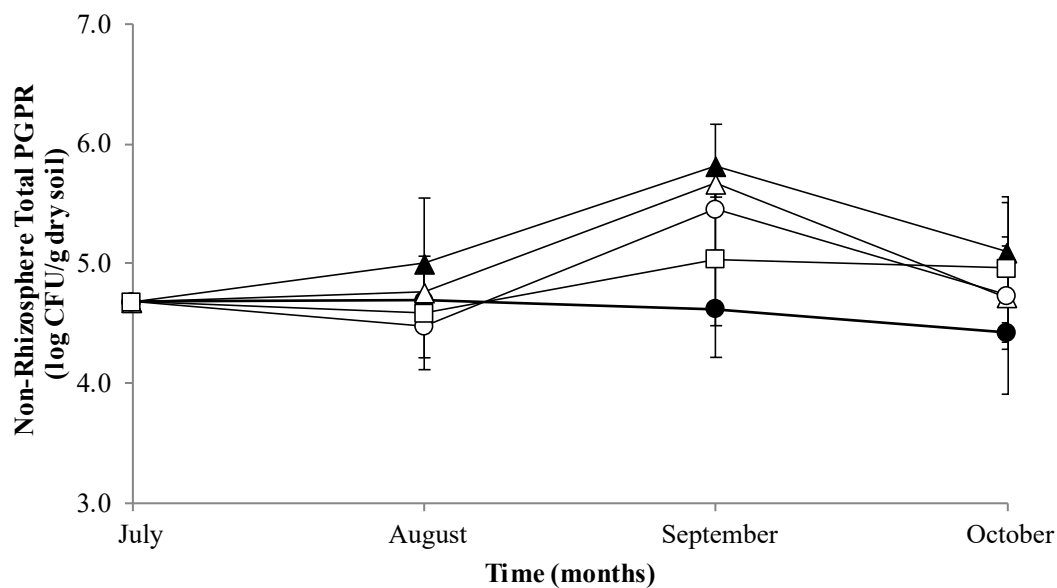
Figure 2-5 Total (A) culturable heterotrophic bacteria; and (B) plant growth promoting rhizobacteria (PGPR) from the non-rhizospheres of four planted treatments and bulk urban brownfield soil, contaminated with petroleum hydrocarbons (PHCs) and various metals, as determined by plate counts on (A) tryptic soy; and (B) DF agar with 1-aminocyclopropane-1-carboxylate (ACC) as the sole nitrogen source. Symbols: ▲ = Sunflower +PGPR; Δ = Sunflower –PGPR; ○ = Mixed grasses +PGPR; □ = Mixed grasses – PGPR; ● = Bulk soil. Data are the means ± standard deviation (n = 3).
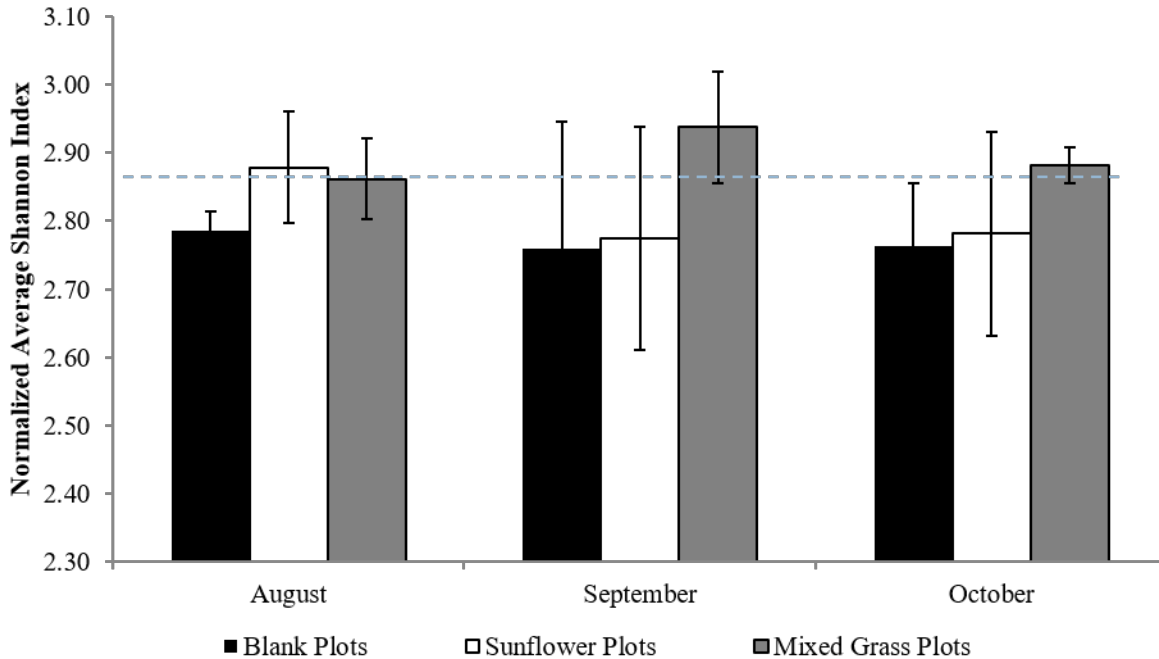
Figure 2-6 Normalized average Shannon's diversity index values based on the number and evenness of the carbon substrates used by the microbial community on the Eco-BIOLOG plates in sunflower, mixed grass, and blank field plots over the course of the 2009 field study. Neither plant growth promoting rhizobacteria (PGPR) application nor plant species had a significant impact on the carbon substrate utilization (ANOVA Tukey, $p > 0.05$). The dashed line represents the average Shannon's diversity index in July (baseline time point). Data are the means ± standard deviation (n = 3).
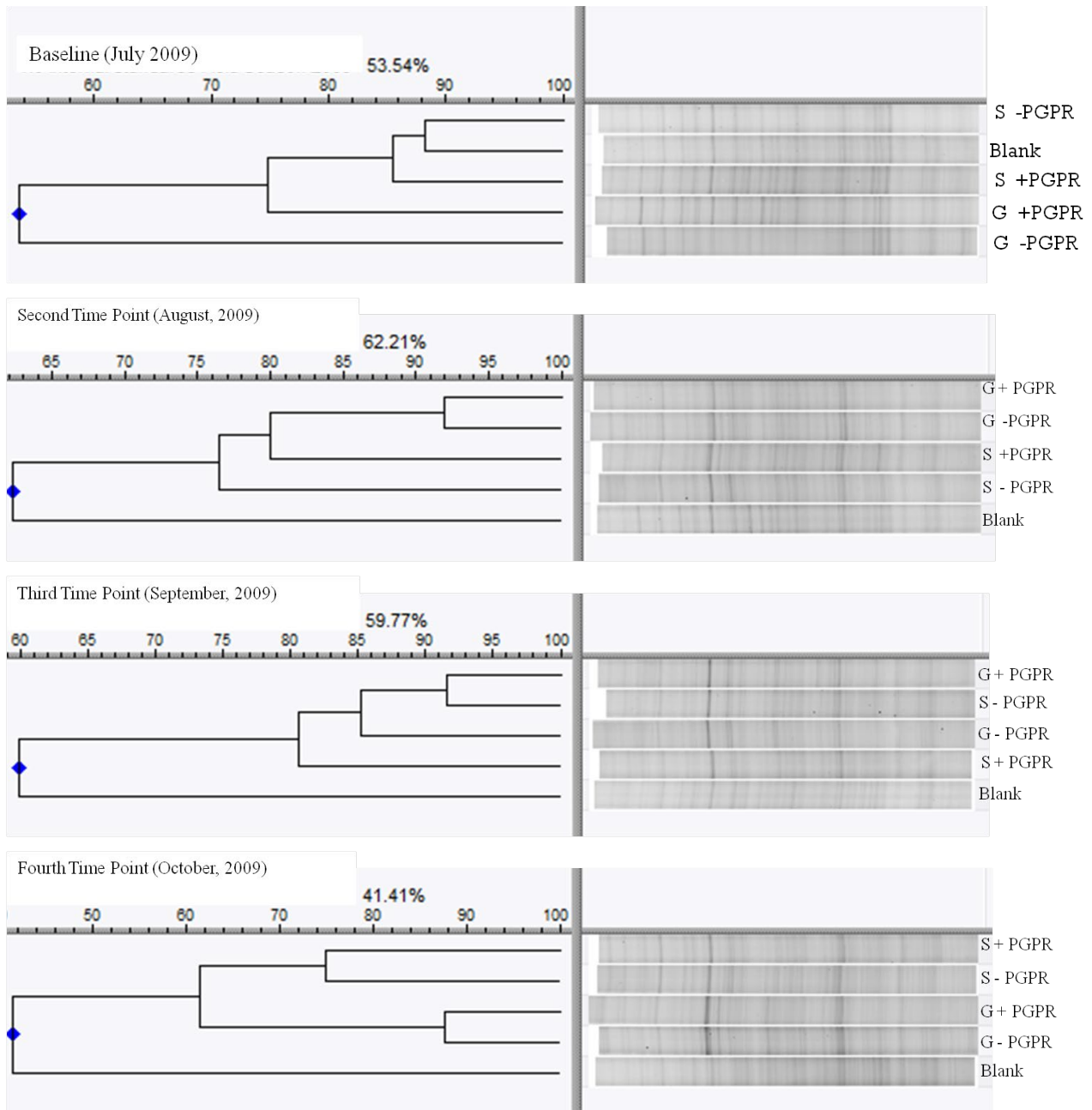
Figure 2-7 Cluster analysis of bacterial denaturing gradient gel electrophoresis (DGGE) profiles using unweighted pair group method with arithmetic mean (UPGMA) based on the similarities of bacterial community structure between the various treatments (+ plant growth promoting bacteria [PGPR] – PGPR treated; -PGPR – seeds untreated with PGPR; Blank – unplanted control; G – mixed grasses; S – sunflower) throughout the 2009 field season (September, August, September, and October).
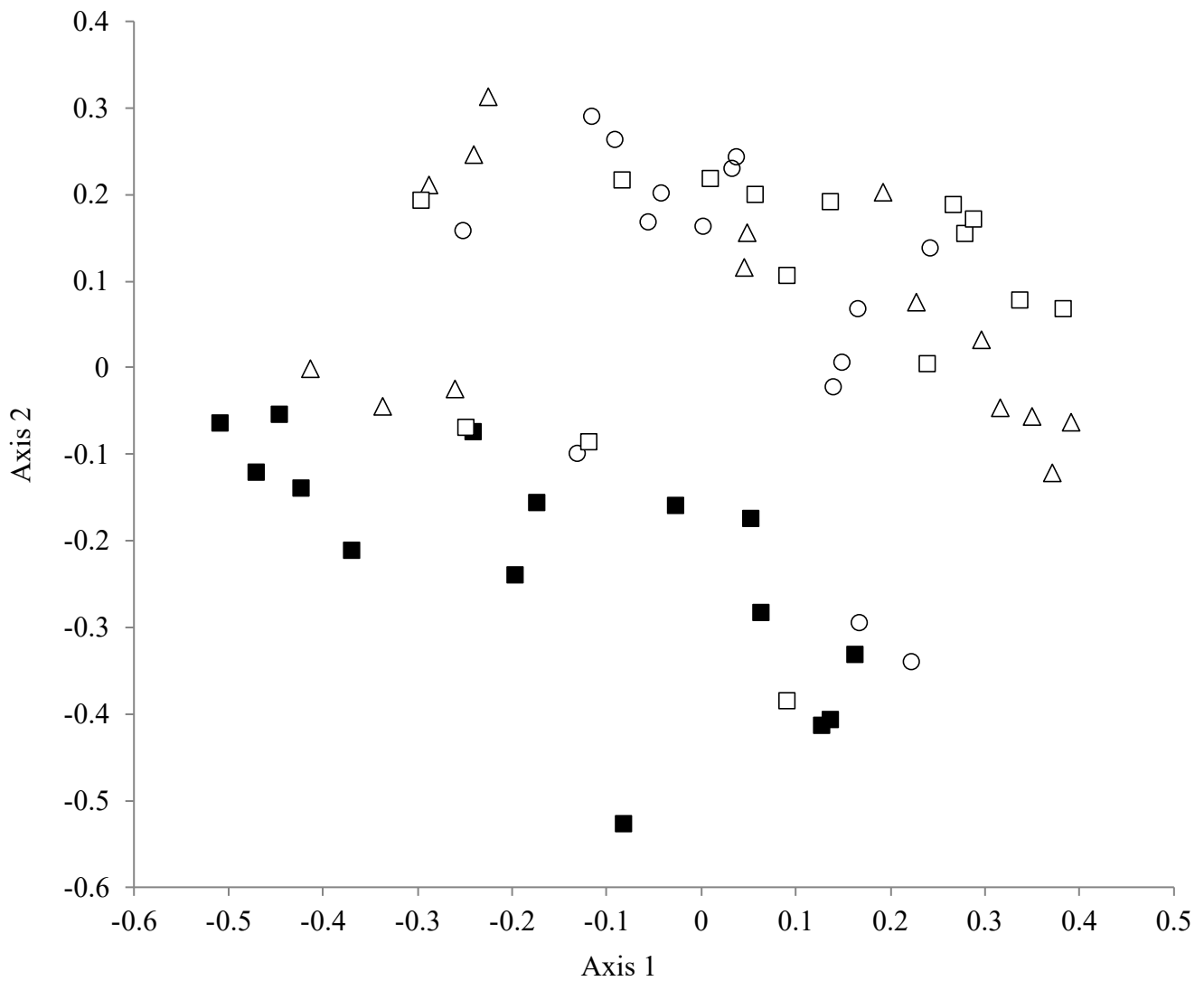
Figure 2-8 Pooled cluster analysis diagrams for ordination of bacterial communities from denaturing gradient gel electrophoresis (DGGE) fingerprints from all treatments (mixed grasses, sunflowers, blank plots, treated with plant growth promoting bacteria [PGPR], no PGPR) over the course of the 2009 field season. Symbols: ■ = July; ○ = August; △ = September; □ = October.

the course of the field season. In general, the DGGE profiles of the bacterial community collected during the first time point (July) clustered with the untreated plots collected later in the 2009 field season (August, September, October) (Figure 2-8). The planted treatments for the second, third, and fourth time points clustered together (Figure 2-8). At the end of the field season, the DGGE profiles clustered by plant species rather than PGPR treatment (Figure 2-9).

2.5 Discussion

2.5.1 Suitability of Plant Species

PEPS has been successfully applied to remediate total petroleum hydrocarbons, salt, PAHs and DDT on rural sites (Chang, 2007; Gurska et al., 2009; Huang et al., 2004a;b; 2005); however, this is the first time PEPS has been applied on an urban brownfield. Several plant species that had not been previously used within PEPS were selected for use to address the multiple contaminants of concern. Of the five plant species tested for field use, only sunflowers and mixed grasses were considered suitable for phytoremediation based on germination rates and plant biomass production. Switchgrass (*Panicum virgatum*) and sugar beets (*Beta vulgaris*) had germination rates less than 5%, while the pumpkins (*Cucurbita pepo*) produced low above ground biomass (48 g d.w./m^2) and were plagued with pest problems such as powdery mildew and cucumber bugs. The powdery mildew may have resulted from the wet weather conditions observed in 2008. Although these plant species have shown potential in controlled environments, they may not be appropriate for brownfield sites with poor growing and soil conditions. Whitfield Aslund *et al.* (2007) used pumpkins for in-situ phytoremediation of a former transformer manufacturing location in Etobicoke, Ontario. However, field plots were amended with fertilizer, an inorganic bulking agent, and a minimal amount of top soil prior to planting (Whitfield Aslund *et al.*, 2007). In this instance, the pumpkin growth observed exceeded that of other studies, with a maximum shoot length of greater than 7 m and a plant biomass of greater than 4 kilograms (kg). Therefore, additional soil amendments may be required for these plant species to be successful on urban brownfields. The above ground biomass production of the sunflowers in 2008 was 77 g d.w./m^2; however, increasing the planting density in 2009 increased biomass production to 462 g d.w./m^2. Total (roots and shoots) biomass production of the mixed grass plots was 564 and 273 g d.w./m^2 in 2008 and 2009, respectively. A lower seeding rate and number of fertilizer applications may have contributed to the lower biomass production in 2009. Sunflowers have previously demonstrated fairly high phytoextraction potential on brownfield soils contaminated with multiple metals (Madejon *et al.*, 2003). Based on high biomass

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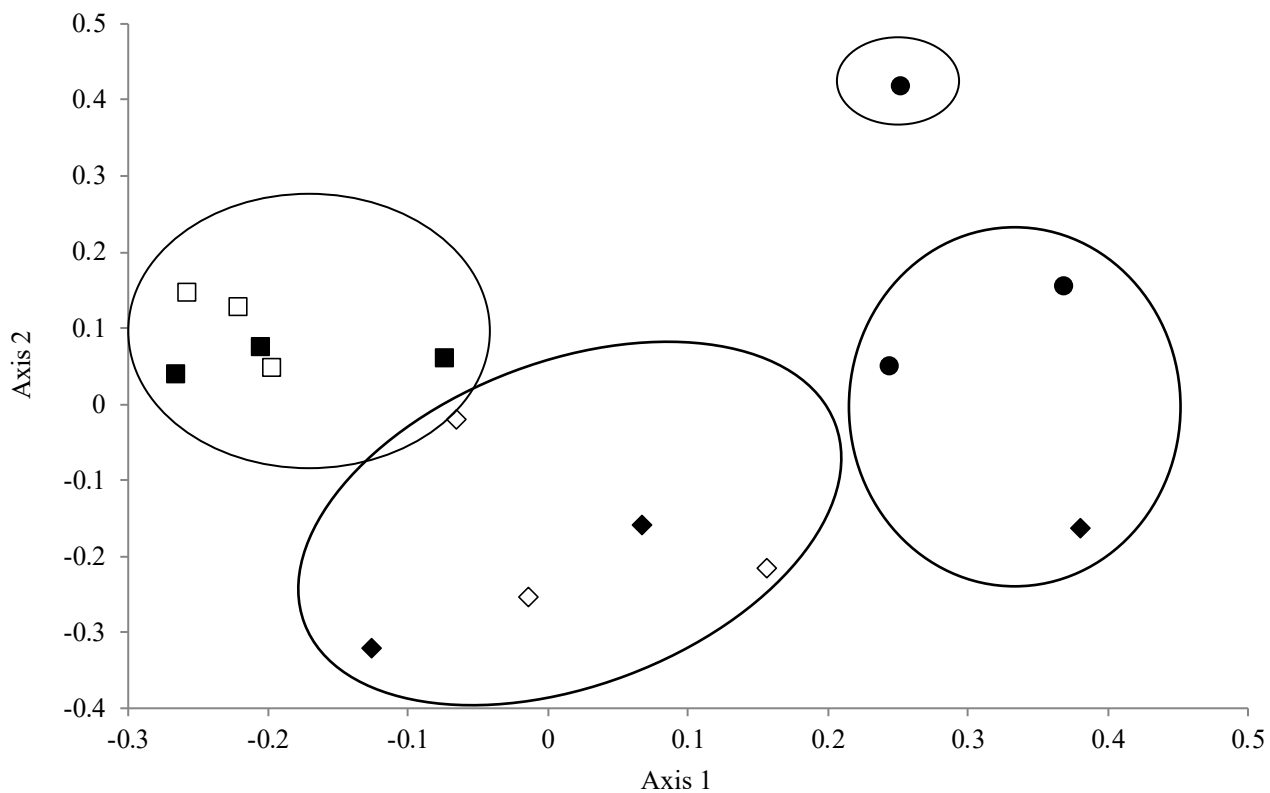
Figure 2-9 Pooled cluster analysis diagrams for ordination of bacterial communities from denaturing gradient gel electrophoresis (DGGE) fingerprints from all treatments in October of the 2009 field season. Symbols: □ = Mixed grasses + plant growth promoting rhizobacteria (PGPR); ■ = Mixed grasses - PGPR; ◇ = Sunflowers +PGPR; ◆ = Sunflowers - PGPR ; ● = Blank. Data was grouped based on the unweighted pair group method with arithmetic mean (UPGMA) dendrograms. Field plots were tested in triplicate (n=3).

production rates, and their ability to phytoextract metals, sunflowers should be considered as an additional plant species for use in the PEPS. Future work may address modifying conventional agricultural methods to enhance phytoremediation, for instance, combining plant species (intercropping), such as mixed grasses and sunflowers, to target multiple contaminants and increase overall root biomass. Intercropping has resulted in improved nutrient availability and increased yield which can lead to an increase in plant biomass production and overall plant performance (Kidd *et al.*, 2015). In addition, intercropped species can access different nutrients in the soil and impact contaminant bioavailability enhancing the phyoextraction potential of phytoremediation (Kidd *et al.*, 2015).

Another consideration for the selection of plant species for phytoremediation on urban brownfields is the attractiveness of the selected plant species to the general public. For example, during the initial field season when the site adjacent to the test plots was undergoing construction, numerous sunflowers were harvested from the field site. Therefore, additional measures may be required to restrict access to urban brownfields undergoing phytoremediation to prevent the public from the harvesting the plants. This is particularly important when the contaminants of concern include metals or other contaminants that accumulate in the above ground plant tissue.

2.5.2 Agronomic Practices

At the end of the 2008 field season, the seedlings treated with PGPR transplanted to the field, for both sunflowers and pumpkins, produced significantly more biomass than their counterparts that were directly seeded at the Site (Table 2-3). However, this effect was not reproducible during the 2009 field season. Based on the additional cost and labour, to grow the seedlings in a greenhouse and then transplant them to the field, transplanting seedlings does not appear to be a cost-effective strategy to enhance PEPS.

2.5.3 PGPR Treatment

PGPR did not have a consistent significant effect on biomass production in any of the plant species tested at the Site. Although PGPR appeared to enhance the above ground biomass of sunflower and pumpkin seedlings in 2008, this effect was not reproducible during the 2009 field season with triplicate test plots. PGPR are known to enhance plant growth directly through their metabolism (providing phytohormones or fixing nitrogen) or by modulating plant hormone levels (Ahemad and Kibret, 2014; Glick, 2012; Dos Santos and Maranho, 2018). PGPR that produce ACC deaminase promote plant root elongation, shoot growth, and enhance rhizobial nodulation (Ahemad and Kibret, 2014). PGPR with ACC-deaminase are proposed to reduce high ethylene concentrations produced in response to environmental or contaminant stressors (Glick *et al.*, 1998). However, the ability of PGPR to ameliorate the stress response is impacted

by the plant species sensitivity to ethylene and the ability of non-indigenous PGPR to compete with the native microflora and colonize the plants' root system. During the course of the field trials weather conditions were optimal, with adequate rainfall in both the 2008 and 2009 field season. The soil texture was ideal and fertilizer was applied twice per growing season to enhance plant growth.

Plant toxicity studies on soils collected from the field site showed minimal adverse effects compared to appropriate reference soils. Agnello *et al.* (2016) noted that growing plants on an urban brownfield site with mixed contaminants (PHC and metals) at concentrations over the applicable regulatory guidelines did not impact biomass production. Therefore, although the concentrations of metals and F3 were above the applicable regulatory guidelines, it is possible that contaminant concentrations were not high enough to elicit a stress response in the plants. Furthermore, it is possible that PGPR may not significantly enhance plant growth unless a stress response occurs (Liddycoat *et al.*, 2009). A significant PGPR effect at the end of the field season in 2008 may have been observed in seedlings as a result of stress caused by transplanting the seedlings to the field.

2.5.4 PHC Remediation

No significant PHC remediation was observed during the 2008 field season. The highly heterogeneous nature of the site during the initial field season made quantifying remediation challenging. Variability in initial contaminant levels results in data scatter, which can make it difficult to show statistically significant effects during field trials (Gerhardt *et al.*, 2009). This has been noted in other studies, where high variability in initial PHC concentrations, due to hot spots in the field plots, showed no statistically significant remediation despite large absolute differences in PHC degradation (Phillips *et al.*, 2009). Due to the presence of aged PHC, remediation may have been underestimated in the first year of PEPS, as desorption of previously unextractable hydrocarbons may have occurred (Phillips *et al.*, 2008). Conversely, petroleum remediation during the initial field season may have been hindered by the poor quality of the subsurface soil. Future studies may address the application of compost or increased fertilizer application rates as Palmroth *et al.* (2006) found these enhanced phytoremediation at a site with mixed contaminants. Furthermore, Phillips *et al.* (2006; 2009), found that using a mixture of grass species significantly hindered PHC remediation during the initial growing season in both field and growth chamber trials compared to single plant grass treatments. However, during the second growing season using a mixture of grass species did not have a significant impact on PHC remediation compared to single plant grass treatments (Phillips *et al.*, 2006; 2009). In general, the evaluation of phytoremediation is

challenging due to heterogeneity of the soil properties (e.g., chemical and hydraulic properties) combined with the spatial variability of the contaminants (Nedunuri *et al.*, 2000).

During the second field season of the current study, significant PHC remediation occurred in sunflower and mixed grass plots where PGPR were applied (Figure 2-3), while no significant remediation occurred in plots with plants alone. The normalized PHC concentrations decreased by an average of 25% in the planted plots treated with PGPR, by 2 to 13% in the planted plots treated without PGPR, and by 8% in the unplanted controls, compared to the initial PHC concentration at the beginning of the field season. This indicates that phytoremediation alone has limited potential to remediate PHCs on urban brownfield sites. These results are consistent with those observed by Gurska *et al.*, (2009) where PEPS was applied on a landfarm site to remediate PHC. Significant PHC remediation ($p<0.05$) was observed for plots treated with PGPR, while PHC remediation in plots without the PGPR treatment was not significantly different from the control (Gurska *et al.*, 2009). Similar results have also been observed with other plant species (e.g., corn and ryegrass) and PGPR combinations to remediate PHC (Asghar *et al.*, 2017; Plociniczak *et al.*, 2017; Hou *et al.*, 2015).

Although PGPR did not have a significant impact on biomass production in any of the selected plant species, significant petroleum remediation was observed in both sunflower and mixed grass plots treated with PGPR, implying better root performance and higher rhizosphere activity. Enhanced PHC remediation with PGPR treatment may result in more efficient bioremediation of the organic contamination in the soil. PGPR can produce biosurfactants, which can increase the bioavailability of PHC to plants or other hydrocarbon degrading rhizobacteria (Plociniczak *et al.*, 2017). This may be particularly important at sites with weathered PHCs where the hydrocarbons are sequestered in the soil via partitioning into organic matter or diffusion into the soil micropores (Northcott and Jones, 2001; Reeves *et al.*, 2001; Husemann *et al.*, 2004; Tang *et al.*, 2012). This is further supported by Hou *et al.* (2015) who found that a specific bacteria guild, comprised of petroleum degraders and biosurfactant producers, was positively correlated with higher molecular weight petroleum degradation in weathered PHC contaminated soil, rather than bacterial community diversity. PGPR may also produce plant growth promoting metabolites, liberate sequestered plant nutrients from soil, fix nitrogen, and establish the foundations of the soil nutrient cycle (Gkorezis *et al.*, 2016), which may improve plant growth and enhance PHC remediation. Therefore, although PGPR treatment did not have a significant impact on biomass production or the microbial community, PGPR may have enhanced plant root performance and higher rhizosphere activity resulting in a higher rate of PHC degradation.

An average remediation rate of ~25% was observed in 2009 for plots planted with seeds treated with PGPR, which is consistent with remediation rates reported for other field sites treated with PEPS (Gurska *et al.*, 2009). Siciliano *et al.*, (2003) observed a 30% decrease in PHC in bulk soil following two years of remediation at a site contaminated with aged hydrocarbons, which is consistent with remedial rates observed by Plociniczak *et al.* (2017), Palmroth *et al.*, (2007), Hutchinson *et al.*, (2001), Tang *et al.*, (2010), and Couto *et al.*, (2012). Shirdam *et al.*, (2009) noted approximately 60% remediation under varying conditions and amendments. However, PHC phytoremediation rates can vary greatly (4 to 49%) in planted soils, as noted by Phillips *et al.* (2006) and Cai *et al.* (2010). This may be further compounded by the presence of aged petroleum hydrocarbons as microbial communities preferentially degrade less weathered, more available organic hydrocarbons, rather than older recalcitrant PHCs that are incorporated into the soil structure (Cowie *et al.,* 2010).

2.5.5 Microbial Community

To understand the impact of the introduction of phytoremediation and the introduction of a non-indigenous PGPR on the native microbial community, several microbial indices were considered. Microbial count data indicated that phytoremediation had a positive impact on total cultural bacteria and PGPR in the rhizosphere. At the end of the field season, the planted treatments had significantly higher microbial counts when compared to the unplanted controls. The decrease in microbial counts observed in September may have been a result of low moisture content in the field soils, consistent with Phillips *et al.*, (2009) who found that heterotrophic biomass was positively correlated with local soil moisture content. Furthermore, Grayston *et al.*, (2001) and Mulder *et al.* (2003) noted that the season and climate can significantly influence the soil microbial community in the shallow soil horizons. PGPR treatment did not have a significant impact on heterotrophic or PGPR microbial counts. This is consistent with Wang (2008b), who found no significant differences in total bacterial numbers between seeded soil with and without PGPR treatment during PEPS on a landfarm using both culturable and quantitative molecular methods. The effect of PGPR on plants appears to be independent of total bacterial or PGPR numbers. For example, Plociniczak *et al.* (2017) found that PGPR biomass in soil decreased over the course of the treatment despite observing significant effects of PGPR application on plant biomass production. PGPR had a significant impact on plant biomass production on a landfarm where PEPS was applied (Gurska *et al.*, 2009) despite no significant differences in total bacterial numbers being noted between treatments (Wang, 2008b). Although total biomass is considered a baseline microbial parameter, previous studies indicate that it is not very sensitive (Winding *et al.*, 2005).

Unlike microbial biomass, CLPP using Eco-BIOLOG plates, examines the profile of potential substrate utilization reactions of a microbial community (Winding *et al.*, 2005). Use of CLPP is widespread and has shown sensitivity, reproducibility, and power distinguishing environmental stresses (Winding *et al.*, 2005). Neither PGPR application nor plot treatment had a significant impact on the carbon substrate utilization of the microbial community, which suggests that the use of exogenously added PGPR did not have a significant impact on the microbial community. Although the function of microbial communities, as assessed via CLPP, has been shown to be very sensitive to environmental changes, this method is dependent on the growth of cells under specific conditions within the Eco-BIOLOG plates, and does not resemble the natural environment (Winding *et al.*, 2005). Kirk *et al.* (2005) found that culture independent methods (DGGE) were able to detect shifts in the bacterial community, whereas Eco-BIOLOG plates did not.

The resulting banding patterns of the DGGE profiles were highly conserved between treatments, suggesting that the microbes originate from the same bacterial pool (Wang, 2008b). This is similar to observations reported by Wang (2008b) following PEPS application on a landfarm. DGGE is only able to detect 1-2% of the microbial community, and therefore, is representative of the dominant species in the microbial community (Kirk, 2004; Winding *et al.*, 2005). Typically a microbial species must represent at least 1% of the entire population to be visible within the DGGE banding pattern (Winding *et al.*, 2005). Phillips *et al.*, (2006) found differences between plants species during a phytoremediation trial based on the fluctuating presence of minor bands. Therefore, it is possible that the impact of plants during phytoremediation may be more significant in terms of overall microbial community composition rather than 1 or 2 dominant species (Phillips *et al.*, 2006), which results in similar banding patterns between treatments. Microbial diversity of subsurface soil has been shown to be much lower than corresponding surface soil samples (Zhou *et al.*, 2004; Fierer *et al.*, 2003; La Montagne *et al.*, 2003) due to changes in available resources and the changing physical environment (e.g., oxygen). The test plots were comprised of subsurface soil that was smoothed into test plots at the surface, which may have impacted the microbial diversity at the site.

Based on DGGE, using the 16S rDNA gene, the plant species utilized for phytoremediation had a larger impact on the microbial community than the PGPR treatment. This is consistent with Wang (2008b) who found no significant difference between the DGGE banding patterns between plots treated with and without PGPR at a landfarm (Wang, 2008b). This was further supported by phospholipid fatty acid (PLFA) analysis completed at the same landfarm, which found that the PLFA distribution at the PGPR treated plots was not significantly different from that of the untreated control plots (Cowie *et al.*, 2010).

Piromyou *et al.* (2011; 2013) also concluded that the application of PGPR to forage corn or Chinese kale did not impact the dominant species in microbial community structure, which were instead, strongly influenced by plant development, specifically age. Plociniczak *et al*. (2017) noted that inoculation with PGPR caused significant temporary changes in the bacterial biomass, but did not change the general microbial community composition based on PLFA analysis. This further supports the conclusion that the introduction of non-indigenous PGPR does not have a significant impact on the native microbial community.

2.6 Conclusions

Although PEPS has been successfully applied to remediate a wide range of contaminants on rural sites (Chang, 2007; Gurska *et al.*, 2009; Huang *et al.*, 2004a;b; 2005) this was the first time PEPS has been applied on an urban brownfield site. Although several plant species were tested, only sunflowers showed high germination rates and biomass production. Sunflowers have also been shown to phytoextract metals as well as degrade PHCs. Therefore, sunflowers should be considered as an additional plant species for use in PEPS. Although past studies have indicated that high metal concentrations can hinder or inhibit organic-contaminant degrading microorganisms, PEPS was successfully able to remediate PHCs by 25% during the 2009 field season. No significant PHC remediation was observed in the planted plots without PGPR, suggesting that phytoremediation alone has limited potential to remediate PHCs on urban brownfields. Although PGPR did not consistently enhance biomass production in the plant species the PHC remediation on PGPR treated plots suggests enhanced root performance and higher rhizosphere activity. Although the main goal associated with phytoremediation is the removal of contaminants from the soil, the restoration of soil quality, measured via the composition of microbial communities, is also an important consideration. Phytoremediation significantly increased the number of microbes in the rhizosphere over the field season. The results of the CLPP and DGGE analysis suggest that the plant species utilized for phytoremediation influenced the microbial community rather than the PGPR treatment. Therefore, the introduction of non-indigenous PGPR via PEPS does not have a significant impact on the native microbial community. Future work to enhance PEPS by modifying conventional agricultural methods is recommended. Although this study showed that the transplant seeding method did not consistently enhance PEPS, intercropping sunflowers and mixed grasses may provide a unique combination to address the mixed contaminants commonly found on urban brownfields.

Chapter 3

Direct Soil Contact Values for Ecological Receptors Exposed to Weathered Petroleum Hydrocarbons (PHC) Fraction 3

3.1 Overview

Concerns have been raised that the developed fine- and coarse-grained Ecological Tier 1 Canada-wide standards (CWS) for petroleum hydrocarbons (PHC) fraction 3 (F3; >nC16-nC34) based on freshly spiked soils are overly conservative. In some cases this has resulted in unnecessary and costly remediation. Consequently, recent guidelines were developed using field soils and relevant endpoints (e.g., toxicity). The applicability of the current guidelines to sites with historical PHC contamination warrants further investigation as studies with a limited number of PHC concentrations and a ranked response approach were relied upon to derive the current CWS. The purpose of the current study is to examine the toxicity of weathered PHC (mostly F3) in a coarse-grained soil to derive direct soil contact values for ecological receptors. Coarse grained field and reference soils were obtained from a landfarm site where sludge had been spread for approximately 35 years. Toxicity tests using plants were conducted following standardized test protocols developed by Environment Canada. Endpoint effective concentrations (EC)/and inhibitory concentrations (IC) at the 25 percent (%) effect level were calculated to derive soil standards for F3 in coarse-grained soil protective of plants exposed through direct contact with soil. The values derived for the weathered F3 of 659 and 1,961 milligrams per kilogram (mg/kg), respectively, for agricultural/residential and industrial land use are higher than the current ecological Tier 1 CWS for F3 in soil (300 mg/kg for agricultural/residential and 1,700 mg/kg for commercial/industrial) and support the derivation of remediation targets higher than the current guideline. Additional studies with a more sensitive test species (i.e., earthworms) are recommended to confirm this conclusion.

3.2 Introduction

The Canadian Council of Ministers of the Environment (CCME) estimates that there are over 250,000 potential or actual sites contaminated with PHCs in Canada (CCME, 2008). Other sources suggest this number could be over 1 million sites (Meridian Environmental, 2007). Furthermore, of the known contaminated sites listed on the Federal Contaminated Sites Inventory (FCSI), PHC was identified as a contaminant of concern at 55 % of the sites (CCME, 2008). Soil contamination may occur during petroleum extraction, refining and distribution, transportation, accidental spills, or deliberate application

of PHC products to soil at landfarming facilities. The remediation and management of these PHC impacted sites are a concern and challenge for stakeholders as the number of sites and extent of contamination makes this a multibillion dollar issue in Canada (CCME, 2008).

Endpoints of toxicity studies, with plants and other soil organisms, using crude oil mixtures have been shown to vary up to four orders of magnitude due to variations in PHC composition, weathering and physical/chemical characteristics of the soil (Visser *et al.*, 2003; Van Gestel *et al.*, 2001). Therefore, CCME regulates PHCs based on four separate fractions. The four fractions are based on review of petroleum product composition, hydrocarbon fate, and human toxicity thresholds which were initially developed by the Total Petroleum Hydrocarbon Criterion Working Group (Potter and Simmons, 1998; Gustafson *et al.*, 1997). The four fractions are determined based on elution time on a gas chromatography column; however, a number of compounds, such as benzene, toluene, ethylbenzene, and xylenes are addressed independently. The chain length of the n-alkane defines the range of each fraction as follows: PHC fraction 1 (F1; >nC6–nC10), fraction 2 (F2; >nC10-nC16), fraction 3 (F3; >nC16-nC34), and fraction 4 (F4; nC35+). Of these fractions, F1 has been shown to be the most toxic to ecological receptors, followed by F2, F3, and F4 (CCME, 2008; Dorn *et al.*, 1998, Cermak *et al.*, 2010, Van Gestel *et al.*, 2001). A significant effort was made to collect and analyze ecotoxicological data for soil organisms from fraction-specific toxicity studies to develop the direct contact ecological soil quality guidelines that were established by CCME in 2001 (CCME, 2008). The initial guidelines established in 2001 were derived from the 25th percentile of the species sensitivity distribution of available ecotoxicological data (effective concentration [EC]50/ lethal concentration [LC]50), for plants and soil invertebrates, using freshly spiked field and artificial test soils and adjusting for analytical recovery for F3 (CCME, 2008).

The detrimental effects of PHCs on plants and other soil organisms can be also attributed to changes in the physical and chemical properties of soil following PHC contamination in addition to PHC toxicity (Robertson *et al.*, 2007; Mikkonnen *et al.*, 2012). PHC impacts soil texture, which is a fundamental soil property to which all others are related to (Nemes and Rawls, 2006). In addition, PHC has been shown to decrease water availability and limit water and gas exchange impacting plant growth (Roberston *et al.*, 2007; Adam and Duncan, 2002). The level of impact that PHC has on soil quality is greatly dependent on the duration of PHC exposure (Wang *et al.*, 2010); and therefore freshly spiked artificial test soils may not be representative of the toxicity of field soils. In addition, toxicity data based on freshly spiked PHC may not be indicative of the environmental risk posed by well-weathered PHCs resulting in unnecessary remediation. Weathering and aging of petroleum can sequester hydrocarbons in soil through partitioning

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into organic matter and diffusion into soil micropores (Northcott and Jones, 2001, Reeves *et al.*, 2001, Husemann *et al.*, 2004, Tang *et al.*, 2012). As a result, PHC becomes biologically less accessible. This changes the available composition of the PHC and lowers toxicity. The relative composition of polycyclic aromatic hydrocarbons (PAHs), n-alkanes, and isoprenoids has been used as a method to quantify the degree of weathering (CCME, 2008, Didyk and Simoneit, 1989, Rogues *et al.*, 1994, Wang *et al.*, 1995). However, the toxicity of these individual classes of PHCs is not known. Furthermore, to determine the degree of weathering compositional data for the original product that was released at a specific spill site is required; however, this information is often not available for historically contaminated spill sites.

Angell *et al.* (2012) proposed soil standards for weathered F2 in fine-grained soils (338 mg/kg for commercial/industrial land use), based on plant and soil invertebrate toxicity testing, that are higher than the current PHC CWS derived from studies using fresh product (262 mg/kg for commercial/industrial land use). Concerns were raised that the F3 ecological standards developed in 2001 may be overly conservative as a lack of toxicity, to plants and other organisms, has been observed at several bioremediated sites with concentrations of F3 above the PHC CWS (CCME, 2008). In response, the most recent CCME guidance document (2008) relied on field trials and alternate approaches, such as the ranked response distributions, to derive the current F3 guidelines. A Ranked Response Approach (RRA) was developed to derive guideline values from single concentration field studies by presenting the data as a response relative to the control for a range of species and endpoints. A field soil was considered to meet the guideline if the 25th percentile (agricultural/residential land use) or 50th percentile (industrial/commercial land use) of the responses relative to the reference soil for all species and non-redundant endpoints was equal to or greater than 75% (CCME, 2006). CCME (2008) derived direct soil contact values for F3 for medium/fine textured soil for residential and industrial/commercial land use of 1,300 mg/kg and 2,500 mg/kg, respectively. Direct contact values of 300 mg/kg and 1,700 m/kg were established for F3 for coarse textured soil for residential and industrial/commercial land use, respectively. A long-term field study conducted by Visser (2005) was heavily relied upon due to the large number of species used for the toxicity tests, relevant endpoints (crop yields and invertebrate populations), chronic duration, and analytical methods used (CCME, 2008). Fresh Alberta Federated Crude oil was applied to sandy loam and clay loam field plots at application rates ranging from 1.2% (12,000 parts per million [ppm]) to 3.7% (37,000 ppm) to derive medium-fine and coarse direct soil contact values for ecological receptors. Although the CCME (2008) soil contact values are based on field soils using relevant endpoints, the RRA was used as a limited number of PHC application rates were available. Furthermore, subsequent to plant toxicity bioassays by Visser (2005), Environment Canada (EC) established

standardized test protocols for measuring emergence and growth of plants exposed to contaminants in soil (EC, 2007).

The PHC CWS for F3 requires further examination due to a lack of observed toxicity to plants and other organism at sites with concentrations of F3 above the PHC CWS. Although the most recent CCME guidance document considered recent data obtained from field trials and alternative approaches (e.g., RRA), the suitability of the current PHC CWS for F3 for weathered F3 needs to be investigated using standardized protocol and dose response curves to propose a guideline value. The purpose of the current study was to examine the toxicity of weathered F3 to plant species using a well aged (> 20 years) soil with high historical PHC concentrations. A direct soil contact value was derived for ecological receptors using standardized test protocols established by EC (2007) and a dose response approach.

3.3 Materials and Methods

3.3.1 Soil collection and characterization

Soil was collected from the Ah horizon (0 – 0.3 metres [m]) from a landfarm site located west of Toronto, Ontario. Petroleum sludge had been spread at the site for approximately 35 years before ceasing in 2006; following which the soils were allowed to naturally weather. Reference soil was collected adjacent to the site. Prior to soil collection, the existing plant growth and root mat was removed. Following soil collection, the soil was filtered through a 6 millimetre (mm) screen to remove large debris and indigenous fauna. The screened material was stored in 20 litre (L) plastic buckets.

Based on preliminary in-house petroleum hydrocarbon concentrations determined by University of Waterloo (Waterloo, Ontario), control soil was mixed with PHC impacted soils to generate an exposure series comprised of 10 exposure concentrations as well as field-collected reference soil (negative control soil) for a total of 11 treatments. The aim of the exposure series was to capture PHC concentrations below the existing Tier 1 standards, close to the current standard, and above the standards. Due to a large variation in PHC content in the field soil, select buckets of field soils were mixed with control soils to achieve the desired log concentration series. The experimental design supports the use of regression analyses to determine the toxicity endpoints (Stephenson *et al.*, 2000).

Physical (Table 3-1) and chemical (Table 3-2) characterization of the contaminated soils and controls was conducted. Some of the physical parameters, such as particle size distribution, soil texture, organic matter (percent [%]), total organic carbon content (%), cation exchange capacity, total phosphorous (ppm), total nitrogen (%), were characterized by SGS Agrifood Laboratories (Guelph, ON, Canada). The

Table 3-1 Physicochemical characteristics of the soils used for plant toxicity testing. Soils consisted of a field collected reference soil (O0), a petroleum hydrocarbon (PHC) fraction 3 exposure series (O1 to O10) derived by mixing reference soil with PHC impacted soil from a landfarm site located west of Toronto, Ontario, and an artificial control soil (AS). The various physicochemical characteristics of the soils were determined by SGS Agrifood Laborotories (Guelph, Ontario, Canada) or the University of Waterloo.

Soil Properties	Reference Soil	Petroleum Hydrocarbon Impacted Soil Exposure Series										Artificial Soil (AS)
	O0	O1	O 2	O3	O4	O5	O6	O7	O8	O9	O10	
pH[1,2]	5.1	5.1	5.0	5.8	5.8	6.3	6.7	6.8	6.6	6.7	6.5	7.5
Electrical Conductivity (dS/dm)[1]	1.8	2.0	2.0	1.0	1.9	2.1	2.7	4.5	4.9	4.8	5.4	0.7
Total Organic Carbon (%)[2]	2.2	1.3	2.1	2.5	2.6	2.9	3.7	8.3	4.8	6.3	9.0	2.2
Organic Matter (%)[2]	4.0	2.3	3.7	4.5	4.7	5.1	6.7	14.9	8.7	11.3	16.2	4.0
Total Phosphorous (ppm)[2]	534	524	549	581	584	611	766	669	861	917	1214	55
Total Nitrogen (%)[2]	0.34	0.30	0.28	0.24	0.27	0.23	0.30	0.35	0.36	0.45	0.60	0.05
Water Holding Capacity (%)[1]	NC	52	50	50	52	50	47	43	47	44	46	64
Water Repellency (M EtOH)[1]	3.8	2.5	2.5	2.7	2.6	2.4	2.8	2.8	3.9	3.8	4.1	0
Cation Exchange Capacity[2]	5.9	5.6	5.9	6.7	7.9	8.8	9.4	9.5	10.3	12.6	12.0	8.7
Sand (%)[2]	80	76	76	78	79	79	76	71	71	71	68	76
Silt (%)[2]	15	17	17	17	16	17	17	21	21	22	26	8
Clay (%)[2]	5	7	7	5	5	4	7	8	8	7	6	16
Soil Texture[2]	Loamy Sand	Sandy Loam	Sandy Loam	Loamy Sand	Loamy Sand	Loamy Sand	Sandy Loam	Sandy Loam	Sandy Loam	Sandy Loam	Sandy Loam	Sandy Loam

[1]   Soil property determined through University of Waterloo analysis

[2]   Soil property determined by SGS Agrifood Laborotories (Guelph, Ontario, Canada)

NC – Not calculated

Table 3-2 Petroleum hydrocarbon (PHC), polycyclic aromatic hydrocarbon (PAH), and metal concentrations in the soils used for plant toxicity testing. Soils consisted of a field collected reference soil (O0), a petroleum hydrocarbon (PHC) fraction 3 exposure series (O1 to O10) derived by mixing reference soil with PHC impacted soil from a landfarm site located west of Toronto, Ontario, and an artificial control soil (AS). Chemical concentrations were determined by Maxxam Analytics.

Chemical Concentrations (mg/kg)	Reference Soil	PHC Impacted Soil Exposure Series										Artificial Soil
	O0	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	
Petroleum Hydrocarbons (PHCs)												
Fraction 2	<10	<10	17	<10	**99**	62	**330**	**410**	**520**	**160**	**635**	<10
Fraction 3	95	180	280	**390**	**1200**	**1300**	**3500**	**6200**	**5400**	**7900**	**12000**	27
Fraction 4	51[A]	130	130[A]	240	550[A]	640	1700[A]	3100[A]	2000	3900[A]	5350	17
Polycyclic Aromatic Hydrocarbons (PAHs)												
Acenaphthene	<0.005	<0.005	<0.005	<0.01	<0.02	<0.02	<0.02	<0.02	<0.02	<0.10	<0.10	-
Acenaphthylene	ND	0.0051	0.0052	<0.01	<0.02	<0.02	0.034	0.040	0.050	<0.10	0.10	-
Anthracene	0.015	0.019	0.018	0.025	0.035	0.055	0.11	0.15	0.17	0.24	0.30	-
Benzo(a)anthracene	0.0083	0.010	0.012	<0.05[B]	<0.02	<0.05[B]	<0.2[B]	<0.2[B]	<0.2[B]	<0.2[B]	<0.5[B]	-
Benzo(a)pyrene	0.012	0.017	0.016	0.032	0.035	0.046	0.061	0.082	0.21	0.11	0.30	-
Benzo(b/j)fluoranthene	0.016	0.017	0.019	0.027	0.026	0.025	0.034	0.029	0.067	<0.10	0.13	-
Benzo(g,h,i)perylene	0.048	0.067	0.072	0.12	0.22	0.35	0.64	0.93	0.99	1.5	1.9	-
Benzo(k)fluoranthene	<0.005	<0.005	0.0052	<0.01	<0.02	<0.02	<0.02	<0.02	<0.02	<0.10	<0.10	-
Chrysene	0.0092	0.0084	0.011	0.014	<0.02	<0.05[B]	<0.05[B]	<0.05[B]	<0.1[B]	<0.10	<0.2[B]	-
Dibenz(a,h)anthracene	< 0.005	<0.005	<0.005	<0.01	<0.02	<0.02	<0.02	<0.02	<0.02	<0.10	<0.10	-
Fluoranthene	0.015	0.016	0.019	0.036	0.026	0.025	0.040	0.029	0.036	<0.10	0.14	-
Fluorene	<0.005	<0.005	<0.005	<0.01	<0.02	<0.02	<0.02	<0.02	0.021	<0.10	<0.10	-
Indeno(1,2,3-cd)pyrene	0.016	0.021	0.021	0.033	0.044	0.064	0.10	0.16	0.20	0.25	0.36	-
1-Methylnaphthalene	0.0055	0.0065	0.0066	0.011	0.021	0.032	0.045	0.060	0.086	0.11	0.15	-
2-Methylnaphthalene	0.0069	0.0084	0.0080	0.013	0.033	0.045	0.061	0.079	0.11	0.16	0.23	-
Naphthalene	<0.005	<0.005	<0.005	0.011	0.058	0.023	0.054	0.049	0.052	<0.10	<0.10	-
Phenanthrene	0.015	0.016	0.018	0.038	0.053	0.059	0.089	0.079	0.10	0.15	0.23	-
Pyrene	0.015	0.016	0.020	0.034	0.033	0.039	0.054	0.057	0.082	0.10	0.18	-

Chemical Concentrations (mg/kg)	Reference Soil	PHC Impacted Soil Exposure Series										Artificial Soil
	O0	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	
Metals												
Antimony	<0.2	0.25	<0.2	<0.2	0.37	0.32	0.60	0.88	0.70	1.5	1.6	-
Arsenic	3.4	3.4	3.9	3.9	4.6	4.3	5.8	7.7	6.3	11	12	-
Barium	37	38	42	41	60	49	71	97	75	140	140	-
Beryllium	0.22	0.20	0.23	0.23	0.25	0.21	0.23	0.23	0.21	0.27	0.22	-
Cadmium	0.20	0.16	0.16	0.16	0.23	0.24	0.30	0.41	0.37	0.66	0.65	-
Chromium	32	32	37	38	53	54	86	130	88	**180**	**200**	-
Cobalt	2.6	2.6	2.9	2.7	3.7	3.2	4.4	6.2	5.1	8.6	8.5	-
Copper	9.2	9.4	11	11	16	17	28	46	31	66	71	-
Lead	19	19	21	25	27	24	33	41	32	60	62	-
Molybdenum	ND	ND	0.58	0.61	1.1	1.2	2.2	3.8	3.0	5.8	6.0	-
Nickel	6.7	6.9	7.6	7.4	11	10	15	24	18	36	37	-
Selenium	<0.5	<0.5	<0.5	<0.5	0.51	0.66	1.1	1.9	1.3	2.4	3.0	-
Silver	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	-
Thallium	0.053	0.061	0.063	0.058	0.10	0.078	0.14	0.18	0.15	0.25	0.26	-
Vanadium	18	19	19	18	24	19	23	32	26	47	49	-
Zinc	47	49	55	53	82	83	130	200	170	330	340	-

-   Soil samples not analyzed for indicated analyte

Bold   Chemical concentrations greater than the Table 3: Full-Depth Generic Site Condition Standards in a Non-Potable Ground Water Condition for residential/parkland/institutional land use with coarse-grained (MOE, 2011).

[A]   During the petroleum hydrocarbon analysis the chromatogram did not reach baseline at C50; therefore, the fraction 4 (F4) values reported may underestimate the F4 concentration in the soil samples.

[B]   The detection limit was elevated due to matrix interferences.

soil texture of the field soils used in the plant toxicity testing was classified as loamy sand or sandy loam, which are considered coarse grained by CCME (2008).

Water repellency was determined by determining the ethanol concentration (0 to 6 millimole [mM] in 0.2 mM intervals) required for the soil sample to take up a drop within 10 seconds (s). Soil was air-dried for 48 hr, sieved through a 2 mm sieve, and allowed to dry for an additional 48 hrs. Aluminum weigh boats (42 millilitre [mL] capacity, 6 centimetre [cm] diameter) were filled with each field soil in triplicate, ensuring adequate surface area. A drop size of 250 microlitre (μL) was administered using a pipette. The time to absorption was recorded, and anything less than 10 seconds (s) was recorded as a positive test result (Roy and McGill, 2002).

Electrical Conductivity ($EC_{1:2}$) measurements were performed in duplicate. Soil was air-dried for 48 hrs and ground using a mortar and pestle. An aliquot of 15 g of soil was mixed with 30 mL double distilled water (ddH_2O) in a 50 mL plastic Falcon culture tube. The mixture was inverted several times and shaken on a shaker table at 200 rpm for 30 min. The electrical-conductivity of the supernatant was measured with an electrical-conductivity meter (Oakton Instruments, Vernon Hills, IL, U.S.A).

pH measurements were determined at the beginning of each plant toxicity test. An aliquot of 25 g of soil was mixed with 50 mL ddH_2O in a 100 mL glass beaker. The mixture was stirred thoroughly with a metal scapula and allowed to settle for 30 minutes (min). The pH was measured with a pH meter (Accumet Basic, AB15 pH meter, Fisher Scientific).

Water holding capacity (WHC) measurements were performed in triplicate (EC, 2007). A soil sample of ~130 grams (g) was placed in a glass petri plate and dried at 105 degrees celcius (°C) for 24 hours (hr). An aliquot of 100 g of oven-dried soil was placed into a 250 mL glass beaker with 100 mL ddH_2O and mixed with a glass rod. The slurry was poured through a glass funnel inserted with a hydrated folded piece of filter paper (185 mm diameter, P8 coarse porosity) placed in a 500 mL Erlenmeyer flask. Any soil remaining in the glass beaker was rinsed into the funnel using as little water as possible. The funnel was covered with aluminum foil and allowed to drain for 3 hrs and then weighed. The WHC for the soil sample was expressed as a percentage of soil dry mass (WHC = [(mass of funnel + hydrated filter paper + wet mass of soil) – (mass of funnel + hydrated filter paper + dry mass of soil)]/dry mass of soil] x 100) (EC, 2007).

Petroleum analysis (F2-F4) to determine the PHC concentration in each exposure series test soil (O0 to O10) was outsourced to Maxxam Analytics, Calgary, Alberta, Canada. To summarize, a 10 g aliquot of homogenized soil was cold-extracted with 1:1 acetone: hexane. The extract was passed through a column

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containing 1 g of activated silica gel and eluted with 1:1 dichloromethane hexane solvent. A matrix spike, spike, and blank were run with each set of samples. Recovery of the laboratory surrogate was within the quality control limits of 50 to 130%. Quantification of the eluted PHCs was determined using gas chromatography with flame ionization detection (FID). The reportable detection limits were 10 mg/kg for F2, F3 and F4. Metal and PAH analysis was also outsourced to Maxxam Analytics.

In-house PHC concentrations were also determined by the University of Waterloo (Waterloo, Ontario) to examine whether PHC concentrations in three treatment soils (O3, O6 and O10) changed during the course of the two and/or three week plant toxicity tests. To summarize, a 10 g aliquot of homogenized soil was extracted with a 1:1 acetone: hexane and passed through a silica gel column to remove biogenics. Quantification of the eluted PHCs was determined using gas chromatography with FID.

3.3.2 Toxicity Testing

3.3.2.1 Soil Treatment Preparation

Soils for the plant toxicity tests were prepared on the day of test initiation as per EC (2007). The moisture content of each batch of test soil and negative control soil was determined and ddH_2O water added until the desired moisture level was achieved. The desired moisture content for each test and control soil was determined by adding water to oven dried soil until soil clumps ranging from 3 to 5 mm in diameter formed (EC, 2007). The percent moisture was calculated and expressed as a percentage of the WHC on a dry weight basis. Immediately following the mixing of a batch of test soil and negative control soil, an identical wet weight of soil equivalent to a volume of ~500 mL (~400 to 550 g) was transferred to each replicate test vessel and leveled using a spoon.

3.3.2.2 Plant Species

Toxicity testing was performed on five different plant species, including 3 dicots and 2 monocots, consistent with EC (2000) and ASTM International (1999). These selected plant species were consistent with those used to develop the Tier 1 PHC standards (CCME, 2001). Definitive plant growth tests were performed with northern wheatgrass (*Elymus lanceolatus*), barley (*Hordeium vulgare*), alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*), and cucumber (*Cucumis sativus*) using the methods and procedures of EC (2007).

3.3.2.3 Toxicity Tests

Plant tests usually consisted of five replicates per treatment. In several instances only four replicates per treatment were used due to limited soil availability. Test soils were hydrated with ddH$_2$O to 29% to 46% of the WHC. Five seeds were planted in each test unit for northern wheatgrass, cucumber, red clover and barley, and 10 seeds were planted for alfalfa. Measured endpoints included emergence (7 days [d] and 14/21 d), shoot and root length (mm), and shoot and root dry mass (mg/plant). Test durations were 14 d for cucumber, barley, and red clover, and 21 d for northern wheatgrass and alfalfa. Toxicity testing was conducted within a temperature controlled growth chamber which maintained 24 ± 3°C and 15 ± 3°, respectively for a light:dark photoperiod of 16:8 hr. The measured light fluency rate was within 300 ± 100 micromole per square metre per second (μmol/m^2·s) (275-370 μmol/m^2·s).

Replicates were grown in 1 L polypropylene test units, filled with ~400 to 550 g wet weight of soil, which were randomly placed within the growth chamber. Lids were removed on day 7 of the test (northern wheatgrass, red clover, alfalfa, cucumber) or after plant heights reached the lid (day 5 for barley). After the lids were removed, plants were watered to near-saturation once daily with ddH$_2$O. Soil hydration levels were measured at the end of the study duration. Test units were randomized daily to limit potential edge effects.

Performance of the plant species in the negative control soil treatment (artificial soil) was evaluated relative to the test method validity criteria consistent with EC test methods (EC, 2007). Reference toxicity tests were conducted with boric acid spiked artificial soil. Boric acid reference toxicity test data was analyzed to ensure that organism sensitivity and performance was constant with time. The negative control treatments during all of the plant toxicity tests passed EC test method validity criteria for percentage of survival of emerged seedlings, percentage of seedlings with phytotoxicity symptoms, mean percentage of emergence, mean shoot length, and mean root length. Reference toxicant tests with boric acid had consistent endpoint results over the course of the plant toxicity tests.

3.3.3 Statistical Analysis

Statistical analysis followed the recommendations of EC (2007). Data for plant emergence (quantal endpoint) was analyzed using logit procedures to determine EC25s/50s (SPSS). Non-linear regression analyses of non-quantal data to determine inhibitory concentrations (IC)25/50s was performed using SYSTAT (Systat Software) (2007). Goodness-of-fit for quantitative endpoints was assessed by line fit to scatter plot (co-efficient of determination), r^2, and the closeness of the confidence intervals. The

quantitative endpoints were assessed for normality (Shapiro-Wilk normality test; $p>0.05$) and homogeneity of variances (analysis of variance [ANOVA], $p>0.05$). When the toxicity data were not normal ($p<0.05$), the data were weighted by the inverse of the variance. For quantitative endpoints which did not meet the assumptions of a model using nonlinear regression, the program ICPIN was used to estimate the IC25 via linear interpolation (Norgerg-King, 1993). As the soil contained F2 to F4, quantal and non-quantal endpoints were modeled against PHC concentrations expressed as F2, F3, F4, F2+ F3, and F2 – F4. As F2+F3 had a better goodness-of-fit for the largest percentage of the quantitative endpoints, it was used to derive the EC/IC25s (CCME, 2006).

Although the concentrations of F2+F3 in the reference soil fall within typical Ontario background concentrations (MOE, 2011), due to the magnitude of PHC in the control soil (105 mg/kg F2+F3) the data were normalized by dividing the PHC concentration in each test soil by the reference PHC concentration (105 mg/kg F2+F3). Following this transformation, soil concentrations were log-transformed prior to analysis. Control values were assigned a log value of 0.0001, which was considered to be the no-effect level.

3.3.4 Estimation of Direct Contact Soil Contact Values

The EC/IC25s for the various plant species were used to generate a distribution of responses, from which the direct soil contact values for ecological receptors were derived for both residential and industrial/commercial land use distributions. The derivation process followed the precedent set by the 2008 CCME protocol, which uses a rank species sensitivity analysis. Regression procedures were applied to the ranks, and the 25th percentile was used to derive soil contact values for agricultural/residential land use, while the 50th percentile was used for commercial/industrial land use.

The data were also organized into a RRA to derive direct soil contact values. This approach entails expressing non-redundant data for each endpoint as a percentage of the reference response and ranking them accordingly (CCME 2008). Non-redundant endpoints (end of test [14 or 21 d] emergence, root weight, root length, shoot length and shoot weight of the test soils were expressed as a percentage of the Site control. The 25th and 50th percentile was determined for each test soil. Consistent with the CCME (2006) approach, a ranked response distribution (RRD) was considered to meet the requirements for agricultural or residential land use if the 25th percentile of the RRD showed a response of at least 75% of the control response. For commercial and industrial land use; the 50th percentile required a response of at least 75% of the control response (CCME, 2006).

3.4 Results

3.4.1 Test Soils

3.4.1.1 Physical and Chemical Properties of Test Soils

The exposure series was created by mixing landfarm soil with reference soil to achieve the desired log concentration series. As a result of this mixing process and nature of the field-collected soils, a range of chemical and physical soil properties were observed across the exposure series (Table 3-1 and Table 3-2). The resulting range of exposure levels in the test soils ranged from 95 mg/kg to 12,000 mg/kg F3. This encompassed levels below the existing Tier 1 standards (e.g., no effects concentrations), close to the existing standards (e.g., 280 F3 mg/kg soil dry wt.), and above the existing standards (Table 3-2). The range of exposure concentrations was limited by the highest PHC concentration collected in the field (~ 12,000 mg/kg F3 mg/kg soil dry wt.). The general PHC composition of the test soils were 4% F2, 64% F3, and 32% F4. However, as noted in Table 3-2, during PHC analysis the chromatogram did not reach baseline at C50; therefore, some of the F4 values presented in the table may underestimate the F4. Exceedances above the existing Tier 1 standards for F2 and F4 were also noted in several of the test soils. The highly weathered nature of the PHC in the test soil can be inferred from the chromatograms (Mikkonen *et al.*, 2012). The aromatics and aliphatics of the PHC for the field soil obtained from the landfarm appear as an unresolved complex mixture (Figure 3-1).

The test soils were also analyzed for PAHs (part of F3) and metals (Table 3-2). PAHs were not detected or well below the Full Depth Generic Site Condition Standards in a Non-Potable Ground Water Condition for residential/parkland/institutional property use and coarse grained soil as outlined by the Ontario Ministry of the Environment (MOE) under Ontario Regulation 153/04 (MOE, 2011). Measurable concentrations of several metals were found in the test soils, with chromium exceeding the Site Condition Standards in two test soils (O9 and O10) (Table 3-2).

In general most of the soil properties (pH, EC, TOC, OC, OM, P, N and CEC) increased with increasing PHC concentrations (Table 3-1). The EC increased from 1.8 dS/dm in the reference soil to 5.4 dS/dm in the field soil with the highest PHC concentration. Measured P, N, and CEC in the field soil with the highest PHC concentration was approximately double that of the reference soil, while TOC and OM were quadruple that of the reference soil. However, these changes in the soil properties did not appear to impact plant growth during the plant toxicity tests. The WR of the test soils increased with increasing PHC concentration while a slight decrease in WHC was found (Table 3-1). During the current study,
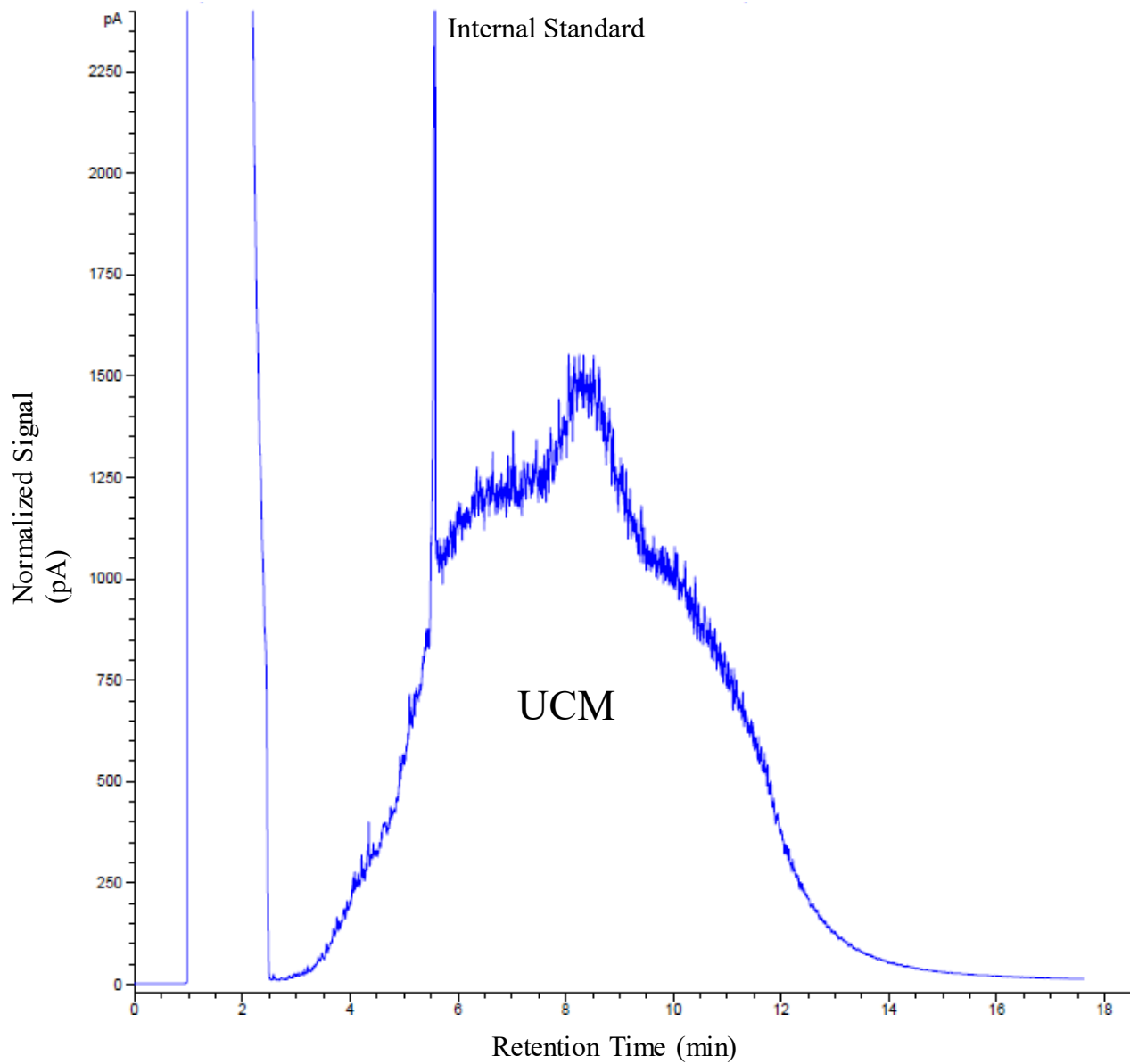
Figure 3-1 Chromatogram of field soil (O10) used to derive the petroleum hydrocarbon (PHC) exposure series in the current study demonstrating the presence of the unresolved complex mixture (UCM) hump. PHC quantification was determined by gas chromatography with flame ionization detection (FID) by Maxxam Analytics (Calgary, Alberta, Canada).

some of the test soils displayed water repellent properties. For instance, water would pool in beads on the surface of the field soil within the test vessel or rapidly percolate through interstitial soil spaces to the bottom of the pots.

3.4.1.2 PHC Stability in Test Soils

The F2 –F4 concentrations in three test soils (low, medium, and high F2 – F4) were measured at the end of the plant toxicity tests (14 d or 21 d) to verify that the PHC in the exposure series did not decrease over the course of the toxicity test. The F2-F4 concentrations remained relatively stable during the toxicity tests for all exposure concentrations (Figure 3-2).

3.4.2 Toxicity

3.4.2.1 Derived Effective Concentrations/Inhibitory Concentrations

The toxicity responses of the plant species were modeled based on F2+F3 (CCME, 2006). Responses were modeled using the log-log, linear, logistic and gompertz regression models. In a few instances the data were weighted by the inverse of the variance (Table 3-3; Table 3-4). In a select number of cases the responses were modeled using the ICPIN computer program (Norgerg-King, 1993).

The calculated EC/IC25s ranged from 132 mg/kg F2+F3 for northern wheatgrass root weight to 56,910 mg/kg F2+F3 for cucumber root length. The average calculated EC/IC25 for the non-redundant endpoints was 7,514 mg/kg. A number of the other derived EC/IC25s (28%) exceeded the maximum F2+F3 concentration used in this study of 12,635 mg/kg (Table 3-3). The calculated EC/IC50s range from 282 mg/kg F2+F3 for northern wheatgrass root weight to 1.45E+15 mg/kg F2+F3 for barley root length (Table 3-4). It should be noted that 60% of the derived EC/IC50s were extrapolated above the maximum F2+F3 concentration that was tested in this study. Repeating this study with a wider range of PHC concentrations would increase the confidence in these extrapolated EC/IC50 values and increase confidence by narrowing the confidence intervals.

In the current study many of the endpoints for various plant species demonstrated a very steep response curve at low concentrations (Appendix A; Figures A-1 to A-5). This was particularly true for northern wheatgrass (shoot and root weight) and barley (root weight) which are the three lowest IC25s (for non-redundant endpoints) reported in the current study at 178 mg/kg, 132 mg/kg, and 247 mg/kg, respectively (Table 3-3). Furthermore, these three lowest IC25s were the only IC25s below the current PHC CWS for agricultural/residential land use of 300 mg/kg, the remainder of the non-redundant endpoints were greater than the current PHC CWS.
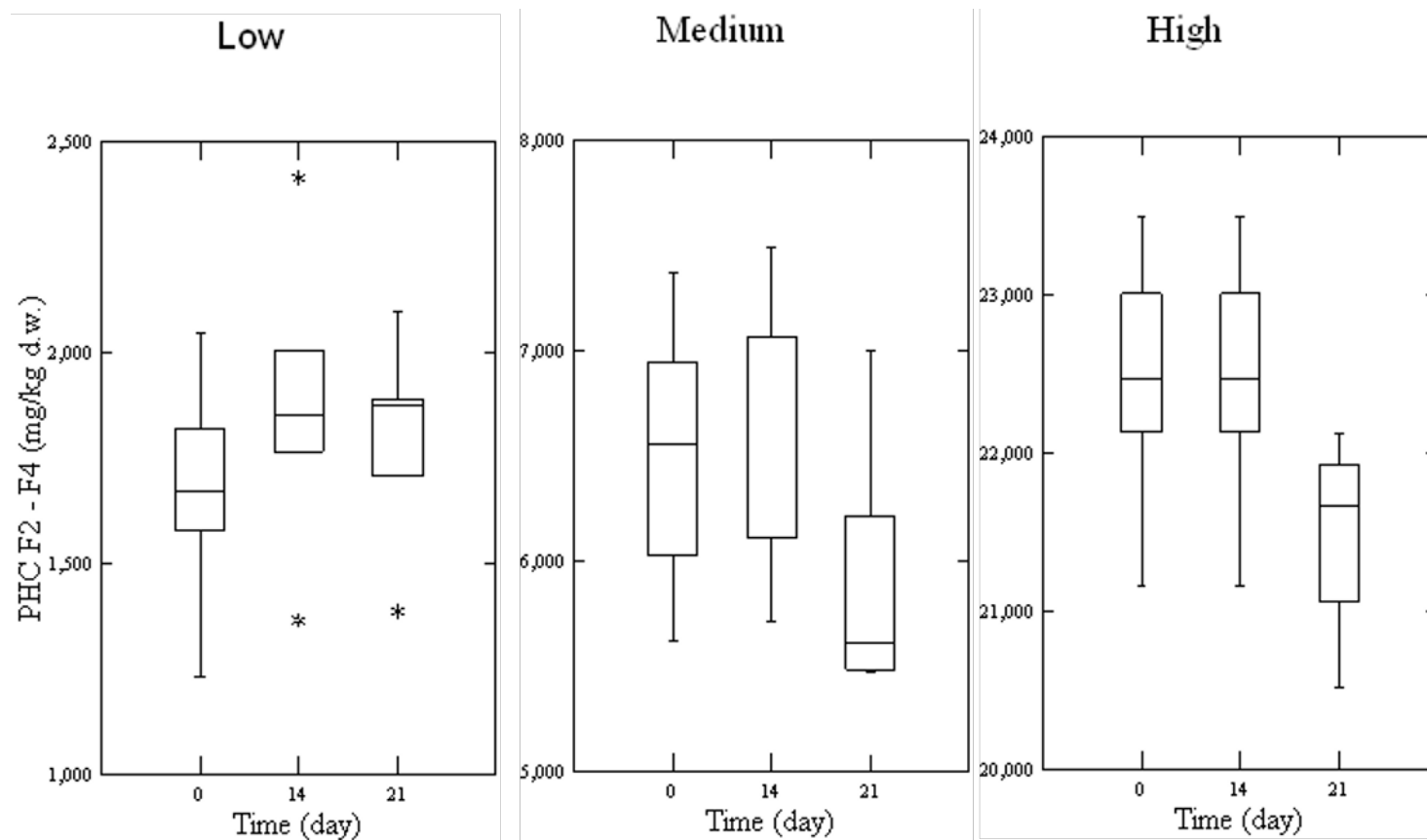
Figure 3-2 Changes in soil petroleum hydrocarbon (PHC) fraction 2 (F2) to F4 (mg/kg d.w.) levels over the course of testing, at the start of a test (day 0) and at the end of the plant tests (14 days or 21 days) for selected low, medium and high exposure concentrations. Sample sizes ranged for the low (n = 5 to 7), medium (n = 5 or 8), and high (n = 4 or 6) exposure concentrations. The vertical rectangles contain the interquartile range. The horizontal lines bisecting the vertical rectangles illustrate the medians of the distributions. The whiskers extending from the vertical rectangles indicate the maximum and minimum values for the data. The astericks indicate extreme outliers.

Table 3-3 Summary of effective concentrations and inhibitory concentrations 25% (ECs/ICs) for red clover, northern wheatgrass, barley, cucumber and alfalfa for six different endpoints (emergence and length and weight for roots and shoots) generated by plant toxicity testing.

Parameter	Model	EC/IC25 (mg/kg F2 + F3)	LCL (mg/kg F2 + F3)	UCL (mg/kg F2 + F3)	r^2	X^2 (df, p value)[A]
Red Clover						
Emergence (7 d)	Log-log	1,103	531	1,839	NA	32.94 (31, 0.372)
Emergence (14 d)	Log-log	3,070	1,490	7,041	NA	26.87 (31, 0.679)
Shoot Length	Linear	1,115	784	1,586	0.768	NA
Root Length	Logistic	17,266	4,991	59,592	0.347	NA
Shoot Dry Mass	Logistic	490	251	955	0.823	NA
Root Dry Mass	Logistic	610	348	1,070	0.877	NA
Northern Wheatgrass						
Emergence (7 d)	Log-log	165	19	418	NA	19.95 (31, 0.94)
Emergence (21 d)	Log-log	2,841	829	26,495	NA	18.30 (31, 0.966)
Shoot Length	Logistic	927	440	1,955	0.865	NA
Root Length		> 12,635				NA
Shoot Dry Mass	Logistic	178	137	231	0.889	NA
Root Dry Mass	Logistic	132	107	164	0.846	NA
Barley						
Emergence (7 d)	Log-log	> 12,635	NC	NC	NA	24.00 (31, 0.811)
Emergence (14 d)	Log-log	> 12,635	N	NC	NA	27.69 (31, 0.637)
Shoot Length	Logistic (w)	2,119	761	5,905	0.758	NA
Root Length	Logistic (w)	22,972	322	1,641,305	0.393	NA
Shoot Dry Mass	Gompertz (w)	686	373	1,262	0.798	NA
Root Dry Mass	Logistic	247	61	998	0.629	NA
Cucumber						
Emergence (7 d)	Log-log	15,439	5,720	925,051	NA	27.04 (31, 0.670)
Emergence (14 d)	Log-log	26,835	8,042	64,287,339	NA	24.80 (31, 0.777)
Shoot Length	Logistic	3,000	1,743	5,178	0.861	NA
Root Length	Logistic	56,910	4,057	798,343	0.515	NA
Shoot Dry Mass	Logistic	2,144	1,170	3,919	0.792	NA
Root Dry Mass	Gompertz	1,381	390	4,889	0.645	NA
Alfalfa						
Emergence (7 d)	Log-log	502	234	844	NA	42.11 (31, 0.088)
Emergence (21 d)	Log-log	2,043	1,015	4,378	NA	20.13 (31, 0.933)
Shoot Length	Gompertz	1,439	1,024	2,024	0.962	NA
Root Length	Gompertz	14,832	9,532	23,131	0.369	NA
Shoot Dry Mass	ICPIN	748	433	1,214	NA	NA
Root Dry Mass	Linear	590	507	686	0.899	NA

A   Chi-square lack of fit (degrees of freedom, p value)

(w)   Indicates that the data have been weighted by the inverse of the variance.

EC/IC25 – effective concentration/inhibitory concentration, 25%, LCL – lower 95% confidence limit, UCL – upper 95% confidence limit, r^2 = coefficient of determination for regression analysis; NC – not calculated, NA – not applicable, ICPIN = the ICPIN computer program (Norgerg-King, 1993)

Table 3-4 Summary of effective concentrations and inhibitory concentrations 50% (ECs/ICs) for red clover, northern wheatgrass, barley, cucumber and alfalfa for six different endpoints (emergence and length and weight for roots and shoots) generated by plant toxicity testing.

Parameter	Model	EC/IC50 (mg/kg F2 + F3)	LCL (mg/kg F2 + F3)	UCL (mg/kg F2 + F3)	r^2	X^2 (df, p value)[A]
Red Clover						
Emergence (7 d)	Log-log	5,371	3,198	11,613	NA	32.94 (31, 0.372)
Emergence (14 d)	Log-log	22,271	9.009	204,956	NA	26.87 (31, 0.679)
Shoot Length	Linear	11,836	5,864	23,944	0.768	NA
Root Length	Logistic	17,266				NA
Shoot Dry Mass	Logistic	2,129	1,089	4,171	0.823	NA
Root Dry Mass	Logistic	2,625	1,582	4,347	0.877	NA
Northern Wheatgrass						
Emergence (7 d)	Log-log	1,098	440	2,397	NA	19.95 (31, 0.937)
Emergence (21 d)	Log-log	46,134	9,152	2.15E+08	NA	18.30 (31, 0.966)
Shoot Length	Logistic	84,176	19,915	356,607	0.865	NA
Root Length		12,635				NA
Shoot Dry Mass	Logistic	518	331	809	0.889	NA
Root Dry Mass	Logistic	282	174	455	0.846	NA
Barley						
Emergence (7 d)	Log-log	>12,635	NC	NC	NA	NA
Emergence (14 d)	Log-log	>12,635	NC	NC	NA	NA
Shoot Length	Logistic	1,349,551	12,308	147,634,990	0.758	NA
Root Length	Logistic	1.47E+15	1.16E-09	2.01E+39	0.393	NA
Shoot Dry Mass	Gompertz	25,833	5,119	130,374	0.798	NA
Root Dry Mass	Logistic	401,966	670	2.42E+08		NA
Cucumber						
Emergence (7 d)	Log-log	144,539	25,636	1.37E+09	NA	27.04 (31, 0.670)
Emergence (14 d)	Log-log	260,187	33,262	1.80E+12	NA	24.80 (31, 0.777)
Shoot Length	Logistic	45,310	1.77E+04	1.15E+05	0.861	NA
Root Length	Logistic	56,910				NA
Shoot Dry Mass	Logistic	9,532	5,932	15,318	0.792	NA
Root Dry Mass	Gompertz	13,842	4,923	38,921	0.645	NA
Alfalfa						
Emergence (7 d)	Log-log	502	234	844	NA	42.11 (31, 0.088)
Emergence (21 d)	Log-log	38,482	13,119	439,925	NA	20.13 (31, 0.933)
Shoot Length	Gompertz	8,398	6,717	10,500	0.962	NA
Root Length	Gompertz	26,375	9,488	73,314	0.369	NA
Shoot Dry Mass	ICPIN	2,005	913	3,402	NA	NA
Root Dry Mass	Linear	3,313	2,450	4,479	0.899	NA

A   Chi-square lack of fit (degrees of freedom, p value)
EC/IC50 – effective concentration/inhibitory concentration, 50%, LCL – lower 95% confidence limit, UCL – upper 95% confidence limit, r^2 = coefficient of determination for regression analysis; NC – not calculated, NA – not applicable, ICPIN = the ICPIN computer program (Norgerg-King, 1993)

3.4.2.2 Sensitivity of Species and Endpoints

Based on the geometric means of the five toxicity endpoints, northern wheatgrass was the most sensitive plant species followed by red clover and alfalfa (Table 3-3). Barley and cucumber were the least sensitive plant species and had the largest seed sizes of the five plant species used in the plant toxicity testing.

IC/EC25 values for the individual plant species varied by up to 2 orders of magnitude (Table 3-3). However, a similar trend in sensitivity for each endpoint was noted for all plant species (Figure 3-3). For most plant species root dry mass was the most sensitive endpoint followed by shoot dry mass, with the exception of red clover, in which these endpoints were reversed. For most plant species, concentrations that resulted in a 20% decrease in shoot or root length resulted in a greater than 50% decrease in root and/or shoot biomass (Table 3-3). For all species, root length was the least sensitive endpoint. Overall, endpoint sensitivity was as follows: root dry mass > shoot dry mass > shoot length > seedling emergence > root length. A similar pattern was observed for the calculated IC/EC50s; however, the IC/EC50s in most cases were extrapolated outside the range of PHC concentrations tested in this study with extremely large confidence intervals. Although seedling germination was a very insensitive endpoint when evaluated at the end of the study duration (14 d or 21 d), this endpoint was relatively sensitive in northern wheatgrass, red clover, and alfalfa on day 7 with EC25s falling in the same range as those observed for root and shoot biomass or shoot length (Table 3-3). However, as noted by the EC25s for seedling emergence at the end of the study duration, in most cases germination was delayed but not inhibited by the presence of PHCs (Table 3-3).

3.4.3 Comparison of the Direct Soil Contact Values

3.4.3.1 Distributions of Response

Based on the distributions of response (Figure 3-4), the toxicity data support a guideline value of 686 mg/kg F2+F3 for agricultural and residential land use with coarse textured soil, based on the 25th percentile of the responses. A guideline value of 2,043 mg/kg F2+F3 was determined for commercial/industrial land use, based on the 50th percentile of the responses. Based on the proportion of F2 to F3 in the test soils, this equates to guideline values of 659 mg/kg and 1,961 mg/kg for PHC expressed as F3 for agricultural/residential and commercial/industrial land use, respectively.
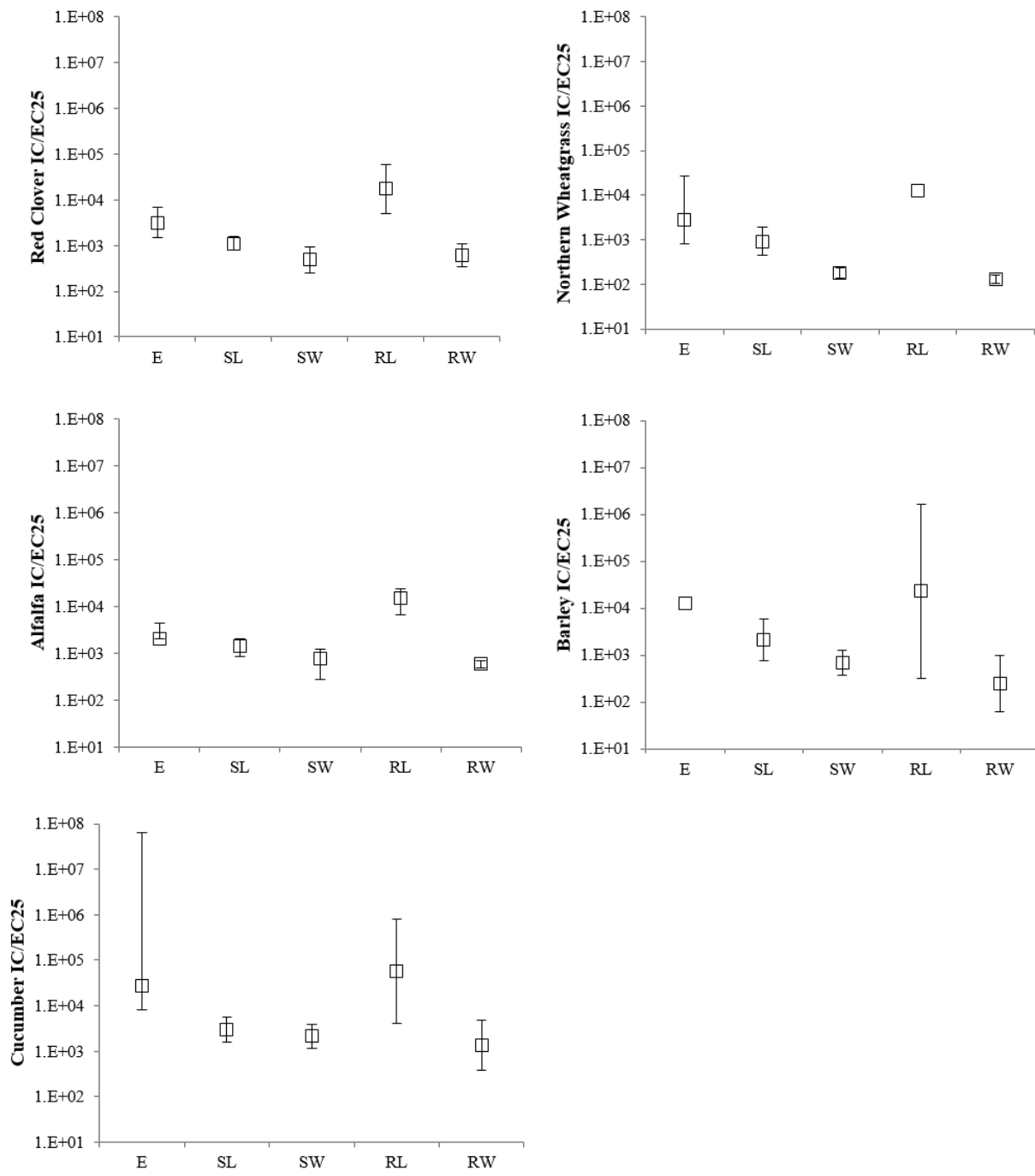
Figure 3-3 Inhibiting/effective concentration for a 25% percent effect (IC/EC25) and 95% confidence interval (vertical bars) for five plant species exposed to petroleum hydrocarbon (PHC) contaminated soils in definitive chronic toxicity tests (E = 14/21 day emergence, SL = shoot length, SW = shoot weight, RL = root length, RW = root weight).
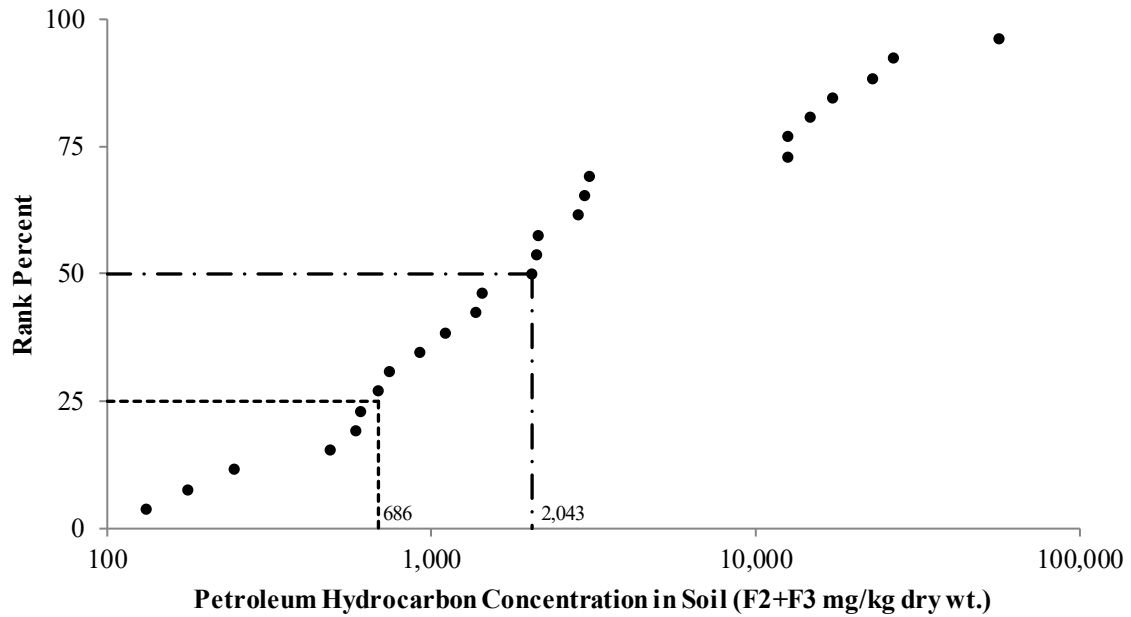
Figure 3-4 Ranked response distribution of rank values for weathered petroleum hydrocarbon fraction 2 and 3 (F2+F3) using effective concentrations/inhibitory concentrations, 25% (EC/IC25s) for the non-redundant endpoints of five plant species. Threshold effect concentrations for the 25th (agricultural and residential land use) and the 50th percentiles (commercial and industrial land use) were 686 and 2,043 mg/kg F2+F3 soil dry weight, respectively.

3.4.3.2 Ranked Response Distributions

The 25th and 75th percentile of the RRA for the PHC impacted soil exposure series are presented in Table 3-5. Consistent with the CCME (2006) approach, a ranked response distribution (RRD) was considered to meet the guideline requirements for agricultural or residential land use if the 25th percentile of the RRD showed a response of at least 75% of the control response. Therefore, based on the comparisons with the reference soil (O1), the toxicity data support a guideline value ranging between 400 mg/kg and 1,300 mg/kg F2+F3 for agricultural or residential land use, as test soil O4 did not meet the requirements (Table 3-5). Based on the proportion of F2 to F3 in the test soils, this equates to a guideline value of 385 – 1,250 mg/kg for agricultural or residential land use.

   For commercial and industrial land use the 50th percentile of the RRD required a response of at least 75% of the control response to meet the guideline requirements (CCME, 2006). Based on the comparisons with the reference soil, the toxicity data support a guideline value ranging between 1,350 mg/kg and 3,800 mg/kg F2+F3, as test soil O6 did not meet the requirements. Based on the proportion of F2 to F3 in the test soils, this equates to guideline value of 1,300 – 3,675 mg/kg for PHC expressed as F3 for commercial/industrial land use.

3.5 Discussion

3.5.1 Physical and Chemical Properties of Test Soils

Coarse textured soil (sandy loam or loamy sand) was used for the plant toxicity testing in the current study. The highest PHC concentration tested (12,000 mg/kg F3 mg/kg soil dry wt) was dictated by the highest PHC concentration collected in the field. The majority of the PHC in the test soil was F3 (64%); however, as noted in Table 3-2, some of the F4 values presented in the table may underestimate the F4 concentration as the chromatogram did not reach baseline at C50. Therefore, the total percentage of F3 may be less than 64% in some samples. As exceedances above the F2 and F4 CCME Tier 1 Guidelines were noted, responses of the plant species may be attributed to more than one PHC fraction. Co-contaminants such as PAHs and metals are not expected to have an impact on the plant responses observed in the current study, as the PAHs were not detected or well below the applicable soil guidelines. Furthermore, the metals were all measured at concentrations less than component values based on plant and soil invertebrate toxicity data (MOE, 2011). Modeling the plant responses to the observed chromium concentrations resulted in very low correlation values.

Table 3-5 The 25th and 75th percentile of the ranked response distribution (RRD) using the ranked response approach (RRA) for the petroleum hydrocarbon (PHC) impacted soil exposure series.

	PHC Impacted Soil Exposure Series									
Percentile of the RRA	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
25th	93%	83%	83%	**62%**	**68%**	**49%**	**52%**	**37%**	**49%**	**44%**
50th	98%	90%	90%	79%	80%	**59%**	**68%**	**58%**	**60%**	**58%**

Bold – Test soils fail to meet the guideline requirements as the indicated percentile is less than 75% of the control response.

A range of physical properties was observed in the test soil exposure series (Table 3-1). Most of the soil properties (pH, EC, TOC, OC, OM, P, N and CEC) increased with increasing PHC concentrations which is consistent with other reports (Trofimov and Rozanova, 2003; Wang *et al.*, 2010; Mikkonen *et al.*, 2012). The increase in TOC can be attributed directly to the increase in PHC concentration in the soil. The increase in EC may be attributed to the crude oil-derived sludges and other refinery wastes spread on the landfarm, which often contain salts (Mikkonen *et al.*, 2012). The WR of the test soils increased while WHC slightly decreased with increasing PHC, consistent with the results of Roy and McGill (2000). The water repellent properties observed, such as pooling or rapid percolation through soil spaces, are similar to those observed by ESG International (2003) with soils containing greater than 20,000 mg/kg F4. The decrease in water retention capacity and impaired soil hydraulic properties are related to PHCs in the soil (Roy and McGill, 2000; Roberston *et al.*, 2007). PHC forms hydrophobic films on the exterior surfaces of soil aggregates reducing the wettability and increasing the structural stability of the soil (McGill *et al.*, 1981; Certini, 2005). The water repellent nature of soils can be highly resistant to weathering (Roy and McGill, 2000). During a field study conducted by Visser (2005) in which crude oil was applied to sandy loam at an application rate of 1.2% (12,000 ppm), water repellency after 24 months of weathering was considered extreme even with low total PHC concentrations of 2,200 mg/kg. Following an additional year of treatment with fertilizer, crop planting, and tillage and/or fallowing, all water repellency had been eliminated (Visser, 2005).

Given that the test soils were treated with on-going PHC applications for approximately 35 years, it is expected that the PHC would have resulted in changes to the soil properties, including soil texture and the physical properties, as the level of impact that PHC has on soil quality is greatly dependent on the duration of PHC treatment (Wang *et al.*, 2010). In contrast, Visser (2005) found that for most physical and chemical soil properties there were no distinct differences between control and oil treated field plots when PHC was applied at a rate of 1.2%. In the case of Visser (2005) PHC concentrations rapidly decreased over a period of 12 months, with a 78% decrease in total PHCs.

3.5.2 Toxicity

The calculated EC/IC25s ranged from 132 mg/kg F2+F3 for northern wheatgrass root weight to 56,910 mg/kg F2+F3 for cucumber root length. The calculated EC/IC50s ranged from 282 mg/kg F2+F3 for northern wheatgrass root weight to $1.45E+15$ mg/kg F2+F3 for barley root length. Many of the endpoints for several plant species demonstrated a very steep response curve at low F2+F3 concentrations, particularly northern wheatgrass (shoot and root weight) and barley (root weight) (Appendix A). These endpoints and species represent the three lowest IC25s from the current study. Similar steep response

curves were observed by ESG (2003) for all three plant species tested (northern wheatgrass, alfalfa, and barley) and most of the endpoints that were considered (root and shoot length and weight).

As consistently shown in the literature, the comparison of toxicity to other studies is difficult as toxicity is a result of many factors, including: the source of petroleum, soil type and other properties, species tested, endpoints considered, co-contaminants, and the degree of aging and weathering of the petroleum (Salintro *et al.*, 1997; Alexander, 1995; Cermak, 2012). Furthermore, the toxicity of crude oil and the various petroleum fractions is highly variable; therefore, the comparison of toxicity values is problematic (Cermak, 2012). In general, the IC25/IC50s values reported in the current study are similar to those previously reported in the literature. For example, Stephenson *et al.*, (2000) reported IC20s ranging from 50 to 48,700 mg/kg for alfalfa, barley and northern wheatgrass, during studies with similar durations to those in the current study. IC50s ranged from 1,100 to 53,300 mg/kg F3 (Stephenson *et al.*, 2000). ESG (2003) reported IC50s for F3 ranging from 610 mg/kg to 54,1000 mg/kg for northern wheatgrass, alfalfa and barley.

Given the weathered nature of the PHC in the test soils, the IC25 values found here were lower than predicted by other studies and may be a result of an underestimation of the PHC concentration in the test soils. Mikkonen *et al.*, (2012) found that polar metabolites and heavy complexed compounds accumulate in the surface soil (0 to 20 cm) of landfarms despite tillage. Furthermore, total petroleum hydrocarbons (non-polar C10-C40) accounted for only 12% of the total soil extractable material and grossly underestimated the weathered contamination (Mikkonen *et al.*, 2012). Visser (2005) concluded that the CCME method of PHC quantification may significantly underestimate the concentrations of PHC in sandy loam soils as the silica gel clean-up may remove crude oil hydrocarbons in addition to polar materials from plant residues. Therefore, a gravimetric analysis may provide a more representative PHC concentration as it may capture the polar and complexed degradation products typically found on a landfarm site (Mikkonen *et al.*, 2012; Wang and Fingas, 1997).

The differential sensitivity of plants to PHC toxicity is well known (Adam and Duncan, 2002; Chaineau *et al.*, 1997). Northern wheatgrass was the most sensitive of the plant species tested here followed by red clover and alfalfa. Barley and cucumber were the least sensitive plant species, partly due to their large seed size. Large seeds are capable of generating root and shoot growth under adverse conditions by relying on stored energy sources instead of the surrounding environment (Clark *et al.*, 2004; Kapustka, 1997). This can make them less sensitive to PHC impacts. Alfalfa and red clover may have produced nitrogen via root nodules enhancing plant growth under poor soil conditions. A similar trend in species

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sensitivity was observed in chronic toxicity tests performed by ESG (2003). Northern wheatgrass was the most sensitive, followed by alfalfa and barley.

Although IC/EC25 values for individual plant species varied, root dry weight was the most sensitive endpoint, following by shoot dry mass in all species, except for red clover in which these were reversed. As similarly observed by Cermak et al., (2010), PHC concentrations that resulted in minimal decreases in root and shoot length resulted in substantial (>50%) decreases in root and/or shoot biomass compared to the reference soil (Table 3-3). Overall endpoint sensitivity was root dry mass > shoot dry mass > shoot length > seedling emergence > root length. Delayed germination was noted in the current study, in line with previous observations by Adam and Duncan (2002). PHCs were proposed to prevent or reduce water and oxygen from entering the seeds by acting as a physical barrier, extending the lag phase prior to germination (Adam and Duncan, 2002). This is consistent with the current study EC25s for seedling emergence as in most cases germination was delayed but not inhibited. Previous studies have found that root and shoot biomass were the most sensitive compared to other endpoints such as root or shoot length (ESG, 2003; Clark et al., 2004, Cermak et al., 2010; Saterbak et al., 1999; Visser, 2005); however, no single endpoint alone is sufficient to describe the effects of PHCs on plants (Clark et al., 2004). For instance, seedling emergence was found to be insensitive to PHC. Although historically thought to be a sensitive endpoint often utilized in toxicity testing, numerous studies have consistently reported seedling emergence as the least sensitive endpoint (Cermak et al., 2010; Dorn et al., 1998; ESG, 2003; Banks and Schultz, 2005, Song et al., 2006). Seedling emergence is an insensitive endpoint as the seedlings receive nutrition from storage materials effectively isolating them from the surrounding environment (ESG, 2003; Kapustka, 1997). In addition, some chemicals, such as PHCs may not be taken into the seed (Kapustka, 1997). Contrary to previous studies, the current study found that root length was the least sensitive to PHC. However, a limited number of studies in the literature were conducted following methods in-line with EC. Many of the previously conducted studies used petri plates for test vessels with a limited amount of test soil combined with short exposure durations (4 – 5 d). These conditions may not accurately predict the impact of PHC on root lengths in larger test vessels with more appropriate test durations, such as those proposed by EC (2007). Furthermore, Saterbak et al. (1999) found that root length was poorly correlated with physical chemical properties and PHC content, in line with the current study.

3.5.3 Direct Soil Contact Values

The initial PHC CWS for agricultural/residential and commercial/industrial land use were 400 mg/kg F3 and 1,700 mg/kg F3, respectively. These existing guidelines were calculated from a species sensitivity

distribution of 50th percentile (IC/EC/LC$_{50}$) F3 toxicity data. The data set combined or removed any redundant data in line with CCME (2000) protocols. The agricultural/residential land use guideline value was derived from the 25th percentile of the combined plant and soil invertebrate data set, while the commercial/industrial guideline value was based on the 50th percentile of the plants only database (CCME, 2006). A factor of 0.31 was applied to each of these values to derive the coarse soil guidelines to account for the analytical recovery of F3 from soil. During the five-year review of the guideline values (CCME, 2006), it was recommended that the agricultural/residential guideline value be decreased to 300 mg/kg F3 to be protective of soil invertebrates, based on new ecotoxicological data (Visser, 2005) (Table 3-6). It was concluded that there was insufficient new data available to recommend updating the commercial/industrial land use guideline of 1,700 mg/kg. Based on the distributions of responses in this study, guidelines values of 659 mg/kg and 1,961 mg/kg for PHC expressed as F3 for agricultural/residential and commercial/industrial land use, respectively, were recommended (Table 3-6).

Based on this study, one can conclude that the current guideline value for agricultural/residential land use is over protective of plant growth, in line with the previous study conducted by Visser (2005), and would support the derivation of a site specific remediation objective (SSRO) higher than the current guideline value. The current study also supports the conclusion that the current guideline of 1,700 mg/kg is over protective of plant growth at commercial/industrial sites. However, Visser (2005) previously reported that soil invertebrates, specifically earthworms (*Eisenia andrei*), were more sensitive to F3 than plants. Although residual F3 concentrations resulted in no toxicity to plant growth or springtail (*Folsomia candida)* survival or reproduction, a significant decrease in earthworm reproduction was observed during laboratory tests (Visser, 2005). Furthermore, in field plots containing low residual F3 concentrations (325 mg/kg) a significantly lower number of springtails were observed. In line with Visser (2005), McCallum (2014) found that the site soils used in the current study had no adverse effect on springtail adult survival or juvenile production during laboratory studies. Therefore, prior to establishing a SSRO, additional toxicity tests should be completed with soil invertebrates (specifically earthworms) to confirm that the recommended value is protective of all soil invertebrates. Furthermore, a number of the derived EC/IC25s (28%) and EC/IC50s (60%) were extrapolated above the maximum F2+F3 concentration that was tested in this study. Repeating this study with a wider range of PHC concentrations would increase the confidence in these extrapolated EC/IC50 values, and by extension the derived direct soil contact value, by narrowing the confidence intervals.

Table 3-6 Summary of the Canadian council ministers of the environment (CCME) Canada-wide soil standards for petroleum hydrocarbon fraction 3 (milligrams per kilogram [mg/kg]) and the soil quality guidelines derived in the current study based on the distribution of responses and ranked response approach.

Land Use	CCME Canada-Wide Soil Standards for Petroleum Hydrocarbon Fraction 3 (mg/kg)		Derived Soil Quality Guidelines for Petroleum Hydrocarbon Fraction 3 (mg/kg)	
	Fine grained soil texture	Coarse grained soil texture	Distribution of Responses	Ranked Response Approach
Agricultural/Residential	1,300	300	660	>390 and <1,200
Commercial/Industrial	2,500	1,700	1,960	>1,300 and < 3,500

The detrimental effects of PHCs on soil organisms can be attributed to changes in the physical and chemical properties of soil following PHC contamination in addition to PHC toxicity (Robertson *et al.*, 2007; Mikkonnen *et al.*, 2012). The landfarm soil used in the current study was exposed to high PHC concentrations for approximately 35 years due to continual application of sludge. Based on the duration of the exposure, major changes to the soil properties including soil texture and the physical properties would have occurred. However, this prolonged exposure to high PHC concentrations may not be representative of many environmental sites with PHC contamination, as PHC concentrations typically drop rapidly following contamination. PHC depletion typically occurs in two phases. In the first phase, there is rapid loss of PHCs via volatilization, microbial decomposition, and irreversible sorption to clays and organic matter (Visser, 2005). In the second phase, a slower degradation rate occurs as the residual PHCs are transformed by the microbial biomass and incorporated into the soil matter (Visser, 2005). This is consistent with what has been observed in the literature during greenhouse and field trials with soils spiked with PHC products. For instance, hydrocarbon bioremediation in artificially weathered soils with initial concentrations of 35,700 to 122,200 mg/kg ranged from 10 to 90% PHC reduction in two soil types (Salintro *et al.* 1997). Rate losses as high as 13 to 81% PHC/month were reported (Salintro et al., 1997). Hutchinson *et al.*, (2001) observed a drop in PHC concentrations of petroleum sludge produced at an oil refinery of 49% in the first 6 months of a phytoremediation trial in the unvegetated control. At the end of one year, PHC concentrations had dropped to 57% of the initial concentration (48,800 mg/kg PHC [C10-C35]). Finally, the field study conducted by Visser (2005), spiked soils with Alberta Federated crude oil at a concentration of 12,000 mg/kg reported dissipation rates of 100%, 96%, 78% and 37% for F1, F2, F3, F4, respectively, after 12 months. The total mass loss of PHCs was 78% and 82% , respectively, after 12 months and 36 months of remediation (Visser, 2005). Therefore, as a result of the altered physical and chemical properties of the soils used in the current study from the prolonged exposure to high PHC concentrations, the proposed guideline values may be in fact be conservative for many PHC contaminated sites in Canada.

Multi-concentration studies, such as performed in this study, are preferred for toxicity testing as they allow for the calculation of traditional toxicity endpoints (e.g., IC/LCx). However, during field trials or when working on a historically contaminated site where no truly representative reference soil is available, it may not be possible or practical. Therefore, the CCME (2006) proposed a RRA as a tool to determine if the responses seen in toxicity tests with a limited number of treatments comply with or fail to meet the specified criteria. Using a RRA, the current study derived guideline values of 390 – 1,200 mg/kg and 1,300 – 3,500 mg/kg for PHC expressed as F3 for agricultural/residential land use and

commercial/industrial land use, respectively (Table 3-6). Although these guideline values are in line with those discussed above, a limiting factor of the RRA is the number of treatments available and their respective PHC concentrations. In many cases, the RRA will only indicate that an appropriate guideline value is either above or below a certain value. Depending on the PHC concentrations, this may or may not be useful from a guideline derivation or SSRO perspective. Even applying this approach to the current multi-concentration study resulted in a large range for the proposed guideline values. Additional PHC concentrations in the toxicity test performed here would enable this range to be narrowed to a more specific concentration.

3.6 Conclusion

This study was performed to determine if weathered F3 in soil obtained from a landfarm site elicited adverse effects during plant toxicity testing. The EC/IC25s ranged from 132 to 56,910 F2+F3 mg/kg. When these values are used to generate a distribution of response, the proposed agricultural/residential and commercial/industrial standards for F3 are 659 and 1,961 mg/kg, respectively. The guideline values derived using the ranked response distribution responses were 385 – 1,250 mg/kg and 1,300 – 3,675 mg/kg for PHC expressed as F3 for agricultural/residential land use and commercial/industrial land use, respectively. Although this approach allows for the derivation of a guideline value with limited exposure concentrations during field trials or on historically contaminated sites where no representative reference soil is available, the large range of the proposed value may not be useful from a guideline derivation or SSRO perspective. The derived standards indicated that the current CCME guideline values for direct soil contact for agricultural/residential land use (300 mg/kg) for coarse textured soil are protective of plant growth and support the conclusion that the current industrial guideline of 1,700 mg/kg is protective of plant growth at commercial/industrial sites. Additional toxicity studies using a more sensitive test species (i.e., earthworms) are recommended to further evaluate if the current PHC soil contact values are overly conservative for weathered F3. Furthermore, a number of the derived EC/IC25s and EC/IC50s were extrapolated above the maximum F2+F3 concentration that was tested in this study. Repeating this study with a wider range of PHC concentrations would increase the confidence in these extrapolated values, and by extension the derived direct soil contact value, by narrowing the confidence intervals. The guideline values derived in the current study may be conservative given the prolonged exposure of the test soils to high PHC concentrations due to continuous sludge application, which contributed to changes in the physical and chemical properties of the soil. Changes in the physical and chemical properties of the soil as a result of PHC exposure can result in detrimental effects on plants and soil invertebrates, in addition to

the direct effects of PHC (Roberston *et al.*, 2007, Mikkonnen *et al.*, 2012). Prolonged exposure to high PHC concentrations may not be representative of many environmental sites with PHC contamination, as PHC concentrations have been demonstrated to drop rapidly within several years following contamination (Visser, 2005). Additional studies are recommended with field soils from historically contaminated sites with conditions more similar to a spill situation, where rapid bioremediation has occurred.

Chapter 4

Conclusions

With over 30,000 contaminated brownfield sites in Canada, innovative cost-effective remedial technologies are required to allow for the redevelopment of these properties. Plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation system (PEPS) has been shown to effectively remediate a wide range of contaminants on rural sites. However, this study was the first application of PEPS on an urban brownfield. Although a number of new plant species were considered to address the complex mixture of contaminants often found on urban brownfields, only sunflowers are recommended for consideration as an additional plant species for PEPS based on high germination rates, biomass production, and the ability to phytoextract metals and degrade petroleum hydrocarbons (PHCs). Past studies have observed that the presence of metals can inhibit organic-contaminant degrading microorganisms; however, PEPS was successfully able to remediate PHCs by 25 percent during the 2009 field season. Phytoremediation alone appears to have limited potential to remediate PHCs on urban brownfields as no significant remediation was observed in the planted plots without the PGPR treatment. PEPS relies upon the use of a non-indigenous rhizobacteria; therefore, the impact of PEPS on the composition of the soil microbial communities is an important consideration. Phytoremediation significantly increased the number of the microbes in the rhizosphere over the course of the field season. Community level physiological profiles (CLPP) and denaturing gradient gel electrophoresis (DGGE) analysis suggests that the plant species selected for phytoremediation had a larger influence on the microbial community than the PGPR treatment. Therefore, the introduction of non-indigenous PGPR via PEPS does not have a significant impact on the native microbial community. Future work to enhance PEPS by modifying conventional agricultural methods is recommended. Although this study showed that the transplant seeding method did not consistently enhance PEPS, intercropping sunflowers and mixed grasses may provide a unique combination to address the mixed contaminants commonly found on urban brownfields. Therefore, based on the completed field trial, PEPS shows promise as an effective tool for urban brownfield remediation.

Due to the large number of sites contaminated with PHCs, it is imperative that appropriate clean-up standards are applied to avoid unnecessary costly remediation. The current study investigated the impact of weathered PHC fraction 3 (F3) in coarse-textured soil from a landfarm site on various plant species using standardized plant toxicity testing methods developed by Environment Canada (EC). When the

resulting endpoints (lethal concentrations [LC], effective concentrations [EC], and inhibitory concentrations [IC]) were used to generate a distribution of responses, the resulting agricultural/residential and commercial/industrial standards for F3 were 659 and 1,961 milligrams per kilogram (mg/kg), respectively. The derived standards for coarse-textured soil indicate that the current Canadian Council Ministers of the Environment (CCME) guideline values for direct soil contact for agricultural/residential land use (300 mg/kg) and commercial/industrial land use (1,700 mg/kg) are protective of plant growth at residential and commercial/industrial properties. Guideline values developed using the ranked response distribution ranged from 385 to 1,250 mg/kg for residential land use and from 1,300 to 3,675 mg/kg for industrial land use. Although the ranked response distribution approach allows for the derivation of guideline values when limited exposure concentrations are available or on historically contaminated sites where no representative control soil is available, the large range of the proposed standards may not be practical to derive a guideline or establish site-specific remedial objectives. Future toxicity studies using a more sensitive test species (i.e., earthworms) are recommended to further evaluate if the current F3 soil contact guidelines are overly conservative for weathered F3. A number of the derived EC/IC25s and EC/IC50s were extrapolated above the maximum F2+F3 concentration in this study. Repeating this study with a wider range of PHC concentrations would increase the confidence in these extrapolated values, and by extension the derived direct soil contact value. Due to the prolonged exposure of the test soils to elevated PHC concentrations, the guideline values derived in the current study may be conservative due to changes in the physical and chemical properties of the soil. Changes in the physical and chemical soil properties, as result of the high PCH concentrations, can have a detrimental effect on plants and soil invertebrates in addition to the direct effects of PHC. Furthermore, prolonged exposure to high PHC concentrations may not be representative of many environmental sites with PHC contamination, as PHC concentrations have been demonstrated to decrease quickly within several years following contamination. Future studies should focus on using field soils from historically contaminated sites with conditions more similar to a spill situation, where rapid bioremediation occurred.

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Appendix A
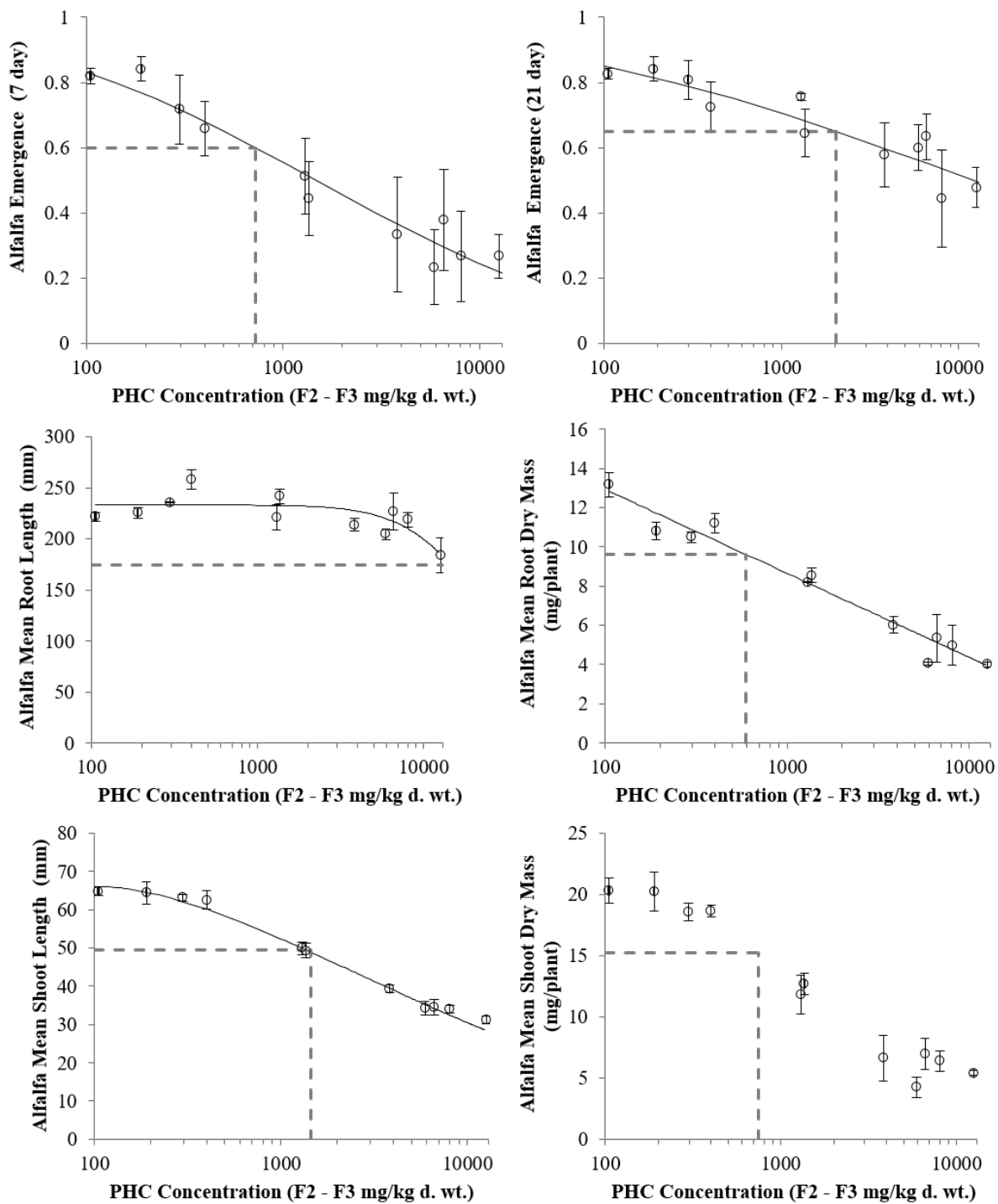## Modeled Endpoints of Plant Toxicity Testing

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Figure A-4-1 The modelled effect of petroleum hydrocarbon (PHC) fraction 2 and 3 (F2+F3) (milligrams per kilogram dry weight [mg/kg d. wt.]) contaminated soil on alfalfa germination, root length, root mass, shoot length, and shoot mass. Dashed lines represent the EC/IC25 derived for each endpoint.
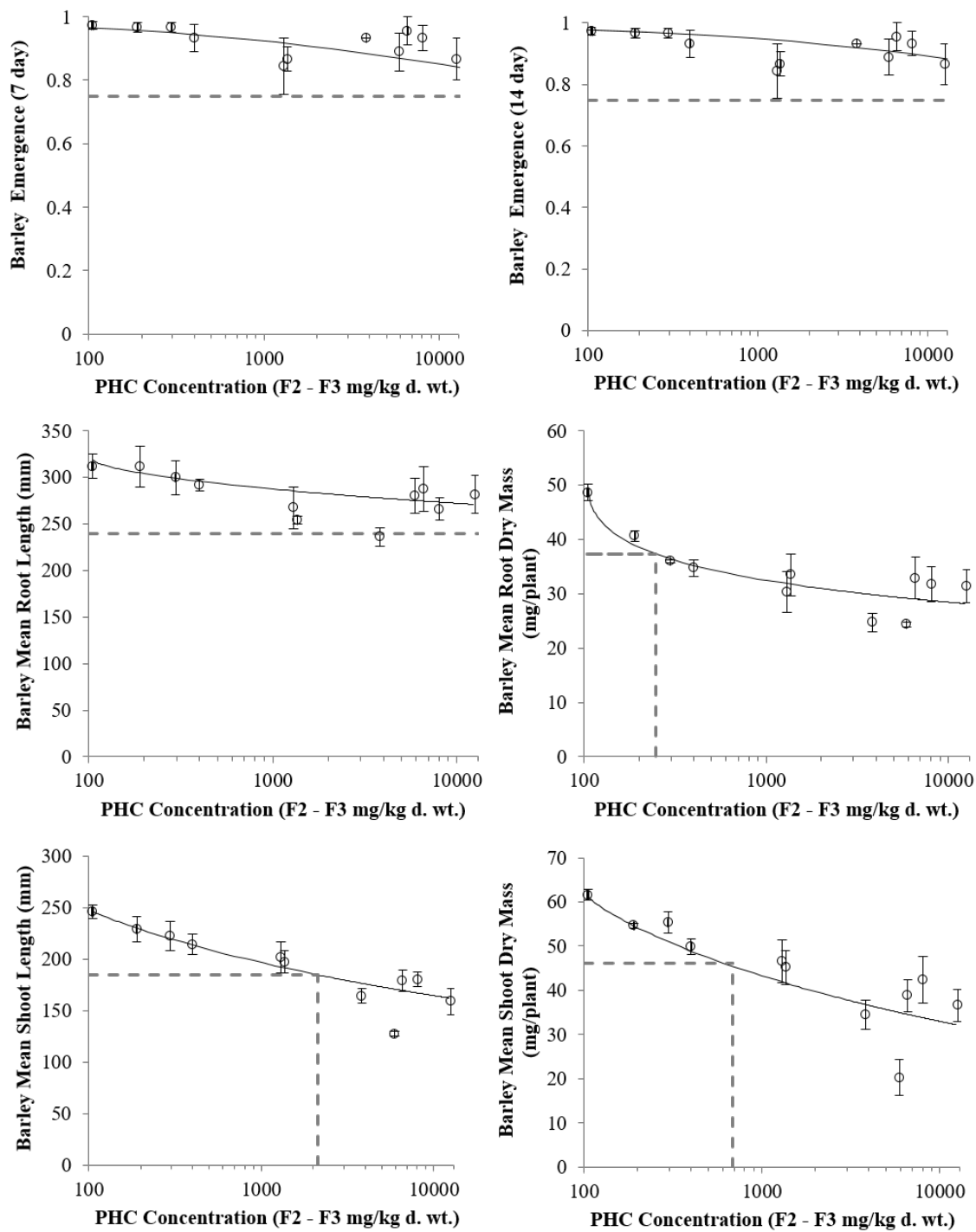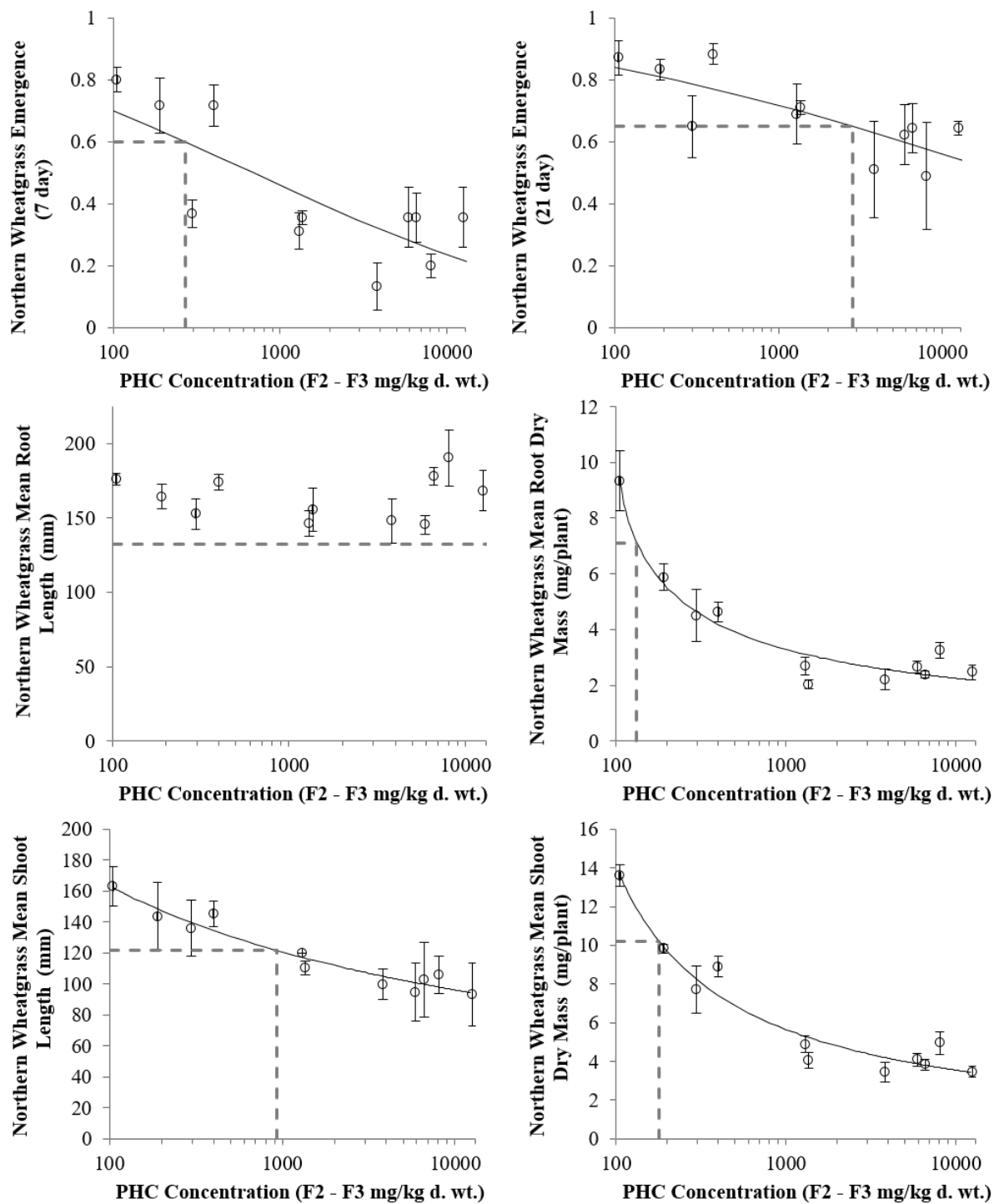
88

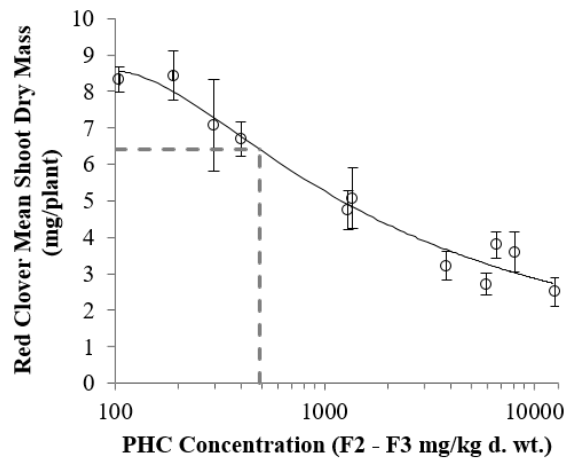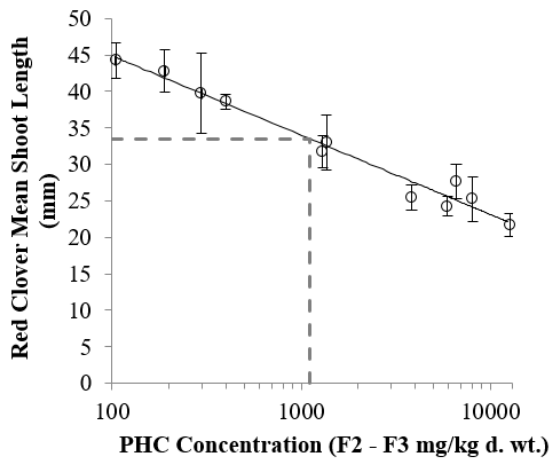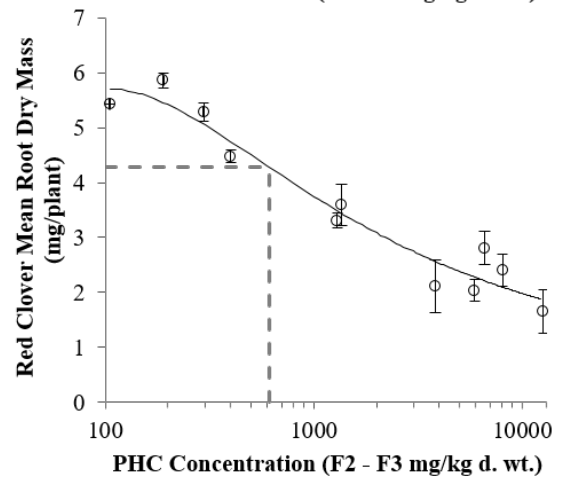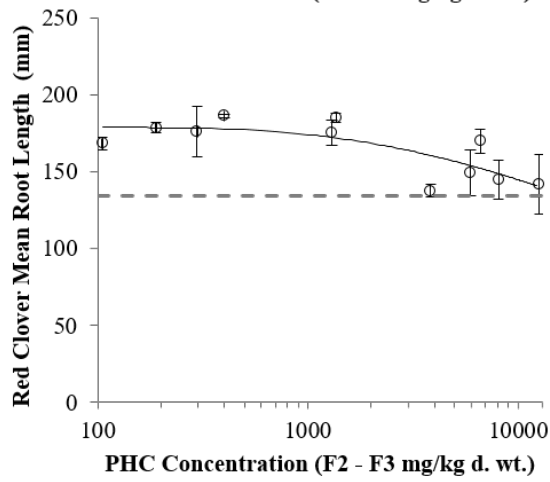Figure A-4-2 The modelled effect of petroleum hydrocarbon (PHC) fraction 2 and 3 (F2+F3) (milligrams per kilogram dry weight [mg/kg d. wt.]) contaminated soil on barley germination, root length, root mass, shoot length, and shoot mass. Dashed lines represent the EC/IC25 derived for each endpoint.
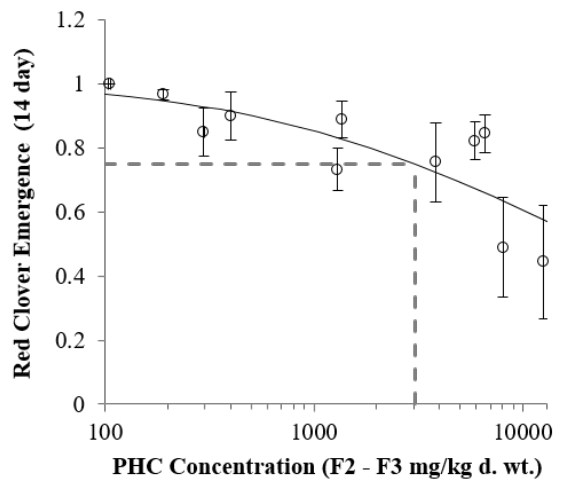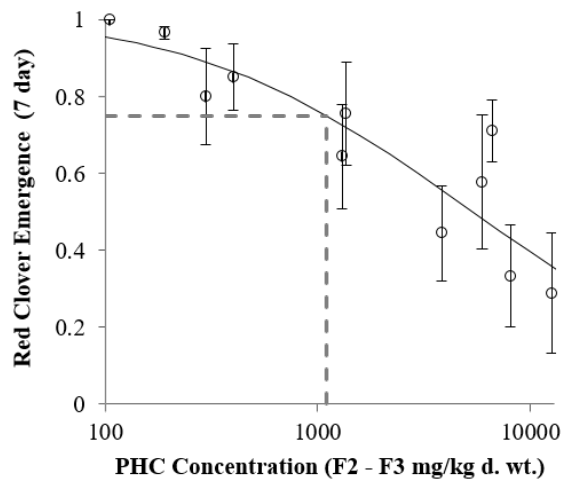
89

Figure A-4-3 The modelled effect of petroleum hydrocarbon (PHC) fraction 2 and 3 (F2+F3) (milligrams per kilogram dry weight [mg/kg d. wt.]) contaminated soil on northern wheatgrass germination, root length, root mass, shoot length, and shoot mass. Dashed lines represent the EC/IC25 derived for each endpoint.

90

Figure A-4-4 The modelled effect of petroleum hydrocarbon (PHC) fraction 2 and 3 (F2+F3) (milligrams per kilogram dry weight [mg/kg d. wt.]) contaminated soil on red clover germination, root length, root mass, shoot length, and shoot mass. Dashed lines represent the EC/IC25 derived for each endpoint.
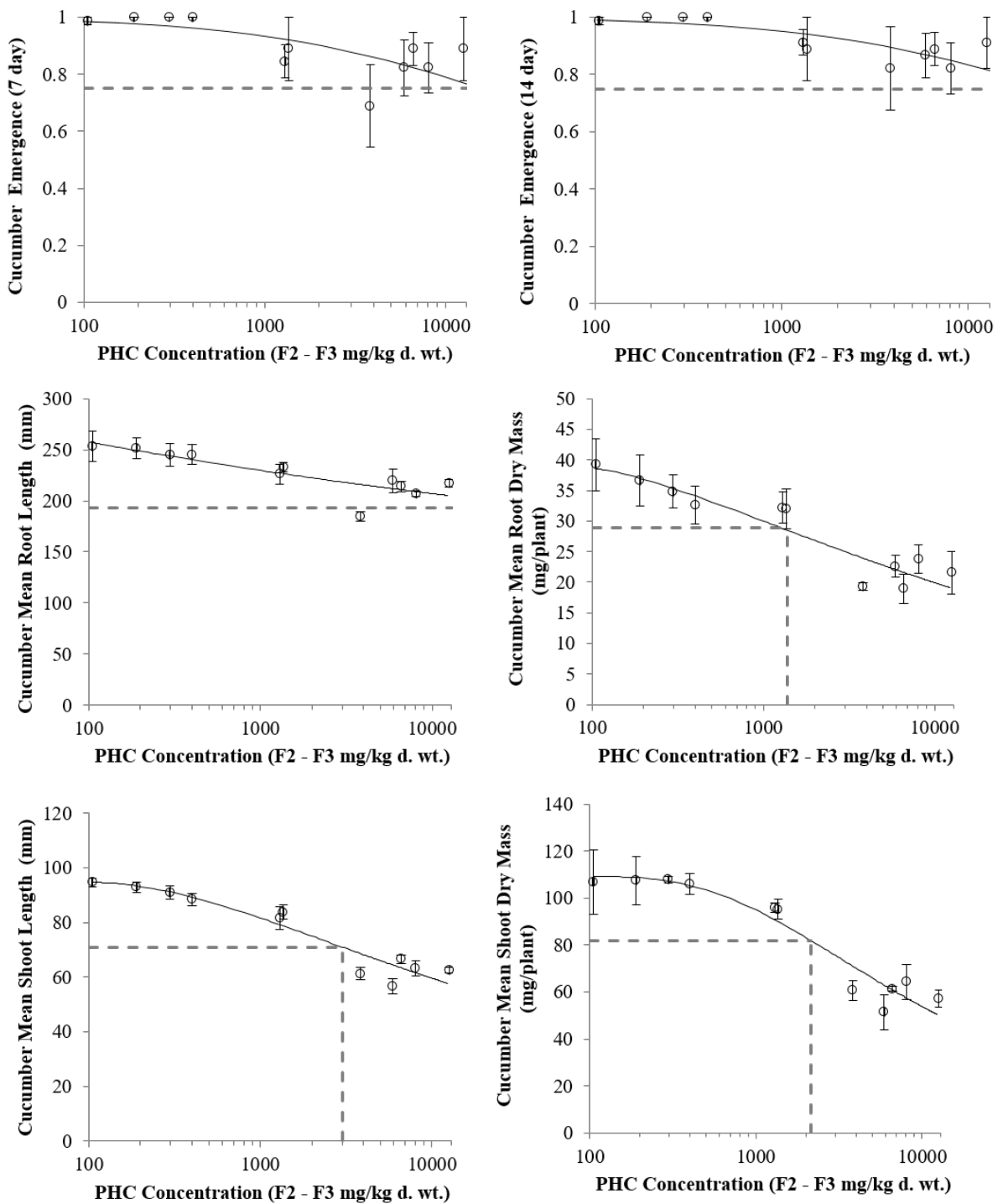
91

Figure A-4-5 The modelled effect of petroleum hydrocarbon (PHC) fraction 2 and 3 (F2+F3) (milligrams per kilogram dry weight [mg/kg d. wt.]) contaminated soil on cucumber germination, root length, root mass, shoot length, and shoot mass. Dashed lines represent the EC/IC25 derived for each endpoint.