Quantitative Analysis of Petroleum Hydrocarbons (PHC) in Soils and Chemically Augmented Plant Growth Promoting Rhizobacteria Enhanced Phytoremediation Systems

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

Petroleum hydrocarbons (PHC) are a complex mixture of organic compounds, which can negatively impact terrestrial ecosystems. The Canadian Council of Ministers of the Environment (CCME) provides recommendations on the allowable concentrations of PHC in Canadian soils. The preferred method for PHC analysis according to the CCME is solvent extraction with 1:1 acetone/hexane (1:1 AH) to recover PHC from soils, followed by quantification via gas chromatography with a flame ionization detector (GC-FID). However, the 1:1 AH solvent often co-extracts naturally occurring biological organic compounds (BOC) along with PHCs from soils. This co-extraction of BOC could lead to an incorrect PHC concentration in soil. One potential PHC soil remediation technology is plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation systems (PEPS). PEPS use plants and their associated microbiota to remove PHC from soils. This investigation was divided into two sections. First was optimization of PHC quantification methods. Second was the investigation of the efficacy of chemical augmentation with PEPS (CA-PEPS) with respects to increasing the rate of PHC remediation in soils. The PHC concentrations of these soils were tested using the PHC quantification methods optimized in the first part of this study.

Two analytical methods were developed in this investigation, to accurately quantify PHCs in soils while minimizing BOC interference. The first method used the 1:1 acetone/hexane solvent mixture to extract PHC from soils, followed by the addition of activated silica to remove BOC from the PHC-extract (1:1 AH silica cleanup). The second method used the solvent dichloromethane along with the *in situ* addition of anhydrous sodium sulfate to dry the soils, and activated silica to remove BOC (DCM+SS+Silica). Both 1:1 AH silica cleanup and DCM+SS+Silica extracted similar amounts of PHC (2809.56 mg/kg and 2895.60 mg/kg, respectively; $P>0.05$) from the weathered PHC-impacted soils, except when soil moisture was higher than 12%. At higher soil moistures the extraction efficiency of the DCM+SS+Silica method decreased by 11.7% ($P=0.013$) while the 1:1 AH with silica cleanup PHC extraction efficiency was unaffected.

PEPS generally remediate PHCs in soils at a slower rate compared to traditional physical cleanup methods (e.g. removal of soils to land fill). In an attempt to increase PHC remediation rates with PEPS, two chemical classes, surfactants and oxygen releasing compounds (ORCs) were investigated. To test this, CA-PEPS greenhouse trials using PGPR-treated seed (*Lollium multiflorum*, annual rye grass), were performed on weathered PHC-impacted soils. Prior to PEPS application, soils were treated with increasing concentrations of surfactants (petroleum sulfonate oil; PSO) and ORCs (calcium peroxide; CaO_2). Increasing PSO concentration (0.00-12.5 µL/g) did not improve ($P>0.05$) PHC remediation rates, and decreased both *L. multiflorum* root and shoot dry biomass relative to PEPS. Furthermore, 100 µL/g of PSO treatment completely inhibited *L. multiflorum* germination resulting in significant decrease in the PHC remediation rate. Conversely, the CaO_2 (3.33 mg/g) treatment increased the PHC remediation rate by 11.0% ($P=0.038$), as well as increasing *L. multiflorum* root and shoot dry biomass by 22.7% ($P=0.016$) and 10.6% ($P=0.086$), respectively, relative to PEPS alone.

The rates of PHC remediation in the CA-PEPS experiments were determined by the methods developed in the first part of this study (Chapter 2). The DCM+SS+Silica extracted 7.80% ($p=0.038$) less PHC from soils then 1:1 AH with silica cleanup. Likely, this was due to the moisture content of the soils, preventing complete PHC extraction with DCM+SS+Silica. When soils were treated with PSO, the removal of PSO from the PHC-extract with activated silica is highly recommended. Otherwise, the GC-FID would overestimate the PHC concentration in the soils, as it cannot distinguish PSO from PHC.

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Dedication

To my parents for their support, as well as my grandparents John H.K. and May Wong for their continued financial support.

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1:1 AH	1:1 Acetone:Hexane
1:1 AH Silica Cleanup	PHC Extraction from Soils using 1:1 Acetone:Hexane Followed by Acetone Removal and Addition Activated Silica
ACC	1-Aminocyclopropane-1-Carboxylate
ACC Deaminase	1-Aminocyclopropane-1-Carboxylate Deaminase
BOC	Biological Organic Compound
CaO_2	Calcium Peroxide
CA-PEPS	Chemically Augmented Plant Growth Promoting Rhizobacteria Enhanced Phytoremediation Systems
CCME	Canadian Council of Ministers of the Environment
CEC	Cation Exchange Capacity
DCM	Dichloromethane
DCM+SS+Silica	PHC extraction from Soils using DCM Anhydrous Na_2SO_4 and Activated Silica
Df	Dilution Factor
DMF	Dimethylformamide
EPA	U.S. Environmental Protection Agency
ePGPR	Extracellular Plant Growth Promoting Rhizobacteria
F1	PHC Fraction 1 (C_1-C_{10})
F2	PHC Fraction 2 (C_{11}-C_{16})
F3	PHC Fraction 3(C_{17}-C_{34})
F4	PHC Fraction 4 (C_{34+})
FID	Flame Ionization Detector
GC	Gas Chromatography
HMW	High Molecular Weight
IAA	Indole-3-Acetic Acid
iPGPR	Intracelluar Plant Growth Promoting Rhizobacteria
IAA	Indole-3-Acetic Acid
LMW	Low Molecular Weight
$MgSO_4$	Anhydrous Magnesium Sulfate
MSD	Mass Spectra Detector
Na_2SO_4	Anhydrous Sodium Sulfate
ORC	Oxygen Releasing Compound
PAH	Poly Aromatic Hydrocarbon
PEPS	Plant Growth Promoting Rhizobacteria Enhanced Phytoremediation Systems
PGPR	Plant Growth Promoting Rhizobacteria
PHC	Petroleum Hydrocarbon
PHC-Extract	Effluent from PHC-Impacted Soil
PSO	Petroleum Sulfonate Oil

Rf	Retention Factor
RO-H2O	Reverse Osmosis Water
SDS	Sodium Dodecyl Sulfate
SE	Standard Error
WHC	Water Holding Capacity
UCM	Unresolved Complex Mixture
ϵ	Dielectric Constant

Chapter 1:

Introduction of Petroleum Hydrocarbons and Phytoremediation

1.1.0 Petroleum Hydrocarbons

Crude oil is a viscous liquid mixture that is mainly composed of hydrocarbons, plus heterocylic organic compounds containing sulfur, nitrogen and oxygen and metals (Lubeck, 1998). Petroleum hydrocarbon (PHC) molecules found in crude oil vary greatly in size and structure. Open chain (aliphatic) carbons are the most abundant structural class of PHCs, followed by cyclic hydrocarbons (e.g. naphthenes) and lastly by molecules with multiple aromatic rings (polycyclic aromatic hydrocarbons; PAHs). It is predicted that there may be over a thousand different PHC molecules in crude oil (Lubeck, 1998). The diversity of PHC molecules makes identification of all compounds in PHC mixtures unrealistic (CCME, 2012). Instead the Canadian Council of Ministers of the Environment (CCME) separates PHCs into four fractions (F1, F2, F3 and F4) based on carbon chain length (CCME, 2012). This classification system allows for comparisons to be made between different PHC mixtures in contaminated environments. The F1 (C_1-C_{10}) and F2 (C_{11}-C_{16}) fractions are composed of short, single chain hydrocarbons which, due to their low molecular weight (LMW), are highly volatile. The F3 (C_{17}-C_{34}) and F4 (C_{34+}) fractions are composed of long single or branched chains and aromatic hydrocarbons with low volatility. The F3 fraction is mostly comprised of single chain hydrocarbons with some PAH, and can be transported away from a spill site by water, albeit to lesser extent than F1 and F2. The F4 fraction is comprised mostly branched and aromatic hydrocarbons that are generally immobile in soils due to their high molecular weight (CCME, 2012).

1.1.1 Weathering of PHC in Soil

Weathering is defined as chemical or physical changes to a molecule by abiotic and/or biotic factors (CCME, 2008). This affects how PHC molecules behave in soils (CCME, 2008). The most

significant abiotic mechanisms of PHC weathering in soil are volatilization, photooxidation, aging and sequestration (McGill *et al*., 1981; Ghore *et al.*, 1983). Volatilization is the vaporization of molecules into a gaseous state (McGill *et al*., 1981). Furthermore, volatilization tends to decrease with increasing molecular weight of PHCs, due to London dispersion forces acting to keep the PHC molecules in the liquid state (McGill *et al*., 1981). Photooxidation is oxidation from radiation (mostly UV radiation), which generally increases the susceptibility of PHC molecules to biodegradation (Ghore *et al*., 1983). Only PHCs at or close to the soil surface are susceptible to photooxidation as photons cannot penetrate more than approximately 1 mm into the soil (McGill et *al.*, 1981; Hawkins, 2012). Sequestration is the binding of PHC molecules to organic matter (humic substances) in the soil through intermolecular forces (McGill et *al.,* 1981). Humic substances mainly sequester small aromatic and carbonyl compounds; rarely do they sequester aliphatic PHCs (McGill *et al.*, 1981; Robertson *et al*., 2007). Aging is a decrease in PHC bioaccessiblity over time due to the movement (diffusion, capillary action or gravitational flow) of PHC molecules into micro- and macro-pore spaces in the soil (McGill *et al.,* 1981).

Biotic weathering is the metabolism (biodegradation) of PHC molecules by soil organisms (Atlas, 1981; Sierra-Garcia *et al.*, 2013). Generally, low molecular weight PHCs (F1 and F2) are more readily biodegraded by soil organisms than high molecular weight hydrocarbons (F3 and F4, branched alkanes, and PAHs; Atlas, 1981). The vast majority of biotic PHC weathering is carried out by aerobic microbes, while anaerobic microbes contribute little to PHCs weathering in soils (Sierra-Garcia *et al.*, 2013; Northrup and Cassidy, 2008). Overall weathered soils tend to have higher concentrations of HMW PHCs compared to unweathered soils, due to their slower rates of weathering (Alexander, 2000, Atlas, 1981; McGill *et al.*, 1981).

1.1.2 Soil Properties

Soil is a heterogeneous matrix composed of organic matter, minerals, air and water. The composition of these components greatly influences properties of the soil which can vary from soil to soil (Yong *et al.*, 2012). Organic matter can be classified as either unaltered organics or transformed organics

(Orsi, 2013; Yong *et al.*, 2012). Unaltered organics are biogenic compounds that have not undergone any chemical transformation process in the soils (Orsi, 2013; Yong *et al.*, 2012). In contrast, transformed organic compounds of biogenic origin have undergone biological and chemical reaction, and are no longer structurally similar and contain no properties of the original parent compounds. These transformed organics often originate from decayed plant matter and are often referred to as humic substances. They compose the bulk of organic matter in soils.

Air and water is the total amount of gas and water respectively contained within the soil. Their exact contribution to the overall soil mass is dependent upon the mineral and organic content of the soil. Minerals are the inorganic crystalline materials such as; clay, oxides and hydrous oxides of iron, sulphates, phosphates, sulphides, carbonates, and silicon; and non-crystalline materials such as; iron hydroxide, aluminum hydroxide and silicon hydroxide (Yong *et al.*, 2012).

Soil is classified based on soil particle size (diameter), clay is <0.002 µm, silt is 0.002-0.05 µm and sand is 0.05-2.00 µm. (Plaster, 2013; Osman, 2013). The relative composition of clay, silt, and sand particles determines the soil type. The soil type greatly influences the properties of the soil such as specific surface area, water holding capacity, bulk density and pore size all of which affect how PHCs behave in the soil (Plaster, 2013).

The specific surface area (SSA) is the total surface area at a given weight of dry soil; thus SSA increases as soil particle size decreases (clay soil>silt soil>sand soil) (Plaster, 2013; Osman, 2013). Higher SSA provides more area for liquid and chemicals (e.g., water or PHC) to sorb onto, thereby increasing their retention within the soil matrix. As such, clay soils tend to retain more PHC as compared to sandy soils. The latter would allow PHCs to move more readily through the soil into ground water (Simanzhenkov and Idem, 2005).

The Water Holding Capacity (WHC) of a soil is defined as the ability of a soil to retain water, by overcoming the downward force of gravity (Hudson, 1994; Osman, 2013). WHC tends to increase with SSA. As such, clay soils generally retain more moisture than sandy soils (Simanzhenkov and Idem, 2005). WHC is also greatly affected by the organic matter content of soil even though it accounts for only

a small percentage of the total soil composition. According to Hudson (1994) a 1.0% increase in organic matter improves the soil WHC by 3.7%. This is due to the porous nature of organic matter which increases SSA, as well as the number of micro-pore capillaries (Hudson, 1994). Generally soils with higher WHC limit the downward migration of PHC; this causes PHCs instead to migrate laterally due to the hydrophobic repulsion between PHC compounds and water in the soil (Fingas, 2015).

The Bulk Density of soil is calculated as the mass of dry soil (g) relative to the volume that the dry soil occupies (cm^3; Equation 1.1) and is heavily influenced by soil texture (Osman, 2013; Plaster, 2013). Bulk density increases as soil particle size decreases (clay soil>silt soil>sand soil; Plaster, 2013).

$$\boldsymbol{Bulk\ Density\ of\ Soil}\ (g/cm^3) = \frac{\boldsymbol{Weight\ of\ Dry\ Soil}\ (g)}{\boldsymbol{Volume\ of\ Dry\ Soil}\ (cm^3)} \qquad \textbf{Equation 1.1}$$

Soils with lower bulk density have a greater volume of pore spaces, which can be occupied by water, air or other compounds (Osman, 2013; Plaster, 2013). Pore spaces are classified as being either macro-pores, which have large diameters (>75µm), or micro-pores, which have small diameters (5-75 µm; Plaster, 2013). Generally the ratio between macro to micro-pore spaces decreases as the bulk density of the soil increases. Soils with higher bulk density tend to hold more recalcitrant PHC contaminants compared to low bulk density soil due to the lower bioavailability of PHCs trapped within micro-pores (Gerhardt *et al*., 2015; Volkering *et al*., 1998). Furthermore high bulk density soils compared to low bulk density soils, have less air in the soil matrix and higher WHC which will generally lower PHC degradation rates (Plaster, 2013).

PHC behavior in soils varies greatly and is heavily influenced by soil type (McGill et *al.,* 1981). In general PHCs in clay soils compared to sandy soils tend to be less mobile and bioavailable as a result of the soils high WHC, SSA and bulk density (Gerhardt *et al*., 2015; Maletic *et al*., 2003). Higher WHC of clay soils content impedes the downward migration of PHC into soils, due to the repulsion between water in soils and PHC (Fingas, 2015). As well, clay soils can sorb large amounts of PHCs due to soil particles high SSA (Fingas, 2015). This limits their downward migration and bioavailability to PHC

degrading microbes (Gerhardt *et al*., 2015). Finally, the decreased aeration in clay soils due to their high bulk density limits the biotic PHC weathering (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014).

In contrast to clay soils, the low bulk density and SSA of sandy soils provide lower surface area for PHC sorption thus increasing its mobility. Furthermore, sandy soils have the low WHC which increases the downward migration of PHCs due to the lack of repulsion from water. Overall, sandy soils tend not to retain as much PHC as clay soils and contain less weathered PHC contaminants (Fingas, 2015).

1.1.3 Canadian Guidelines for PHC in the Environment

The Canadian Council of Ministers of the Environment (CCME) has developed a three tier system with specific guidelines on allowable PHC concentrations for each of the four PHC fractions they have defined (CCME, 2008). Tier 1 is the baseline of acceptable PHC concentrations in the soil according to land use (Agricultural., residential/parkland, commercial., industrial; Table 1.1).

Table 1.1: Allowable levels of PHC in Surface Soils (Tier 1)

Land Use	Soil Texture	F1, C_6-C_{10} (mg/kg)	F2, C_{11}-C_{16} (mg/kg)	F3, C_{17}-C_{34} (mg/kg)	F4, C_{34+} (mg/kg)
Agricultural	Fine	210 (170[b])	150	1300	5600
	Coarse	30[b]	150	300	2800
Residential/Parkland	Fine	210 (170[b])	150	1300	5600
	Coarse	30[b]	150	300	2800
Commercial	Fine	320 (170[a])	260 (230[a])	2500	6600
	Coarse	320 (240[a])	260	1700	3300
Industrial	Fine	320 (170[a])	260 (230[a])	2500	6600
	Coarse	320 (240[a])	260	1700	3300

Data taken from CCME guidelines (CCME, 2008)
a= Protection for groundwater
b= Assumes contamination near residence
If grain size of soil <75µm it is classified as fine
If grain size of soil >75µm it is classified as coarse

Tier 2 and 3 guidelines, which are adjustments of Tier 1 guidelines, are set by regulators using site specific criteria (CCME, 2008). For Tier 2, human exposure to PHCs must be limited by specific site criteria such as the location being remote geographically. For Tier 3, the site must be undergoing specific site cleanup (e.g, reclamation, phytoremediation etc.) thereby mitigating the risks to the public health and the environment (CCME, 2008).

1.2.0 Current Analytical Techniques for PHC Analysis in Soil

Various analytical techniques can be employed to determine the concentration of PHC in the soil; each has its own benefits and drawbacks (CCME, 2008; Okparanma, 2013) (T able 1.2). However, the inability of certain techniques to distinguish PHCs from naturally occurring biological organic compounds (BOC) present in soil can be problematic (CCME, 2008; Okparanma, 2013). The presence BOC in a sample may lead to an overestimation of PHC in the soil, potentially resulting in expensive and unnecessary PHC cleanup (Wang *et al.*, 2009).

1.2.1 Gravimetric Analysis

Gravimetric analysis is the simplest technique used to analyze PHC in soils. It has a detection limit of 1.4 mg/L (PHC/volume of sample; EPA, 2010). This method involves use of an organic solvent (e.g., hexane, chloroform, toluene; EPA,2010; CCME, 2007) to extract PHC from soils. The resulting extract is collected and the solvent is evaporated (EPA,2010; CCME, 2007). The residue is then weighed and reported (Equation 1.2; EPA,2010; CCME, 2007).

$$PHC\ \left(\frac{mg}{L}\right) = \frac{W_h(mg)}{V_s(L)} \qquad \textbf{Equation 1.2}$$

Where: W_h= Weight of Residue (mg); V_s= Volume of Sample (L)

Although this method is relatively inexpensive to perform, it cannot distinguish between BOC and PHC molecules (EPA,2010; CCME, 2007; Okparanma, 2013). Furthermore this method has very limited accuracy for low molecular weight PHC compounds (F1 and F2) due to the volatilization of

analyte during the solvent evaporation step. Thus, this method is best suited for HMW PHC compounds (F3 and F4) where loss of analyte by volatilization is limited and where BOC interference is not an issue. This method is being phased out in favor of more accurate analytical techniques; however, this method does have the benefit of being economical and rapid (Okparanma, 2013).

1.2.2 Infrared Spectroscopy

Infrared (IR) spectroscopy measures the stretching and bending of bonds within PHC or other molecules when they are excited by an infrared energy source (Okparanma, 2013). The PHCs in impacted soil samples are extracted using a halogenated solvent (which does not interfere with the IR signal), typically Freon-113 (EPA, 1978; Okparanma, 2013). The molecules in the sample extract are then excited by IR (2900 to 3000 cm^{-1}) and detected to quantify the concentration of PHCs (Okparanma, 2013). IR-spectroscopy has the benefit of being fast, while having low cost to perform. However, it cannot differentiate between PHC and BOC molecules (Okparanma, 2013). As well, heavier hydrocarbons (weathered PHC, F3, F4) have poor solubility in Freon (the extraction solvent) resulting in poor recoveries when analyzed by IR-spectroscopy (Schwartz *et al*., 2012). Furthermore, IR-spectroscopy reliance on Freon-113 has limited its appeal as a PHC quantification method due Freon-113's damaging effect on the ozone layer. For these reasons, IR-spectroscopy has largely been replaced by gas chromatography for PHC analysis (Okparanma, 2013;).

1.2.3 Gas Chromatography with a Flame Ionization Detector

Gas-chromatography with flame ionization detector (GC-FID) is recommended by the CCME as the industry standard for quantifying PHCs in soils (CCME, 2001). PHCs are extracted from soils using an organic solvent and quantified based on molecular weight using a GC-FID. LMW compounds elute first from the GC column followed by HMW compounds. When the sample reaches the FID at the terminal end of the GC column it is combusted and the resulting ions are detected by the FID. The strength of signal is proportional to the amount of combusted PHC. The GC-FID has several advantages: (1) it has a low detection limit (50 μg/kg); (2) it is highly sensitive for the detection of hydrocarbon

compounds, with limited interference from inorganic molecules; (3) it can potentially differentiate between un-weathered and weathered PHC molecules. The GC-FID method, however, requires relatively high equipment and operational costs when compared to IR-spectroscopy and gravimetric methods. It is also unable to directly differentiate between BOC and PHC, as well as PHC and PAH compounds. However, suspected BOC and PAH peaks can be identified on the GC-FID trace (CCME, 2001). Despite these disadvantages the GC-FID method is currently the preferred analytical method for PHC soils analysis by the CCME and EPA (CCME, 2008; EPA, 1992).

1.2.4 Gas Chromatography with a Mass Spectroscopy Detector (GC-MSD)

Gas chromatography with a mass spectroscopy detector (GC-MSD) is similar to the GC-FID method; however, a mass spectroscopy detector (MSD) is used in place of a FID (CCME,2008; EPA, 2014). The process of GC-MSD is similar to that of GC-FID, PHC compounds travel through the GC-column at different rates based on their molecular weight. However, at the terminal end of the GC-column, PHC compounds are impacted by electrons which ionize them into molecular fragments (Hoffman and Stroobant, 2013; Fen *et al.*, 1989). A magnetic field then separates PHC molecular fragments based on their mass to charge before being detected (Hoffman and Stroobant, 2013; Fen *et al.*, 1989). The major benefit of GC-MSD over GC-FID is that it can potentially distinguish PHC compounds from BOCs (Okparanma, 2013). However, the GC-MSD method is not as sensitive to PHC compounds, resulting in a higher detection limit (660 µg/kg) when compared to GC-FID (50 µg/kg). Furthermore, GC-MSD is both cost prohibitive for large scale analysis and requires a substantially longer run time per sample relative to GC-FID (Okparanma, 2013; EPA, 2014).

Table 1.2: Analytical Equipment used to Quantify PHC in Soils.

Equipment or Technique	Target Compound	Method Detection Limit (MDL)	Benefits	Disadvantages	Reference
Gravimetric	Non-volatile PHC compounds	1.4 mg/L	-Very inexpensive -Low technical skill	-Prone to error -Cannot distinguish BOC from PHC -Cannot distinguish between PHC fractions	EPA Method 1664, 2010
IR-Spectroscopy	PHC	1.0 mg/L	-Inexpensive run per sample -Fast	-Use of halogenated solvents -difficult to calibrate -insensitive to heavier PHCs -Cannot distinguish BOC from PHC	EPA Method 418.1, 1978
GC-FID	PHC	50 µg/kg	-Highly accurate and sensitive - potentially identifies weathered PHC	-Expensive -High maintenance cost -Cannot directly distinguish BOC from PHC	EPA Method 8015, 2000
GC-MSD	PHC PAH BOC	~660 µg/kg	-Highly accurate and sensitive -Distinguishes BOC vs PHC -Isolates PAH from PHC	-Very expensive -not as sensitive as FID -long runtime per sample	EPA Method 8270, 1998

MDL is expressed as mass of target compound/volume or mass of soil.

1.2.5 The Current CCME PHC Analysis Protocol

The current CCME protocol recommends extracting PHCs from dry soils with non-polar solvents such as hexane or dichloromethane (DCM). However, as soil moisture increases non-polar solvents become less effective at PHC extraction, due to the hydrophobic repulsion of non-polar solvent with water (CCME, 2008). To increase the efficiency of PHC extraction from soils, a polar organic solvent, such as acetone is combined with the non-polar solvents (Semple *et al.*, 2003; Schwab *et al.*, 1999). The current CCME method recommends a 1:1 ratio of acetone to hexane for PHC extraction from soil before being analyzed by GC-FID (CCME, 2008). Unfortunately, acetone also readily extracts the BOCs due to their polar functional groups, which interferes with PHC analysis (Wang *et al*., 2009). Operators can possibly differentiate between PHC and BOC when observing the GC-FID trace but this is subjective and not recommended by the CCME. Thus the BOC co-extraction leads to an overestimation of PHC in soils and possibly resulting in unnecessary and costly remediation (CCME, 2008; Hooper *et al.*, 2013).

1.3.0 Biological Organic Compounds (BOCs)

BOCs are organic molecules derived from living organisms, as opposed to PHCs which are derived from petroleum (Wang *et al.*, 2009). Like PHCs, BOCs are a diverse group of organic molecules, which include sterols, sterones, plant alkanes, humic substances, fatty acids, alcohols, waxes, and wax esters (Wang *et al.*, 2009). Thus, BOCs often contain polar functional groups in contrast to PHC which have very few polar functional groups (Wang *et al.*, 2009). BOCs typically favour odd carbon chain lengths compared to PHCs which show no bias towards even or odd chain length. Furthermore, BOCs are typically found in the F3 region, but may also be present in the F2 and F4 albeit to a lesser extent (Wang et *al.*, 2009; Marzi *et al.*, 1993).

If the biomass is sufficiently high in soils, BOC concentrations may exceed the allowable CCME PHC concentrations (Table 1.1) despite no PHC being present (CCME, 2008; Wang *et al.*, 2009; Hooper *et al*., 2013). For example, several underground diesel fuel storage tanks were suspected of leaking due the sudden detection of PHC compounds in the soil which exceeded regulatory limits (Zemo *et al.*, 1995).

Upon further inspection, the GC-FID trace did not resemble diesel fuel, but rather looked like an innocuous unidentified BOCs likely due to effluent from a nearby spinach farm (Zemo *et al.*, 1995). Thus, it is important to account for BOC when quantifying PHC concentrations in the soil to limit the risk of false positives.

There are several methods to mitigate the interference of BOCs when quantifying PHC in soils. The simplest method is to use representative un-impacted soil to serve as a control for endogenous BOC in the soil (Hooper *et al.*, 2003). This control would serve as a baseline for BOC when determining the PHC concentration in PHC-impacted soils (Equation 1.3). However, this maybe unfeasible for bio-remediated soils as the microorganism and plant amendments are likely to increase the BOC concentration above that of the un-impacted soil (Hooper *et al.*, 2003).

Equation 1.2

$$PHC \text{ in Soils Corrected for BOC } (mg/kg) = [PHC - BOC]$$

Where: PHC = Concentration of PHC in Impacted Soil (mg/kg); BOC = Concentration of Organics in Un-Impacted Soil (mg/kg)

Another method is to determine the carbon preference index (CPI), which is the ratio between organic compounds with an odd number of carbon chain length and organic compounds with an even number of carbon chain length (Wang *et al.*, 2009; Marzi *et al.*, 1993). Thus, CPI greater than 1.0 is indicative of the presence of significant amounts of BOC in soil and is used as a diagnostic tool to determine if BOCs are present in the soil. However, it does not quantify PHC concentrations and has limited effectiveness in weathered soils (Wang *et al.*, 2009; Marzi *et al.*, 1993).

An effective method used to mitigate the interference of BOCs from PHC sample is to pass the extracts through an activated silica column (CCME, 2008). The activated silica binds the polar functional groups of BOC compounds and removes them from the sample. While non-polar PHC molecules are eluted from the silica column and then quantified by an analytical technique (CCME, 2008). Care must be taken to remove polar solvents prior to the column clean-up step so as not to inactivate (foul) the silica. This can be a time consuming process when dealing with large number of samples (Schwab *et al.*, 1999;

CCME, 2008). Despite this, the silica column cleanup is the CCME recommended method in removal of BOC from PHC samples (CCME, 2008).

1.4.0 The Environmental Impact of Terrestrial PHC Contamination

In Canada, an average of twelve PHC spills occur each day amounting to 14,000 tons of PHC spilled annually (Fingas, 2013). The major sources of terrestrial PHC spills in Canada are from pipelines, storage/refineries and oil wells (Fingas, 2013). After a terrestrial oil spill, PHCs penetrate into the ground by gravity and capillary action (McGill *et al*., 1981), occupying both micro- and macropores (Fingas, 2015). As a result the properties of soil are altered, decreasing the cation exchange capacity (CEC), aeration and WHC of the soil, which can negatively impact the health of plants and soil microbes (Uzoije, 2011; Amadi, 1996; Fingas, 2015). As well, PHC-impacted soil can have negative impact on wildlife, livestock, and human health (CCME,2008). Furthermore, the PHC can migrate into ground water and aquatic systems (CCME,2008).

The cation exchange capacity (CEC) is the ability of soil to bind cations (including some plant nutrients) through negatively charged clay or humic substances on the surface of soil particles (Chapman, 1965; Sumner, 1996). In PHC-impacted soils, hydrophobic PHC compounds block cation exchange (CE) sites on the surface of the soil particles decreasing the ability of soil to retain plants nutrients (Uzoije, 2011; Amadi, 1996; Essien and John, 2010). The lack of nutrient in soils stresses plants, resulting in stunted growth or possibly death (Uzoije, 2011; Essien and John, 2010; Njoku *et al.,* 2008). This is further exacerbated by native hydrocarbonoclastic bacteria (bacteria that use PHC as preferred the carbon source) which compete with plants for soil nutrients as they consume PHC (Robertson *et al*., 2007; Njoku *et al.*, 2008). Gas exchange is often limited in PHC-impacted soil due to PHC compounds occupying micro and macro-pore spaces which may cause plant stress due to the limited aeration of root systems (Fingas, 2015; Shukry *et al.,* 2013). Furthermore, plant roots might be damaged by organic acids found in small amounts in some PHC mixtures (Robertson *et al.*, 2007). Lastly, PHC-impacted soils have lower WHC relative to non-impacted soils, as a result of water being repelled from soil particles due to the

hydrophobic nature of PHC (Fingas, 2015). As a result plants growing in PHC-impacted soils are often subject to osmotic or drought-like stresses, due to the lack of water (Robertson *et al.*, 2007). Direct phytotoxicity of PHC is limited, as only small sized PHC molecules (C1-C12) can diffuse through the plant cell wall and imbed themselves in the cell membrane, disrupting its integrity (Baker, 1970).

Soil microbes, compared to plants, are more affected by direct toxicity of PHC compounds compared to changes in soil properties (Efroymson *et al.*, 2004; Robertson *et al.*, 2007). Toxicity towards microbes is dependent upon PHC composition. For example, PHC mixtures with high PAH concentrations tend to be more toxic than PHC mixtures containing only aliphatic hydrocarbons (Robertson *et al.*, 2007). Despite the exact degree of toxicity being dependent on the composition of PHC mixture, a couple of generalizations can be made. PHCs may replace monomers of the microbial membranes disrupting the lipid bilayer resulting in cell stress or death. Furthermore, PHC may inhibit invertases and hydrogenases which are essential enzymes for bacterial metabolism. Finally, the overall microbial diversity may decrease in PHC-impacted soil, often favoring hydrocarbonoclastic bacteria over other microbes (Robertson *et al.*, 2007).

1.5.0 Physical Remediation of PHC-Impacted Soil

In Canada over 60% of contaminated soils are PHC-impacted and they are often left unremediated due to the lack of resources to secure a site or remediation technologies (Sanscartier, 2010). Physical cleanup methods are based on the removal of PHC-impacted soil to remediate a site. Landfilling in which PHC-impact soil is excavated and relocated to a waste disposal site is the most common. Although landfilling is one of the fastest cleanup methods, it does not restore the soil to its preexisting state before the spill (Ezeji *et al.*, 2007). As well landfilling is relatively expensive in comparison to other methods, costing approximately $200–$1500 per ton (Gerhardt *et al.*, 2009).

1.6.0 Bioremediation of PHC-Impacted Soil

Bioremediation employs living organisms to breakdown PHC contaminants in the soil to potentially restore the soil to its previously un-impacted state (Gerhardt *et al.*, 2009). The bioremediation

efficiency of PHC-impacted soils is dependent on bioavailability of PHC molecules to microorganisms (Volkering *et al.*, 1999). Most soil microbes reside in the aqueous phase on the surface of soil particles, thus, increased solubility of PHC compounds in water is correlated to increased bioavailability (Volkering *et al.*, 1999). The solubility of PHC compounds in water is mainly determined by the molecular weight of the PHC molecules and the degree of weathering (Pilon-Smits, 2005; Robertson *et al.*, 2007). HMW PHC compounds have low water solubility relative to LMW PHCs, and are usually tightly sorbed to soil particles (Robertson *et al.*, 2007). As well, PHCs might be absorbed into the soil particle through the weathering processes of ageing and sequestration, limiting their bioavailability. In most cases, recalcitrant PHC contaminants in soils tend to be HMW (F3 and F4) compounds that have undergone a high degree of weathering (Robertson *et al.*, 2007). Lastly, the bioremediation is highly dependent on the titer of microbes capable of degrading PHC, as increased microbial titer in soil correlates with higher rates of PHC degradation (Gerhardt *et al.*, 2009; Jordhal *et al*., 1997).

1.7.0 Phytoremediation of PHC-Impacted Soil

Of the three most common types of bioremediation techniques- microbial remediation, myco-remediation, and phytoremediation- phytoremediation is the most common (Moosavi and Seghatoleslami, 2013). Phytoremediation is a cost effective solution ($25-50 per ton) which uses plants and their associated microbes to degrade PHC contaminants in the soil (Gerhardt *et al.*, 2009). Phytoremediation can remove contaminants from soils by the following four mechanisms; phytoextraction, phytovolatilization, phytostabilization, and rhizoremediation. Each mechanism targets different class of a soil contaminant (Moosavi and Gholamreza, 2013; Zhang *et al*., 2010).

Phytoextraction involves the uptake of target contaminants from the soil by plant roots, which are then transported and sequestered into vacuoles (Moosavi and Seghatoleslami, 2013; Zhang *et al*., 2010). Phytoextraction is typically used for metal and salt remediation, but rarely with PHCs (Moosavi and Seghatoleslami, 2013; Zhang *et al*., 2010).

Phytovolatilization involves the uptake and transport of contaminants by plants, followed by transpiration through the leaves (Moosavi and Seghatoleslami, 2013). Volatile inorganic contaminants such as mercury, selenium and arsenic, as well as some volatile organic compounds such as trichloroethylene can be removed from soils by phytovolatilization (Moosavi and Seghatoleslami, 2013; Zhang *et al*., 2010).

Phytostabilization is the sequestration or immobilization of target contaminants within the rhizosphere and plants thereby limiting the bioavailability of the contaminants (Moosavi and Seghatoleslami, 2013). Phytostabilization is used mostly for metal contaminants such as cadmium, lead, chromium, copper and zinc. It is rarely effective for PHC remediation in soils (Moosavi and Seghatoleslami, 2013; Zhang *et al*., 2010).

Rhizoremediation is the metabolic consumption (degradation) of organic compounds by plants and their associated microbes within the rhizosphere, a one to two millimeters radius from the plant roots (Pilon-Smits, 2005; Dotanyia and Meena, 2015). Rhizoremediation is primarily used for PHC remediation in soils (Pilon-Smits, 2005). Plants rarely take up PHCs from the soil as the vast majority of PHC contaminants are too large to pass through the plant cell wall (Pilon-Smits, 2005). Instead, the role of plants in rhizoremediation is to promote the microbial degradation of PHCs. The plant achieves this by the secretion of phyotosynthates, employing extracelluar enzymes and aerating the soil (Gerhardt *et al.*, 2009; Chaudhry *et al*., 2005). Photosynthates are plant-derived molecules (sugars, organic acids and larger organic compounds) which can be released into the rhizosphere soil through plant roots (Gerhardt *et al.*, 2009). The release of photosynthates has been shown to dramatically increase the microbial density (10-100 fold) within the rhizosphere compared to the surrounding soil; this is known as the rhizosphere effect (Dotanyia and Meena, 2015). The increased microbial density correlates with increased PHC degradation (Dotanyia and Meena, 2015). Plants may also secrete a variety of extracellular-enzymes such as peroxidases, nitrilases, phosphatases, nitroreductases, and cytochrome p450 monoxygenases that aid in the microbial degradation of PAHs (Novotny *et al*.,1997), as well as halogenated hydrocarbons (Susaria *et al.*, 2002), organic molecules with cyanide functional groups (Kaplan *et al*., 2006), organophosphates

(Susaria *et al.*, 2002), organics with nitro-groups (Liu *et al*., 2007) and PAH and aliphatic hydrocarbons (Sandermann, 1992). As well, plant roots physically break up the soil, increasing aeration thereby promoting aerobic microbial growth and PHC degradation (Gerhardt *et al.,* 2009). Overall, plants are essential for rhizoremediation as they increase microbial density in soils (rhizosphere effect) resulting in improved bioremediation rates (Gerhardt *et al.,* 2009; Dotanyia and Meena, 2015; Pilon-Smits, 2005).

1.7.1 Bacterial Degradation of PHC in the Rhizosphere

The majority of bacteria prefer not use PHCs as a carbon source due to PHC's poor solubility in water and lack of polar functional groups resulting in weak chemical reactivity (Sierra-Garcia *et al.*, 2013). However, hydrocarbonoclastic bacteria (e.g., *Pseudomonas* bacteria) use PHC as their preferred carbon source (Sierra-Garcia *et al.,* 2013). These bacteria take up PHC compounds from the soil by diffusion through the cellular membrane, the PHC are then activated through addition of polar functional groups to increase chemical reactivity (Gerdhart *et al.,* 2009). The biodegradation of PHC contaminants in soil may either be anaerobic or aerobic process. However, anaerobic biodegradation does not have a significant impact on phytoremediation of PHC-impacted soils and will not be discussed further (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014). Aerobic biodegradation of PHC contaminants by microbes in the soil can be divided into two categories based on their substrate: aliphatic (straight or branching carbon chains) or aromatic (cyclic carbon chains) hydrocarbons (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014).

Aerobic bacterial biodegradation of aliphatic hydrocarbon compounds is a two stage process which involves the hydroxylation of PHC chain followed by breakdown through β-oxidation (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014). The first step (rate limiting step) the bacteria must have direct contact with the PHC to achieve uptake and hydroxylation of the PHC chain (Rojo, 2010). The aliphatic PHC is hydroxylated either terminally (long chain carbons) or sub-terminally (short chain carbons) by different membrane bound monoxygenase enzymes, which require molecular oxygen to function (Rojo, 2010; Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014). Following hydroxylation,

aliphatic chains are then further oxidized to aldehydes by alcohol dehydrogenases and finally into fatty acids by aldehyde dehydrogenases (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014). The resultant fatty acids are then converted to acetyl-CoA by β-oxidation and used as a carbon source in the citric acid cycle or used as building blocks for biomolecules (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014).

Aromatic PHC compounds, unlike aliphatic PHC compounds are resistant to degradation in the environment due to their resonance stability (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014). Three enzyme superfamilies: Rieske non-heme iron oxygenases, flavoprotein monooxygenases and soluble di-iron multicomponent monooxygenases are known to oxygenate aromatic hydrocarbons (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014). These enzyme superfamilies are encoded by genes from the Protobacteria or Actinobacteria phyla. Aromatic degradation involves two stages; first the aromatic ring must be activated, involving the oxidation of aromatic ring by one of the aforementioned enzymes. The oxidized aromatic intermediate is either a catechol or protocatechol depending on the substrate undergo meta- and ortho-cleavage respectively. Its products (acetaldehyde, pyruvate, and β-ketoadipate) are used as carbon sources in central metabolism for the bacterial (Cao *et al*.,2009; Sierra-Garcia *et al.*, 2013). It is highly unlikely that a single species of bacteria has all the required monooxygenase enzymes to break down every type of hydrocarbon; thus microbial consortia have a higher efficiency at degrading PHC than any single species of bacteria (Rojo, 2010; Sierra-Garcia *et al.*, 2013). However, the bacterial breakdown of petrogenic aromatics may not be required in PHC phytoremediation of soil, due to the low concentration of aromatics in PHC mixtures.

1.7.2 Advantages and Challenges of Phytoremediation

Phytoremediation has several advantages over other remediation methods. It can target a wide range of both organic (PHC, PAH, halogenated organics) and inorganic (metals and salts) contaminants (Zhang *et al*., 2010; Gerhardt *et al.*, 2009). Phytoremediation can be applied both *ex-situ* and *in-situ* in the environment. Since phytoremediation can be applied *in-situ* it is an attractive option in remote locations where physical cleanup methods would be impractical. Phytoremediation has low cost ($25-50 per ton of

soil) relative to other remediation methods and requires minimal maintenance (Gerhardt *et al.*, 2009). Unlike physical methods (such as landfilling), soils are remediated, thus soil disposal sites are not required. Finally, phytoremediation is more aesthetically pleasing than the open pit excavations associated with physical cleanup methods (Gerhardt *et al.*, 2009).

Phytoremediation, however, is not without its limitations. It can be slow in comparison to physical cleanup methods often requiring more than one year to remediate PHC-impacted soils (Ezeji *et al.*, 2007; Gerhardt *et al.*, 2009). PHC remediation is largely limited to the plant root zone (Gerhardt *et al.*, 2009). The selection of plants can be limited to native species, as the introduction of non-native species may be detrimental to a given ecosystem (Chibuike and Obiora, 2014). With *in-situ* phytoremediation, plants are subject to environmental stressors such as, herbivory, pathogens, weather, etc. This can negatively affect both plant growth rates and phytoremediation rates (Gomes, 2012).

1.7.3 Plant Growth Promoting Rhizobacteria (PGPR) Enhanced Phytoremediation System for PHC Impacted Soils

Plant growth promoting rhizobacteria (PGPR) are bacteria that improve overall plant growth largely by mitigating the effects of environmental stressors (Huang *et al.*, 2005; Gerhardt *et al.*, 2009). PGPR enhanced phytoremediation system (PEPS) is the use of PGPR in conjunction with phytoremediation in order to increase the rate of PHC remediation (Huang *et al.*, 2005; Liu *et al.*, 2013).

For a bacterium to be classified as a PGPR the following criteria must be met: 1) the bacterium must have the ability to colonize plant root surfaces; 2) the bacterium must have the ability to survive, multiply and compete with other soil-dwelling microbes; 3) the bacterium must have the ability to increase overall plant growth (Ahemad and Kibret, 2014). Currently, PGPR can be divided into 2 broad categories; intracellular PGPR (iPGPR) which infiltrate plant roots living in specialized nodule cells; and extracellular PGPR (ePGPR) which reside on the root surface. The iPGPR generally increase plant growth by fixing nitrogen gas, and are usually not used for PHC phytoremediation. Unlike iPGPR, ePGPR are preferred in regards to PHC phytoremediation, due to their ability to mitigate environmental

stress through the production 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase and indole-3-acetic acid (IAA; Ahemad and Kilbret, 2014; Arshad *et al.*, 2007). Environmental stressors (e.g., PHC-impacted soil) can cause plants to produce the phytohormone ethylene from its precursor ACC (Figure 1). Ethylene inhibits plant root, shoot, and axillary meristem growth (Baker, 1970; Ahemad and Kilbret, 2014). ACC deaminase is produced by PGPR and can mitigate the ethylene stress responses in plants by hydrolysis of ACC into α-ketobutyric and ammonia (Ahemad and Kilbret, 2014; Arshad et al., 2007). This decreases ethylene production in plants and results in an increased plant growth rate despite presence of some environmental stressors (Ahemad and Kilbret, 2014; Arshad *et al.*, 2007).

Indole-3-acetic acid (IAA) is a phytohormone (Auxin), which can also be produced by PGPR, and can have a wide range of physiological effects on plants (Ahemad and Kilbret, 2014; Mirza *et al*., 2001; Idris *et al*. 2007). IAA increases root length, xylem development, germination rates, tuber formation, root exudate secretions and upregulates plant defense genes. (Figure 1.1; Khalid *et al*., 2004; Ahemad and Kilbret, 2014; Fu and Wang, 2011). Furthermore, IAA limits ethylene stress responses in plants by inhibiting ACC synthase, thus improving the overall plant growth rate (Kim *et al*., 2001; Arshad *et al.*, 2007).

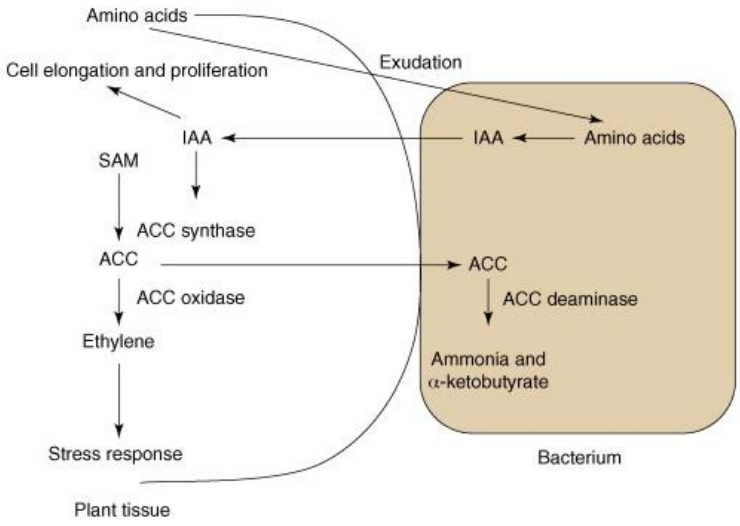

Figure 1.1: The Effects of IAA and ACC Deaminase Produced by ePGPR on Plant Tissue
Both IAA and ACC deaminase are beneficial for plant growth. IAA is produced in ePGPR and is transported into the plant root tissue stimulating cell elongation and proliferation. ACC produced in the plant cells are transported into the ePGPR where it is hydrolyzed into ammonia and α-ketobutyrate by ACC deaminase. This decreases the available ACC in the plant cells, mitigating the ethylene stress response in plant tissues. Diagram taken from Ashad *et al.* (2007).

1.8.0 Chemically Enhanced Phytoremediation

PHC-impacted soil may take several years to be fully phytoremediated, especially in soils with recalcitrant PHC (e.g., PAHs or high molecular weight hydrocarbons; Mulligan *et al.,* 2000). Phytoremediation can be chemically enhanced with the *in-situ* application of chemical additives (oxygen fertilizers and surfactants) to the soil to increase phytoremediation rates (Mulligan *et al.,* 2000). Furthermore, chemically enhanced phytoremediation could be combined with PEPS (Chemically enhanced PEPS) for increased remediation rates.

1.8.1 Oxygen Releasing Compounds

In poorly aerated (e.g., waterlogged and compacted) soils, PHC phytoremediation might be impeded due to low oxygen content, because O_2 is required for both aerobic PHC biodegradation and cellular respiration by PHC degrading microbes (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014).
To limit low oxygen soil conditions and increase biodegradation rates of PHC, oxygen releasing compounds (ORC), which release molecular oxygen in the presence of H_2O, can be added into the soils (Figure 1.2; Goi *et al.,* 2011, Northrup and Cassidy, 2008).

$$XO_{2\,(s)} + 2H_20_{(aq)} \rightarrow X(OH)_{2\,(s)} + H_2O_{2\,(aq)} \qquad (1)$$

$$H_2O_{2\,(aq)} \rightarrow H_2O_{\,(aq)} + O_{2\,(g)} \qquad (2)$$

Figure 1.2: Mechanism for the Production of Oxygen by ORCs.
Reaction 1 shows the production of H_2O_2 from an ORC, where X can either be Mg^{2+} or Ca^{2+}. Reaction 2 shows a disproportionation reaction of H_2O_2 in to H_2O and O_2 (Northrup and Cassidy, 2008).

The three most common ORCs are hydrogen peroxide (H_20_2), calcium peroxide (CaO_2), and magnesium peroxide (MgO_2)(Northrup and Cassidy, 2008; Wang *et al*., 2011). Of these three ORCs, H_2O_2 has the highest solubility in water and rapidly decomposes releasing large amounts of oxygen into the soil (Northrup and Cassidy, 2008). However, the rapid decomposition of an ORC is not ideal, because excess oxygen may escape rather than be used by bacteria for PHC biodegradation. Due its short half-life, H_2O_2 must be regularly reapplied to PHC-impacted soils to be effective (Northrup and Cassidy, 2008). Unlike

H_2O_2, both CaO_2 and MgO_2 have low solubility in water, allowing for the slow release of oxygen into the soil over an extended period of time (Wang *et al.*, 2010; Northrup and Cassidy, 2008).

The majority of ORC-enhanced remediation trials to date have been carried out with laboratory-based microbial bioremediation. In a field study, Davis *et al.,* (1997) showed that PHC-impacted soil (300-400 mg/kg) treated with CaO_2 and MgO_2, had a 68% decrease in PHC concentration relative to the untreated PHC-impacted soil (no ORCs) within the first 13 days. Cassidy and Irvine (1999), investigated the effect of CaO_2 on microbial remediation of bis-(2-ethylhexyl) phthalate (BHEP) in solid state bioreactors over a 20 day period. In the CaO_2 treated bioreactor, the BHEP concentration decreased by 75% while in the untreated control bioreactor it decreased by 26% (Cassidy and Irvine, 1999). Recent research has focused on extending the half-life of ORC by developing ORC-containing soluble polymer beads which release CaO_2 50% slower than CaO_2 applied directly to soil, thus limiting the need for reapplication (Lee *et al.,* 2014; Wu *et al.,* 2014). To date little research has been done on the effects of ORC on phytoremediation.

1.8.2 Surfactant Enhanced Phytoremediation

The bioavailability of PHCs to soil microbes is one of the many rate limiting steps in phytoremediation, as many recalcitrant PHC compounds have low bioavailability (Robertson *et al*., 2007). These recalcitrant PHC compounds are often very hydrophobic, and remain adsorbed to the surface of soil particles or within soil micropores (Das and Chandran, 2011). PHC bioavailability can be increased with the use of surfactants which decrease the surface tension between hydrophobic molecules (PHC contaminants) and the aqueous phase (water) (Volkering *et al.,* 1999; Ron and Rosenberg, 2002). In the context of enhanced bioremediation, surfactants can either be classified as biosurfactants or synthetic surfactants (Kile and Chou, 1990; Mulligan, 2005; Volkering *et al.*, 1998). Synthetic surfactants can either be ionic (anionic or cationic) or non-ionic (neutral) (Volkering *et al.,* 1998). Generally ionic surfactants (e.g., sodium dodecyl sulfate and sodium dodecylbenzenesulfonate) are more effective at solubilization of non-polar compounds than non-ionic surfactants (e.g., Tween-80 and Triton-x100), due

to their higher solubility in aqueous environments (Figure 1.3; Despande *et al.*, 1999). However, unlike non-ionic surfactants, ionic surfactants often have greater toxic interactions to microbial surface proteins due to ionization over certain pH ranges (Volkering *et al.*, 1998; Cserhati *et al.*, 2002). Generally synthetic surfactants have low steric hindrance, and may insert themselves into microbial lipid bilayers more readily compared to biosurfactants. In sufficient quantities synthetic surfactants might solubilize the microbial lipid bilayer resulting in cell death (Volkering *et al.*, 1998). As such, synthetic surfactants are not used as frequently as biosurfactants in conjunction with bioremediation (Mulligan, 2005; Pacwa-Płociniczak *et al.*, 2011). Synthetic surfactants may still be suitable in ex-situ soil washing remediation projects or as a pretreatment where PHC-containing effluent would be collected and disposed of properly (Volkering et *al.*, 1998).

Biosurfactants are naturally occurring and are mostly produced by soil microbes (Jorfi *et al.*, 2014; Mulligan, 2005; Pacwa-Płociniczak *et al.*, 2011). They are usually less toxic than synthetic surfactants possibly because they are more readily degraded and do not easily insert themselves into phospholipid bilayers (Mulligan, 2005). The main classes of biosurfactants are glycolipids, lipopeptides, lipoproteins, fatty acids, phospholipids, and polymeric surfactants (Figure 1.3; Mulligan, 2005; Gautam and Tygai, 2006). The most widely studied biosurfactant is rhamnolipid, a subclass of glycolipids, which is produced by several *Pseudomonas*species (Jorfi *et al.*, 2014; Pacwa-Płociniczak *et al.*, 2011). Rhamnolipids are composed of two rhamnose sugars linked to a β-hydroxydeconic acid or one rhamnose connected to a fatty acid (Mulligan, 2005). Several remediation studies have been carried out to test the efficacy of rhamnolipids as surfactants with varying results. Whang *et al*. (2008) found that rhamnolipid-treated soils had a 97% (un-weathered soil) and 47% (weathered soil) decrease in PHC concentration relative to the untreated control soil. Rahman *et al.* (2003) showed that rhamnolipid-treated unweathered PHC-impacted soils had an 80-85% (C_{22}-C_{31} compounds) and 57-73% (C_{32}-C_{40} compounds) decrease in PHC concentration relative to untreated control soil. Conversely, a one year field study by Szulc *et al.* (2014) found a no significant increase in microbial PHC degradation in rhamnolipid-treated plots relative

to the untreated control soil after 60 days. Currently, the majority of surfactant enhanced research has been focused on microbial bioremediation with minimal research focused on phytoremediation.

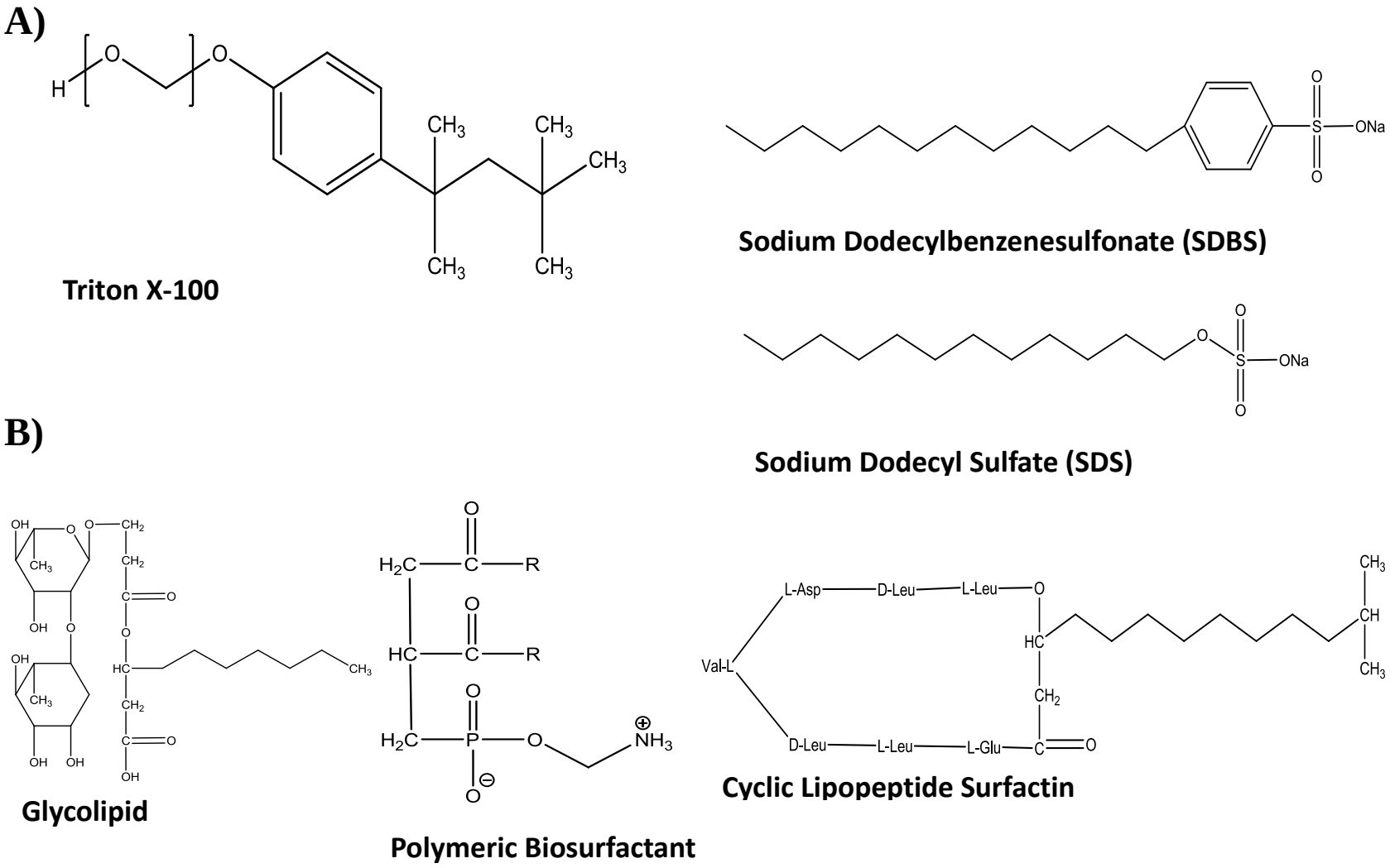

Figure 1.3: Chemical Structures of Surfactants
A) Structures of Synthetic Surfactants: Triton X-100, SDBS and SDS **B)** Structures of Biosurfactants: Glycolipids, Polymeric Biosurfactant, Cyclic Lipopetide Surfactin

1.9.0 Hypothesis and Research Objectives

The investigation of this research is divided into two sections: 1) method development for PHC quantification in soils and, 2) efficacy of chemically enhanced PEPS on weathered PHC-impacted soil quantified using PHC methods developed in section 1. BOC are naturally occurring in soils derived from endogenous soil organisms (plants and microbes) may interfere with quantification of PHC when analyzed by the GC-FID possibly leading to an overestimation of PHC (Wang *et al.,* 2009; Hooper et al., 2013). The current, standard CCME PHC extraction and quantification method does not account for BOC contamination in soil extract samples (CCME, 2012; Wang *et al.,* 2009; Hooper et al., 2013; Zemo *et al.,* 1995). The objective of this research was to develop a method that is effective at extracting PHC from soils as the CCME protocol while limiting BOC interference. To test this objective the following was carried out: (1) Compare the efficacy of different combinations and concentrations of acetone, hexane, and dichloromethane (DCM) on PHC and BOC extraction; (2) Investigate the effectiveness of increasing concentration of activated silica for BOC removal in extract samples.

Chemically enhanced phytoremediation employs the *in-situ* application of chemical additives to the soil to increase phytoremediation rates (Mulligan *et al.,* 2000). Currently, there is limited research on the effects of both CaO_2 and petroleum based surfactants (Petroleum sulfonate oil; PSO) carried out on chemically enhanced PEPS of PHC-impacted soil. Furthermore, most remediation experiments use spiked as opposed to weathered soil contaminants which are resistant to bioremediation and are representative of an actual PHC-impacted soils (Cassidy and Irvine, 1999; Jofri *et al.*, 2013; Hewei *et al.,* 2014; Goi *et al.,* 2011). Both CaO_2 and PSO may increase the rate of PHC phytoremediation relative to unamended phytoremediated soils. To test this hypothesis 3 objectives were pursued: 1) Test the efficacy of PSO and CaO_2 on the rates of PHC phytoremediation in a four-week-long enhanced PGPR-phytoremediation greenhouse trial; 2) monitor plant health by measuring dry biomass and chlorophyll concentration to determine if CaO_2 or PSO had impacts on plant health; (3) Determine the efficacy of the PHC extraction methods developed, on quantification of PHC from chemically enhanced PEPS soil.

Chapter 2:

Analytical Techniques for Petroleum Hydrocarbon Quantification and BOC Mitigation in Impacted Soils.

2.1 Overview

The allowable concentration of petroleum hydrocarbons (PHC) in Canadian soils is recommended by the Canadian Council of Ministers of the Environment (CCME) and set by provincial authorities. Several methods exist for the extraction and quantification of PHC from soils. The preferred method according to the CCME is solvent extraction with 1:1 acetone/hexane (1:1 AH) to recover PHC from soils, followed by quantification via gas chromatography with a flame ionization detector (GC-FID) (CCME, 2008). Unfortunately, the 1:1 AH mixture extracts both PHC and endogenous biological organic compounds (BOC), potentially resulting in an overestimation of total PHC. In this research, two analytical methods were compared for their ability to extract PHC from soils while also limiting interference from BOC. The first method (1:1 AH with silica cleanup) used the 1:1 acetone/hexane solvent mixture to extract PHC from soils, followed by the addition of activated silica to remove BOC from the PHC-soil extract. The second method (DCM+SS+Silica) uses the solvent dichloromethane (DCM) along with anhydrous Na_2SO_4 to dry soils and activated silica to remove BOC. Both methods extracted similar amounts ($P>0.05$) of PHC from the weathered PHC-impacted soils, except when soil moisture was higher than 12.0%. At higher soil moistures the extraction efficiency of the DCM+SS+Silica method decreased by 11.7% ($P=0.013$) while the 1:1 AH with silica cleanup PHC extraction efficiency was unaffected.

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2.2 Introduction

The Canadian Council of Ministers of the Environment (CCME) suggests strict guidelines on the allowable levels of petroleum hydrocarbons (PHC) in soils (CCME, 2008). To quantify the concentration of PHC in soils, PHC are first extracted from soils using an organic solvent often aided by mechanical agitation. The PHC-extracts can be analyzed by a variety of analytical techniques such as gravimetrics analysis, IR-spectroscopy, gas chromatography (GC) with a flame ionization detector (FID), or GC mass spectroscopy (GC-MS; CCME, 2008; Okparanma, 2013). Among these techniques, GC-FID is usually preferred due to its high sensitivity in detecting PHC. When analyzed by GC-FID, PHC-extracts are vaporized and are transported through the GC-column and into the FID located at the terminal end of the column (CCME, 2008). During transport through the GC-column, PHC separate based on boiling points with lower molecular weight compounds eluting from the column first. Upon reaching the terminal end of the GC-column, PHCs are ignited and the resultant ions are quantified by the FID (CCME, 2008).

However, analysis by GC-FID, gravimetrics and IR-spectroscopy cannot distinguish between PHC and biological organic compounds (BOCs). The latter are naturally occurring soil bound organic compounds produced by soil organisms and plants (CCME, 2008; Wang *et al*., 2009; Marzi *et al*., 1993; Yang *et al*., 2013; Hooper *et al*., 2003). Generally, BOC are found among PHC with chain lengths of 17-34 (the F3 fraction of PHC). This can cause an overestimation of PHC in soils, potentially resulting in unnecessary and expensive remediation (CCME, 2008; Wang *et al*., 2009). Currently, GC-MS is the only analytical equipment that can distinguish between PHC and BOC molecules (EPA, 2014). However, it is not widely used, because GC-FID are more sensitive to PHC, analyzes samples faster and is the lower cost option (Okparanma, 2013; EPA, 2014).

Currently, the CCME recommends the cold shake method with 1:1 acetone:hexane (1:1 AH) to extract PHC from soils followed by quantification by GC-FID (CCME,2008). The cold-shake method involves mechanical agitation of PHC-impacted soils in the solvent mixture 1:1 AH at room temperature. However, this method also extracts BOC from soils (if they are present). This is due to the polar solvent (e.g., acetone) solubilizing BOC from soils. To remove BOC contaminants, the CCME recommends

passing PHC-extract through a column of activated silica which binds to the polar functional groups of the BOC, allowing the non-polar PHC to elute (CCME, 2008). However, polar solvents (i.e acetone) must be removed from the PHC-soil extract before it is passed through the silica column. If the polar solvent was not removed from the PHC-extract, it would occupy the majority of binding sites on the silica, drastically decreasing its efficacy (CCME 2008; Schwab *et al*., 1999). As a result, the removal of BOC from PHC-extracts using activated silica (silica cleanup) can be a laborious task (Schwab *et al*., 1999).

The objective of this investigation was to develop a protocol which will accurately quantify PHCs within the soil while limiting interference from BOCs. To accomplish this, there were three objectives: (1) Test the efficacy of different combinations and concentrations of solvents (acetone, dichloromethane, and hexane) on both PHC and BOC extraction. (2) Develop an effective *in situ* silica cleanup protocol for removal of BOCs from extracts of weathered PHC-impacted soils and freshly spiked PHC soils. (3) Compare the efficacy of the 1:1 AH with the *in situ* silica cleanup method to the DCM with an *in situ* silica cleanup method on the PHC extraction using non-simulated weathered PHC-impacted soils.

2.3.0 Materials and Methods

2.3.1. Soil Preparation

Both PHC-impacted and non-impacted soil was collected from a site in Alberta after two full seasons of phytoremediation. Roughly 10.0 kg of each soil type were sieved through a wire mesh (2.00 cm diameter) and vigorously mixed in their respective containers to ensure homogenous distribution of PHC. Soils were then stored at room temperature in sealed containers. In addition to soils from Alberta, artificial soils were also prepared in 20 mL scintillation vials. These artificial soils (4.50 ±0.01 g of silica sand and 0.50 ±0.01 g of peat) were spiked with both 0.50 ±0.01 mL solution of 1:100 diesel to hexane and 1:10 of motor oil to hexane. A 3.00 ±0.01 mL solution of 1:1 acetone:hexane (1:1 AH) was then added to each scintillation vial and then vortexed for 30 seconds to evenly disperse the PHC solution throughout the artificial soil. The cap of each scintillation vial was removed and the solvent was allowed to volatilize in a fume hood for 24 hrs.

2.3.2 Cold Shake PHC Extraction from Soil

PHC in soils are extracted using a modified cold-shake extraction method as recommended by the CCME (CCME, 2008). PHC-impacted soil samples were weighed (5.00-6.00 ±0.01 g) and then placed into 20 mL scintillation vials. Various solvent mixtures were used to extract PHC from soils using the cold-shake extraction method (Table 2.1). In the first extraction 7.50 ±0.01 mL of solvent was added to the sample which was then vortexed for 30 seconds and shaken for 30 minutes at 170 rpm. Vials were then stored in a refrigerator at 4.0°C for 1 hour, to allow soil to settle. The extract/solvent layer was transferred into test tubes which were tightly sealed and returned to the refrigerator to minimize volatilization. In the second extraction, 7.50 ±0.01 mL of fresh solvent was added to each soil sample in the scintillation vials. The samples were then vortexed for 30 seconds and shaken for 30 minutes at 170 rpm. The extract/solvent in the test tube (stored in the refrigerator) was returned to its respective scintillation vial and allowed to settle for 1 hour at 4.0°C. Approximately 1.50 mL of extract/solvent was transferred from each scintillation vial into GC vials and then analyzed using the GC-FID.

Table 2.1: Different Combinations of Acetone, DCM, and Hexane used for Cold Shake PHC Extraction.

1st Extraction (7.50 mL)	2nd Extraction (7.50 mL)
Acetone	Acetone
Acetone	Hexane
1:1 Acetone:Hexane	1:1 Acetone: Hexane
1:2 Acetone:Hexane	1:2 Acetone:Hexane
1:2 Acetone:Hexane	Hexane
1:4 Acetone:Hexane	1:4 Acetone:Hexane
1:4 Acetone:Hexane	Hexane
Hexane	Hexane
DCM	DCM
1:1 DCM:Hexane	1:1 DCM:Hexane
1:1 DCM:Hexane	Hexane
1:2 DCM:Hexane	1:2 DCM:Hexane
1:2 DCM:Hexane	Hexane
1:4 DCM:Hexane	1:4 DCM:Hexane
1:4 DCM:Hexane	Hexane

2.3.3 Preparation of both Activated Silica and Anhydrous Sodium Sulfate

Reagent grade silica (CAS# 1344-09-8, Sigma Aldrich, Oakville, Ontario, Canada) (0.50±0.01 g) and anhydrous sodium sulfate (Na_2SO_4; CAS# 7757-82-6, Sigma Aldrich, Oakville, Ontario, Canada) (0.10±0.01) were weighed using a top loading analytical balance and then transferred into glass test tubes. The test tubes containing silica and Na_2SO_4 were transferred to an oven at 103°C for a period of 24 hours to remove any residual moisture.

2.3.4 Use of Activated Silica for BOC Removal in PHC-Soil Extract Containing Acetone

The cold shake extraction method was used to extract PHC from 5.00-6.00 g soil samples. A volume of 5.00 mL of cold shake 1:1 AH extract was extracted with 10.0 mL of reverse osmosis water (RO-H_2O). The top layer (organic phase) containing PHC, hexane and BOC was then transferred to another test tube containing 10.0 mL of RO-H_2O to maximize acetone removal. The organic layer (PHC, hexane and BOC) were transferred to a test tubes containing 0.10 ±0.01g of anhydrous sodium sulfate (dried at 103°C for 24 hr) to remove any residual RO-H_2O. The samples were then vortexed for 15 seconds and the liquid was transferred to another test tube. Hexane (1.00 mL) was used to wash the anhydrous sodium sulfate to remove any remaining solvent/extract and transferred to the respective labeled test tube. The volume of solvent in the test tube was brought up to 10.0 mL using hexane and then transferred to test tube containing activated silica. Samples were vortexed for 15 seconds and centrifuged until activated silica was pelleted. The supernatant was transferred into GC vials and analyzed using a GC-FID (see below).

2.3.5 DCM Cold Shake Extraction with Addition of Na_2SO_4 and Activated Silica

A modified cold shake extraction method used DCM to extract PHC from soils. PHC impacted soil samples were weighed to 5.00-6.00 ±0.01 g using a top-loading balance (OHAUS TS400, M&L Testing Equipment (1995) INC. Dundas, Ontario) and then placed into 20 mL scintillation vials. Na_2SO_4 (0.50±0.01 g) was added into the scintillation vials which were stored in the refrigerator (4.0°C) for 30 minutes. Activated silica (0.50±0.01 g) was then added to the scintillation vials. For the 1st extraction 7.5

mL of DCM was added to the sample, vortexed (30 seconds) and then placed on a shaker (30 min at 170 rpm). Samples were stored in the refrigerator (4.0°C) for 1 hour, allowing soils to settle. The solvent layer was then transferred into respective test tubes capped with septa and returned to the refrigerator. This process was repeated two more times for a total of three extractions before quantification by GC-FID.

2.3.6 PHC Quantification by GC-FID

Sub-samples from each soil sample were weighed out to approximately 2.00-3.00 g and left to air dry at room temperature for 24 hours. These were then weighed to determine the dry weight and passed through a sieve with 2 cm diameter holes. The gravel was then weighed. Soil mass was corrected for moisture and gravel using Equation 2.1.

Equation 2.1

$$Sample\ Weight\ \left(1-\frac{W-D-G}{W}\right)=Corrected\ Soil\ Weight\ (g)$$

Where: W= Wet Soil Mass (g); D= Dry Soil Mass (g); G= Gravel Mass (g); Sample Weight =Mass of Soil (g).

The GC-FID detector and injector temperature were set to 340°C and 300°C, respectively. The carrier gas was nitrogen (Praxair, CAS #7727-37-9) set a flow rate of 9.96 mL/min. The temperature program used for PHC determination was the following: hold 40°C for 1 minute, increase temperature by 15°C per minute to 300° C, and hold for 20 minutes. From the GC-FID trace the area under the curve known as the unresolved complex mixture (UCM; shown as in red) was measured using GC solutions lite software (Shizmadu Inc; Figure 2.1). Using the soil mass corrected soil moisture and gravel content (Equation 2.1) and the UCM area from the GC-FID trace, the concentration of PHC in soil (mg/kg) was calculated (CCME, 2001) (Equation 2.2).

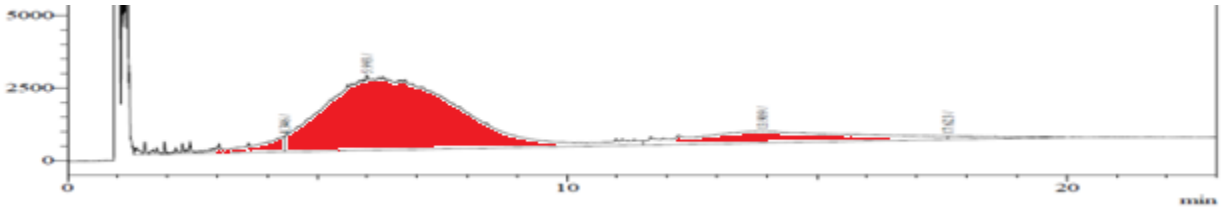


Figure 2.1: Sample GC-FID Trace of PHC-Extract.
The area of the UCM (highlighted in red), represents the total amount of PHC in the PHC-extract. The area of the UCM was used to calculate the PHC concentration in soils (mg/kg) using equation 2.2.

$$\frac{UCM\ x\ Vol\ x\ Df}{Rf\ x\ W} = PHC\ \left(\frac{mg}{kg}\right)$$ **Equation 2.2**

Where: UCM = UCM Area; Vol = Solvent Volume (mL); Df = Dilution Factor; Rf = Retention Factor; W = Corrected Soil Weight (g).

2.3.7 Statistical Analysis

To determine if data sets from each replicate could be combined, data were tested for normality prior to statistical testing with Z-values for kurtosis and skewness between -1.96 and +1.96; and Shapiro-Wilk test P-value greater than 0.05. ANOVA along with LSD *post hoc* tests were used to determine statistical significance (α=0.05) for PHC concentration in soils. All statistical tests were conducted using IBM SPSS Statistics software.

2.4.0.0 Results

2.4.1.0 Cold-Shake Extraction of PHC from Soils using Various Solvent Mixtures

The CCME recommends the cold-shake extraction method to isolate PHC from soils prior to quantification by GC-FID. The cold-shake method has two PHC extraction steps using using 1:1 acetone:hexane (1:1 AH) to maximize PHC recover from soils. Each PHC extraction step involves addition of 7.50 mL of the solvent 1:1 AH to approximately 5.00 g of soil, followed by a mechanical agitation to aid in the breakup of soil aggregates. However, 1:1 AH would co-extracts BOC (if present) along with PHC from soils. Ideally, another solvent combination would be preferred, if it was as effective as 1:1 AH at PHC removal and co-extracted less BOC. To test this, different combinations and ratio of solvents (Acetone, DCM, and Hexane) were used at the different extraction steps (1^{st} 7.5 mL solvent extraction or 2^{nd} 7.5 mL solvent extraction step). In all cases the cold shake extraction was used to recover PHC from soils. Weathered PHC-impacted soil from Alberta was used to determine the PHC extraction efficiency of each solvent combination. As well, un-impacted soil was used to determine the amount of BOC extracted with each solvent combination.

Solvent combinations containing acetone, generally extracted the most PHC and BOC from soils, compared to the other solvent combinations (DCM and Hexane; Figure 2.2). As the ratio of acetone in hexane decreased so did the extraction of both PHC and BOCs from soils. The various DCM solvent combinations were not as effective at PHC extraction from soils compared to the CCME recommended 1:1 AH ($P<0.05$). Furthermore, like acetone, decreases in ratio between DCM and hexane, limited both PHC and BOC extraction from soils. Overall, the most effective DCM solvent combination, was 100% DCM solvent which extracted 32.6% ($P<0.05$) and 45.7% ($P<0.05$) less PHCs and BOC, respectively, than 1:1 AH. Solvent mixtures, with increasing concentration of hexane decreased the overall PHC extraction but had the least BOC co-extraction. Finally, 100% hexane was the least effective solvent, which extracted significantly less ($P<0.05$) PHC and BOC than 1:1 AH. Overall, the CCME 1:1 AH was

the most effective solvent mixture, as it extracted the most PHCs from the soil, despite extracting highest levels of BOC (Figure 2.2).

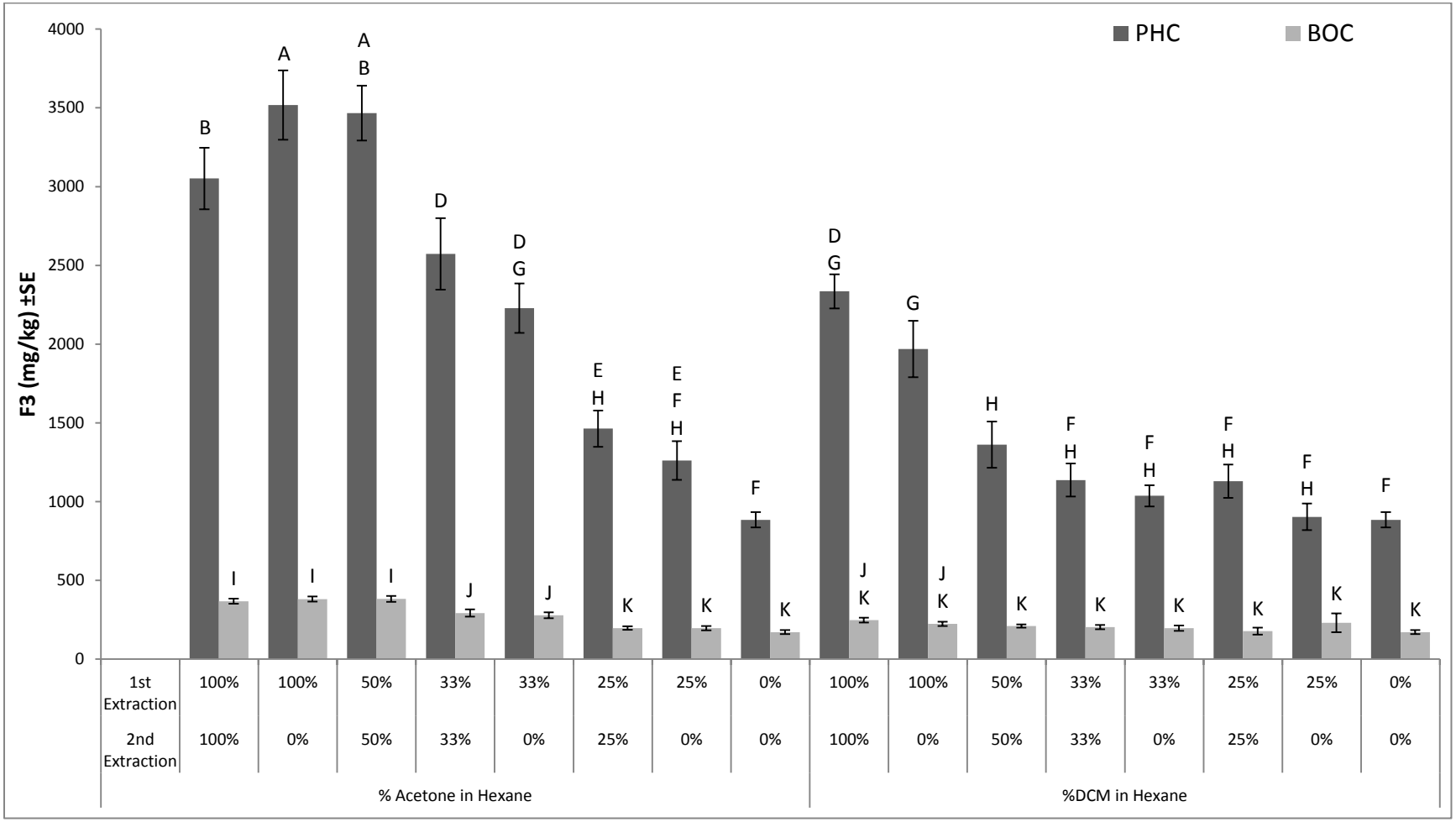


Figure 2.2: PHC Extraction from Soils with Various Solvents

Various concentrations of acetone and DCM in hexane were used to extract PHC+BOC and BOC from PHC-impacted and un-impacted soil respectively. Various solvents combinations were used at different wash steps within the cold shake method. Samples were run in duplicate for three independent replicates (n=6). Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA. Error bars indicates standard error (SE).

2.4.1.1 Appearance of PHC-Impacted Soils after PHC-Extraction

Generally, soil homogenization and lack of soil aggregates after undergoing cold-shake extraction indicates better solvent penetration and improved PHC recovery from soils. Upon completion of the cold-shake extraction with various solvents (Figure 2.2), representative images of PHC-impacted soils were obtained demonstrate the degree of soil homogenization (Figure 2.3). Generally, increased concentrations of either DCM or acetone in hexane improved soil homogenization. However, acetone solvent mixtures were substantially more effective at soil homogenization than DCM solvent mixtures (Figure 2.3).

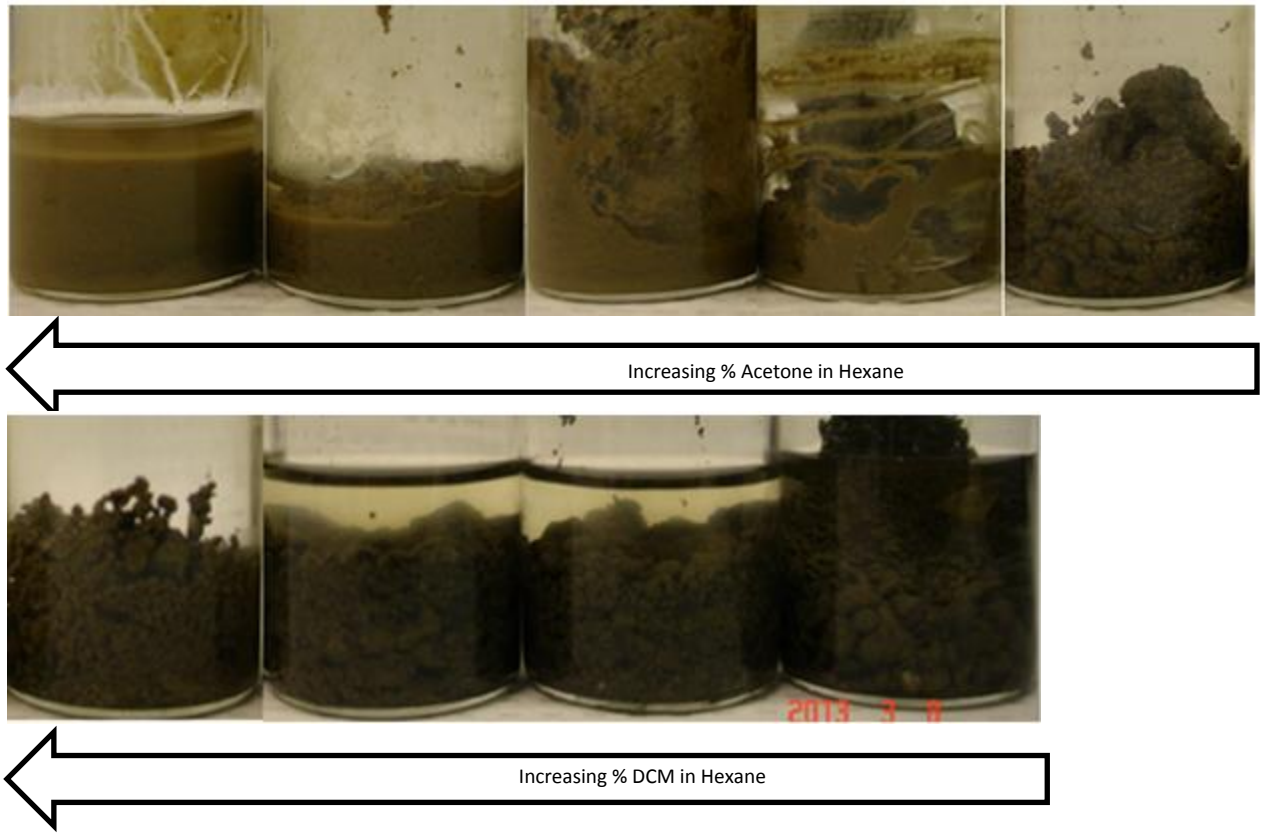


Figure 2.3: Images of PHC-Impacted Soils after PHC Extraction
Representative images of PHC-impacted soil from cold shake extraction with increasing concentrations of acetone (A) and dichloromethane (DCM) in hexane (H).

2.4.1.2 GC-FID Traces of PHC-Extracts from the Cold-Shake Extraction

The GC-FID traces of 5 solvent mixtures and the least effective solvent mixture are provided in Figure 2.4. PHC extracted from soils with acetone solvents had larger unresolved complex mixtures (UCM) and suspected BOC peaks (according to the GC-FID traces) compared to solvent mixtures with DCM (Figure 2.4a-e). Furthermore, lower UCMs and suspected BOC peaks were observed as concentrations of acetone or DCM in solvent mixtures decreased (Figure 2.4a-e). The GC-FID trace of PHCs extracted from soils with hexane alone had the smallest UCM and BOC peaks of all the various solvent combinations (Figure 2.4f).

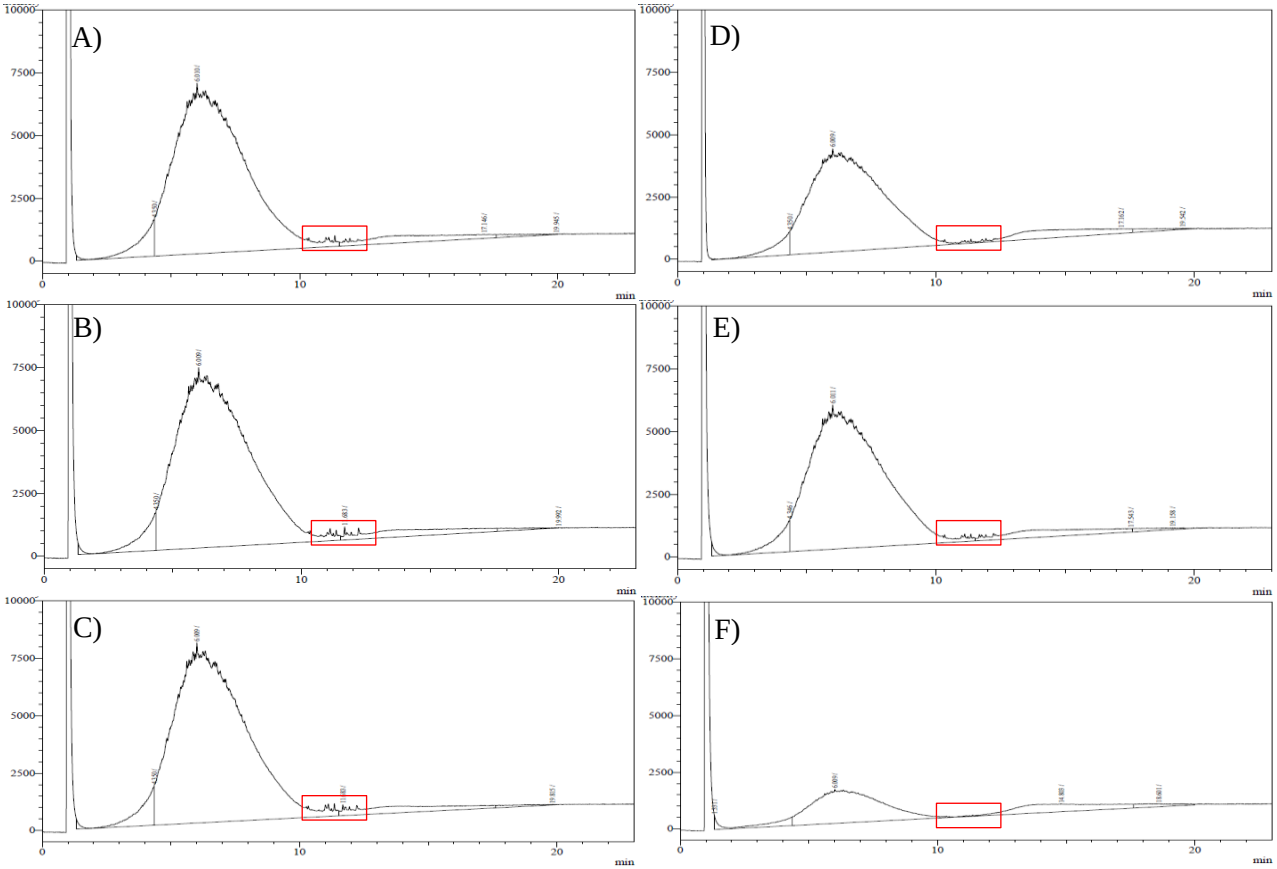


Figure 2.4: GC-FID Traces of PHC Extracted from Soils Using Various Solvents.
Representative GC-FID traces of the PHC-impacted soil after undergoing cold-shake extraction with various solvent mixtures from the experiment presented in Figure 2.2. Suspected BOC peaks highlighted by the red box. **A)** 1st Extraction = acetone, 2nd Extraction = acetone; **B)** 1st Extraction = 1:1 AH, 2nd Extraction = 1:1 AH; **C)** 1st Extraction = acetone, 2nd Extraction = hexane; **D)** 1st Extraction = 1:2 AH, 2nd Extraction = 1:2 AH; **E)** 1st Extraction = DCM, 2nd Extraction = DCM; **F)** 1st Extraction = hexane, 2nd Extraction = hexane.

2.4.2.0 Use of Activated Silica for BOC Removal from PHC-Extracts

The results above indicate that 1:1 AH was the most effective solvent mixture at extracting PHC from soils using the cold shake method. However, it also extracted the most BOC from soils, the BOC should be removed if PHCs are to be accurately quantified by GC-FID. The BOC (co-extracted) in solvent containing extracted PHC from soil (PHC-extract) can be removed with activated silica. However, presence of either acetone or water in the PHC-extract would greatly limit the effectiveness of activated silica with respects to BOC removal (Schwab *et al.*, 1999; CCME, 2008). Thus, a protocol must be devised to remove acetone and water from the PHC-extract, prior to determining the effective concentration of activated silica for BOC removal.

2.4.2.1 Removal of Acetone from the PHC-Extracts with RO-H_2O

Acetone can be removed from the PHC-extract via a liquid-liquid extraction with reverse osmosis water (RO-H_2O), as acetone is miscible in H_2O (Table 2.2). The aqueous layer can be removed resulting in a PHC- extract devoid of significant concentrations of acetone. To determine the amount of RO-H_2O required for acetone removal, increasing volumes of RO-H_2O (7.0, 8.0, 9.0, 10.0 mL) were added to 5.00 mL 1:1 AH samples. The organic phase volume was measured following the addition of RO-H_2O into the sample. Theoretically, the total amount of acetone in the 5.00 mL of 1:1 AH is 2.50 mL, thus the removal of acetone is complete when the volume of the organic phase decreases to 2.50 mL. All RO-H_2O extractions removed significant amounts of acetone (92.0-101.2%) from the 5.00 mL 1:1 AH sample. Based on the data, two 10.00 mL RO-H_2O washes were used for all subsequent work (Table 2.2).

Table 2.2: Volume of RO-H_2O required to Remove Acetone from Organic Phase.

Volume RO-H_2O Wash (mL)	Volume of Organic Phase (mL)	Acetone Removal (%)	Standard Error (mL)
2 x 7.0	2.53	98.8	0.067
2 x 8.0	2.47	101.2	0.033
2 x 9.0	2.70	92.0	0.082
2 x 10.0	2.60	96	0.000

All treatments were conducted in triplicate (n=3).

2.4.2.2 Water Removal from PHC-Extracts Following Acetone Removal

Like acetone, H_2O is polar molecule that can decrease efficacy of silica. Anhydrous Na_2SO_4 were used to remove RO-H_2O from the PHC- extracts. After 2 washes with 10.0 mL of RO-H_2O a final volume of roughly 2.60 mL was reached. It was assumed that approximately 2.50 mL of this liquid was hexane while the remaining 0.10 mL might be residual RO-H_2O. Thus, the amount of Na_2SO_4 must be sufficient in removing at least 0.10 mL of RO-H_2O.

To investigate this, increasing amounts of Na_2SO_4 were added into 2.00 mL of RO-H_2O and the resultant volume was measured. As expected, higher amounts of Na_2SO_4 removed larger volumes of dH_2O from the sample (Table 2.3). Overall, 0.10 g of Na_2SO_4 was decided on for drying (adsorbing H_2O) the PHC extracts, because it removed twice the amount of RO-H_2O expected to be present (0.2 mL of RO-H_2O; Table 2.3). Furthermore, higher amounts of Na_2SO_4 might require multiple hexane washes to remove all the residual PHC adhering to the surface of Na_2SO_4.

Table 2.3: The Amount of Anhydrous Na_2SO_4 Required to Remove 0.10 mL of RO-H_2O

Sample	Amount of Anhydrous Na_2SO_4 (g)	Volume of RO-$H_2$0 (mL) recovered from 2.0 (mL) of RO-H_2O
1	0.10	1.8
2	0.10	1.8
3	0.50	1.7
4	0.50	1.6
5	1.00	1.4
6	1.00	1.5

2.4.2.3 Amount of Activated Silica Required for BOC Removal from PHC- Extracts

Once both the acetone and residual RO-H_2O were removed from the PHC- extract, activated silica could be used to remove the BOC contaminants present in the sample. To determine the amount of activated silica required for BOC removal, increasing amounts of activated silica were added directly into PHC- extract (*in situ*). The PHCs of each sample were then quantified by GC-FID (Figure 2.6) (Note only the F3 is shown as the majority of BOC falls within this fraction).

Generally, higher amounts of activated silica slightly decreased ($P>0.05$) the F3 concentration in PHC-impacted soil (Figure 2.5a). The decreases in F3 (mg/kg) concentration are likely the result of BOC removal by activated silica. To get a more accurate representation of silica effect on BOC, only suspected BOC peaks from the GC-FID trace were integrated using equation 2.2 (Section 2.3.6). This resulted in significant decreases in BOC with activated silica treatments (0.25, 0.50, 0.75, 1.00 g) relative to the untreated control (Figure 2.5b). Overall, 0.50 g (or greater) of activated silica was sufficient to decrease BOC in the PHC- extracts to acceptable levels (i.e nearly full removal of BOC).

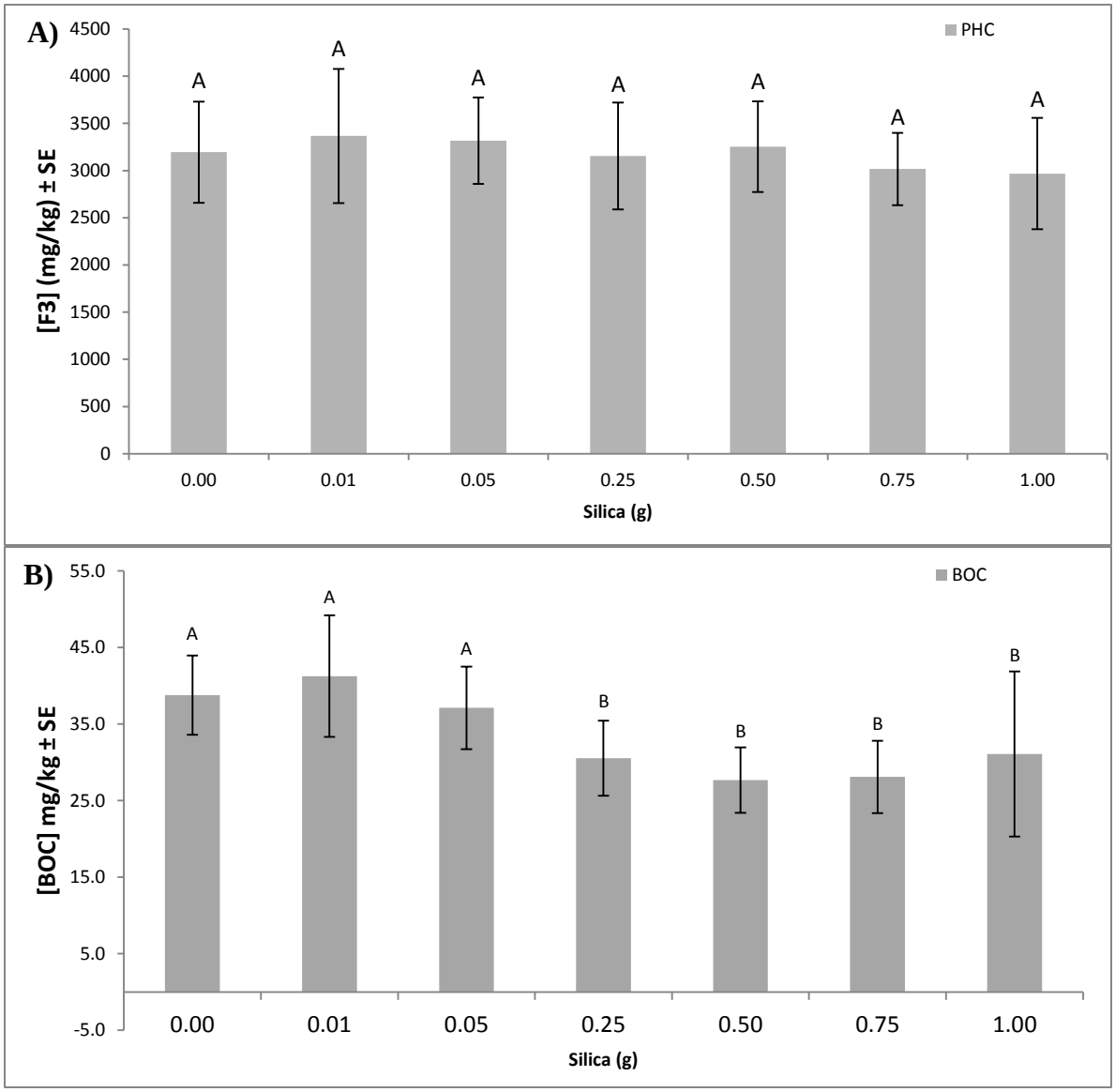


Figure 2.5: Removal of BOC from PHC-Extract of Impacted Soils Using Activated Silica

PHC-impacted soil extracted with the cold shake method using 1:1 AH solvent. After the removal of acetone and water from the PHC-extract, increasing amounts of activated silica were added *in situ*. The resultant PHC-extract was analyzed by GC-FID. **A)** The F3 fraction of PHC-extract with increasing amounts of activated silica. **B)** Suspect BOC peaks in PHC-extract with increasing amounts of activated silica. Error bars represent standard error (SE). All treatments were conducted in triplicate with three independent replicate (n=9). Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA.

2.4.2.4 GC-FID Traces of PHC Samples with Increasing Amounts of Activated Silica

In the above data (See section 2.4.2.3) increased amounts of activated silica were added into PHC-extracts to remove of BOC. The GC-FID traces of this data are shown below with suspected BOC peaks highlighted in red (Figure 2.6). The suspected BOC peaks in the GC-FID traces decreased in intensity as the amount of activated silica added into the PHC-extract increased (figure 2.6). This suggests that BOC was being removed by activated silica.

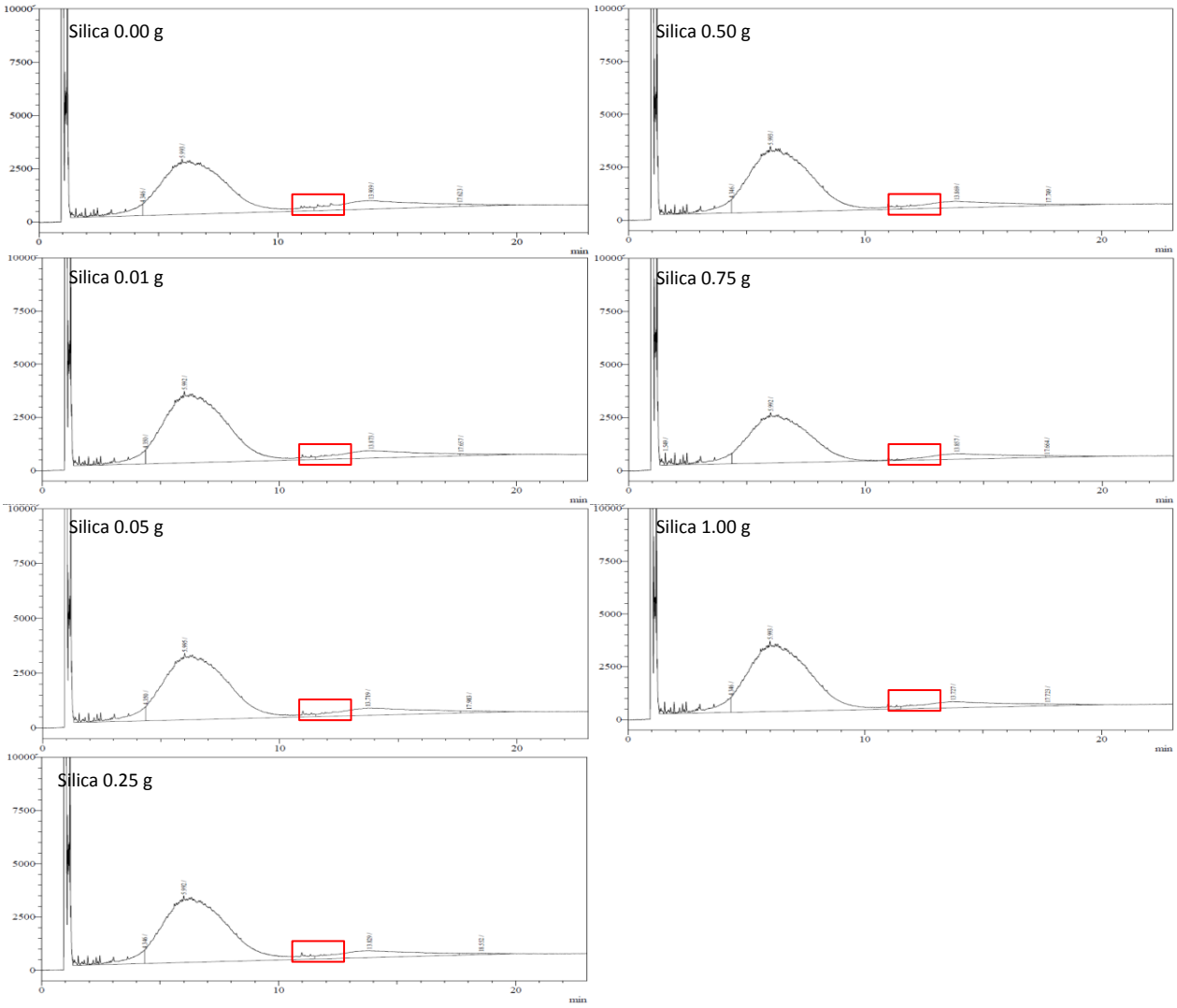


Figure 2.6: GC-FID Traces of PHC-Extract with Activated Silica.
Representative GC-FID traces of PHC-extract following removal of BOC with increasing amounts of activated silica. Red box highlights the suspected BOC peaks.

2.4.2.5 Use of Activated Silica for Removal of BOC from Extracts of Artificial Soil Spiked with PHC

To verify the effects of activated silica on removing BOCs from PHC-extracts, artificial soils spiked with known quantities of PHC (1:10 diesel and motor oil) and BOC (Dried peat) were used (Figure 2.7). This eliminates the variance of BOC and PHC concentrations in weathered PHC-impacted soils. PHC were extracted from samples using the 1:1 AH solvent followed by acetone removal and addition of activated silica (silica cleanup), before being analyzed by GC-FID.

All PHC-extracts undergoing silica cleanup showed a decrease in both F2 and F3 compared to samples without silica cleanup (Figure 2.7). Furthermore, this decrease was more prominent in artificial soil samples containing peat. Surprisingly, silica cleanup treatment decreased F3 of both PHC+Silica and PHC+Peat+Silica samples by 27.6% ($P<0.001$) and 36.3% ($P<0.001$), respectively, compared to their counterparts without silica cleanup. This was substantially more than expected. Interestingly, these samples had roughly the same F2 concentration, suggesting that activated silica was only removing PHC from the F3 fraction. Thus, inspection of the GC-FID traces may provide more insight into the effects of activated silica on BOC and PHC in the PHC-extract (See next section).

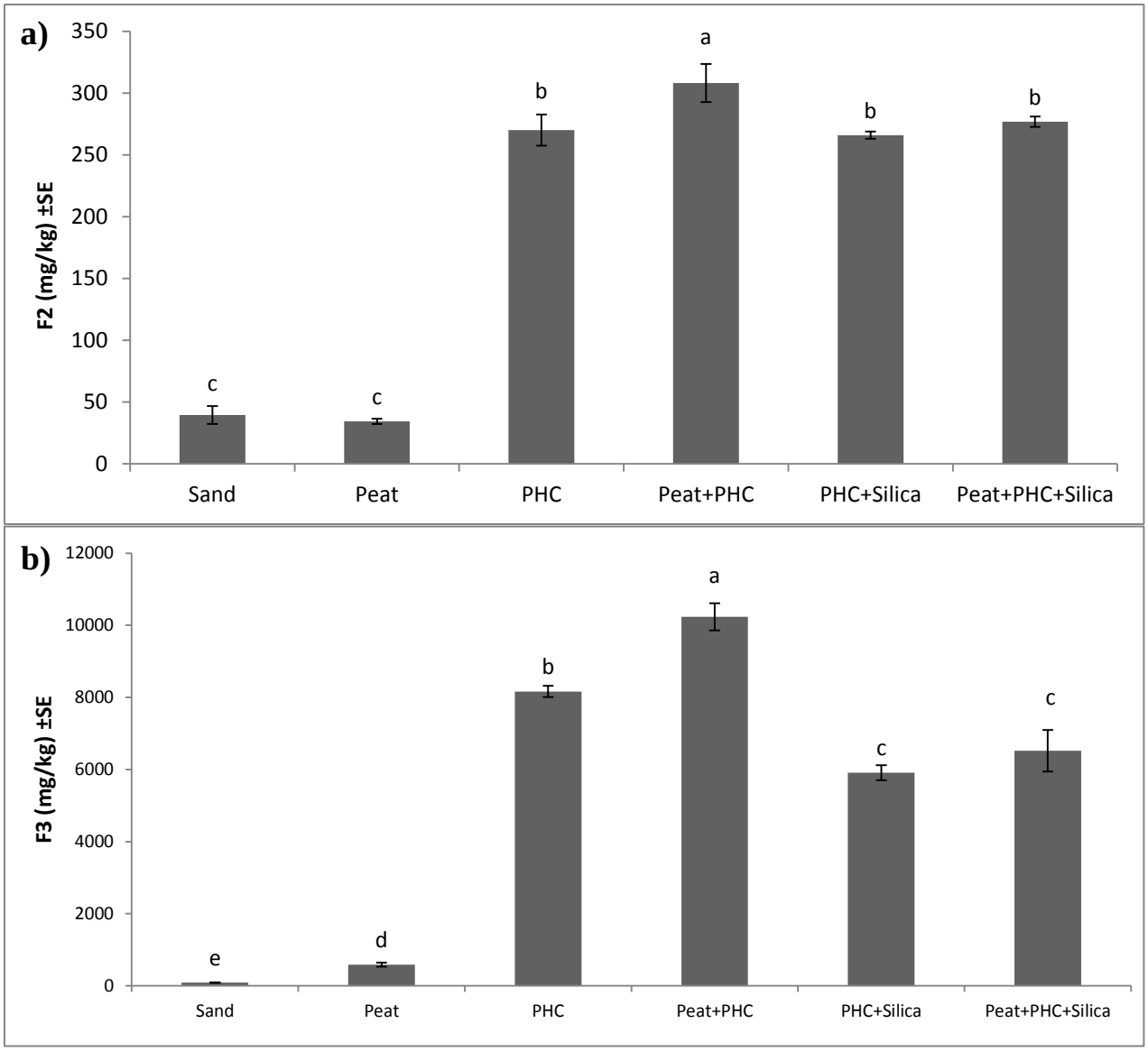


Figure 2.7: Removal of BOC from PHC-Extract of Artificial Soils Using Activated Silica.
Artificial soils samples were composed of sand, with peat and/or PHC (1:10 disel:motor) added into their respective samples. Soils were extracted with 1:1 AH using the cold shake method. The "Silica" PHC-extracts samples underwent the 1:1 AH with silica cleanup to remove BOC. **A)** The F2 concentration in soils. **B)** The F3 concentration in soils. All treatments were conducted in triplicate (n=3).Significance was detected by One-way ANOVA (α =0.05). Identical letters indicate no significant differences between treatments.

2.4.2.6 Artificial Soil GC-FID Traces

To verify the effect of activated silica on BOC removal from the PHC-extract an artificial soil experiment was conducted (See Section 2.4.2.5). The representative GC-FID traces of these PHC-extracts provide more insight into BOC removal with activated silica (Figure 2.8). The location and intensity of these BOC peaks are shown in the Figure 2.8b as the GC-FID trace from the sample only contained peat (BOC) and sand (inert). Thus, any signal from the GC-FID trace was BOC. After the addition of activated silica into the PHC-extract sample (Peat+PHC+Silica; Figure 2.8f), suspected BOC peak intensity decreased by approximately 40% compared to PHC-extract without silica (Peat+PHC; Figure 2.8d). Unexpectedly, a substantial decrease in the unresolved complex mixture (UCM) specifically the F3 region was observed, with the activated silica treatment (Figure 2.8c versus Figure 2.8e). However, no obvious decrease in the UCM of the F2 region was observed with the addition of activated silica, suggesting that activated silica was interacting with the F3 fraction (Figure 2.8c and e). It was later theorized that aromatic compounds and/or polar compounds in the motor oil spike were removed by the activated silica, resulting in the decrease in F3 (explained in detail in the disscussion). Overall, it appears that the 0.50 g of activated silica is sufficent at removing BOC from PHC-soil extract.

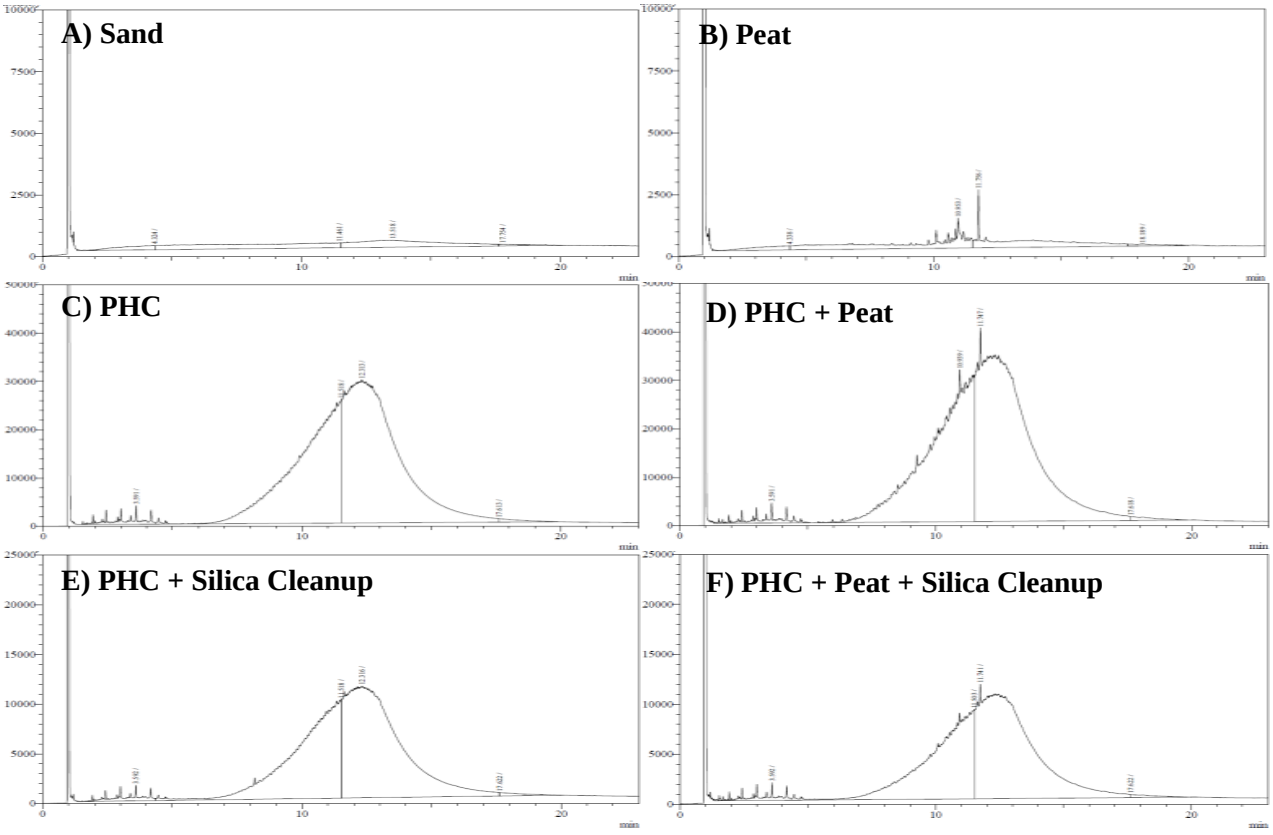


Figure 2.8: GC-FID traces of PHC-Extracts from Artificial Soils.
Representative GC-FID traces of artificial soil with various treatments**. A)** Sand, **B)** Sand + Peat, **C)** Sand + PHC, **D)** Sand + PHC + Peat **E)** Sand + PHC + Silica cleanup **F)** Sand + PHC + Peat + Silica cleanup. All samples were analyzed by GC-FID.

2.4.3.0 Extraction of PHC Using DCM Solvent

DCM was further investigated as an alternative PHC extraction solvent to 1:1 AH as it extracts less BOC and does not foul the silica (Figure 2.9). Unfortunately, unlike using 1:1 AH, PHC extraction with DCM often results in poor PHC recovery due to formation of soil aggregates caused by endogenous water in the soil. Thus, to limit the formation of soil aggregates and improve the PHC recovery from soils with DCM, additional solvent extraction steps (2x 7.50 mL and 3x 7.50 mL), and desiccating agents (Na_2SO_4 and $MgSO_4$) were tested. As well activated silica was either added directly into soils prior to solvent extraction (*in situ*) or into the PHC- extract (*ex situ*) after the DCM extraction. The concentration of PHC recovered from soil using 1:1 cleanup was used as a benchmark (represented as a red line) when comparing alternative DCM extraction methods (Figure 2.9).

Additional DCM extraction step (3x extraction vs 2x extraction) generally improved the overall PHC recovery (Figure 2.9). PHC extraction from soils with DCM was, further improved upon with addition of drying agents ($MgSO_4$ or Na_2SO_4), with Na_2SO_4 being slightly more effective than $MgSO_4$. Generally, the addition of activated silica *in situ* improved PHC recovery from soil, while *ex situ* activated silica decreased PHC recovery with respects to DCM control (2x extraction without drying agents or activated silica). Overall, the most effective DCM extraction treatments, was 3x extraction, with addition of either Na_2SO_4 or $MgSO_4$, and silica (i*n situ*). Both of these DCM treatments extracted roughly ($P>0.05$) the same amount of PHC as the 1:1 cleanup, but 3x DCM extraction with Na_2SO_4 and silica (*In situ*)(DCM+SS+Silica) was ultimately chosen as it recovered slightly more PHC than 3x DCM extraction with $MgSO_4$ and silica (*in situ*) (Figure 2.9).

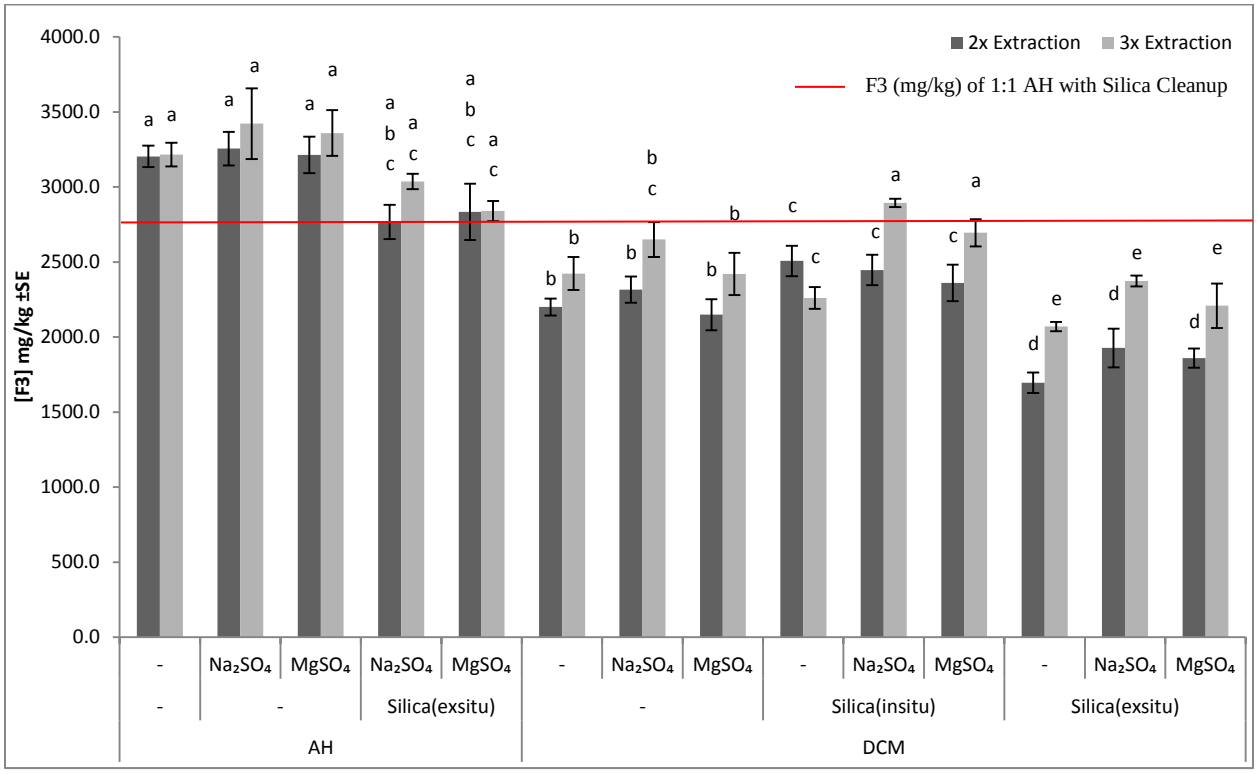


Figure 2.9: Comparison between 1:1 AH and DCM on PHC Extraction of Impacted Soils. The concentration of F3 (mg/kg) extracted from PHC-impacted soil with different combinations of solvents (1:1 AH or DCM); drying agents (Na_2SO_4 or $MgSO_4$); activated silica (*in situ* or *ex situ*), and number of extractions (2x or 3x). Red dashed line repersents the [F3] extracted with 1:1 AH 2x extraction with Na_2SO_4 (*ex situ*) silica cleanup. One way ANOVA was used to test for statistical significance (α=0.05). Error bars represent standard error. All treatments were conducted in triplicate with three independent replicate (n=9). Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA.

2.4.3.1 Comparison between DCM+SS+Silica Method and 1:1 AH with Silica Cleanup Extraction on Two Different Weathered PHC-Impacted Soil

In the previous experiment, DCM+SS+Silica was shown to be as effective at PHC extraction from soils as 1:1 AH with silica cleanup. To further test this DCM+SS+Silica method and 1:1 AH with silica cleanup were used to extract PHCs from authentic PHC-impacted soils from two other sites (Site A and B; figure 2.10). Due to the great variation in PHC concentrations in soils among sample points, the percent recovery of PHC of DCM+SS+Silica method to 1:1 AH with silica cleanup extraction was used instead. As well, the soil moisture of each sample point was compared to the PHC recovery (figure 2.10).

Generally, PHC recovery from soil when using DCM+SS+Silica method decreased as soil moisture increases (Figure 2.10). In some cases, DCM+SS+Silica PHC extraction efficiency was poor (<80.0%) despite low soil moisture (<12.0%). This was could be attributed to various soil types and PHC concentration in the samples. Between the two sites, the average PHC recovery from soil using DCM+SS+Silica method was 11.7% (P=0.013) higher in site B than site A (Figure 2.10). It should be noted that the higher moisture in site A (12.47% H_2O) likely contributed to the decreased PHC recovery from soils, compared to the drier site B (10.13% H_2O; Figure 2.10).

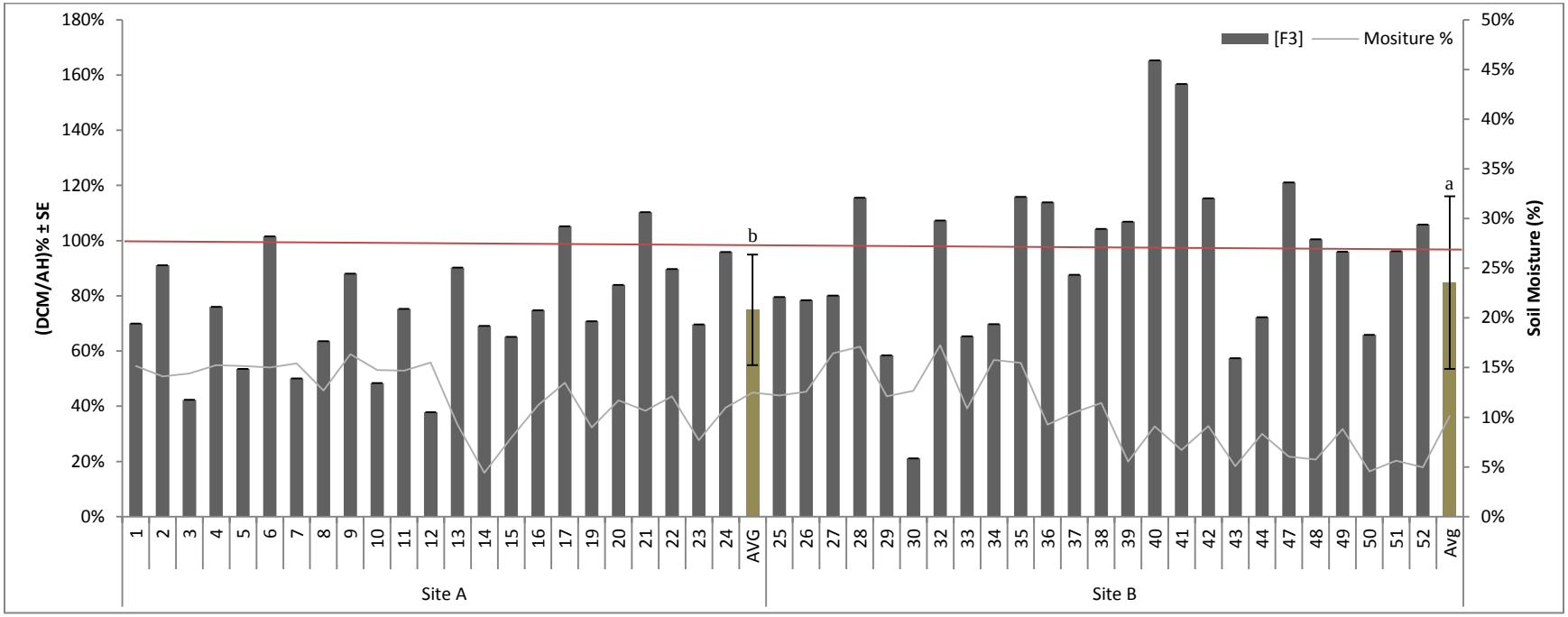


Figure 2.10: PHC Extraction between 1:1 AH with Silica Cleanup and DCM+SS+Silica Method on other PHC-Impacted Soils. Comparison between 1:1 AH with Silica cleanup and DCM+SS+Silica method with regards to PHC extraction from two separate PHC-impacted sites (site A and B). The soil moisture of each sample point within the site was also recorded. Red line repersents equal PHC extraction from soils with both 1:1 AH with silica cleanup and DCM+SS+Silica method. Identical letters indicate groups are not significantly different P>0.05 by Student t-test. Error bars repersents standard error.

2.4.3.2 The Effect of Soil Moisture on DCM+SS+Silica Method and 1:1 AH with Silica Cleanup PHC Extraction

The Figure 2.2 and 2.10, suggests that PHC recovery with DCM solvent decreases with increases in soil moisture content. To better understand the effects of soil moisture on PHC recovery by DCM+SS+Silica extraction, increasing amounts RO-H_2O (0.00, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0%) were added into dried PHC-impacted soil and the soil samples then extracted using the DCM+SS+Silica method, DCM, and 1:1 AH with silica cleanup method (Figure 2.11).

At 0.00% soil moisture DCM, 1:1 AH with silica cleanup, and DCM+SS+Silica extracted roughly the same amount of PHCs (Figure 2.11). However, as soil moisture increases, the PHC recovery from soils using DCM and DCM+SS+Silica decreases. The decrease in extracted PHC was more prominent in DCM treatments compared to DCM+SS+Silica treatments. In contrast, 1:1 AH with silica cleanup extraction was largely unaffected by increases in soil moisture, with the exception of 18.00% and 20.00% soil moisture samples which improved PHC recovery (Figure 2.11).

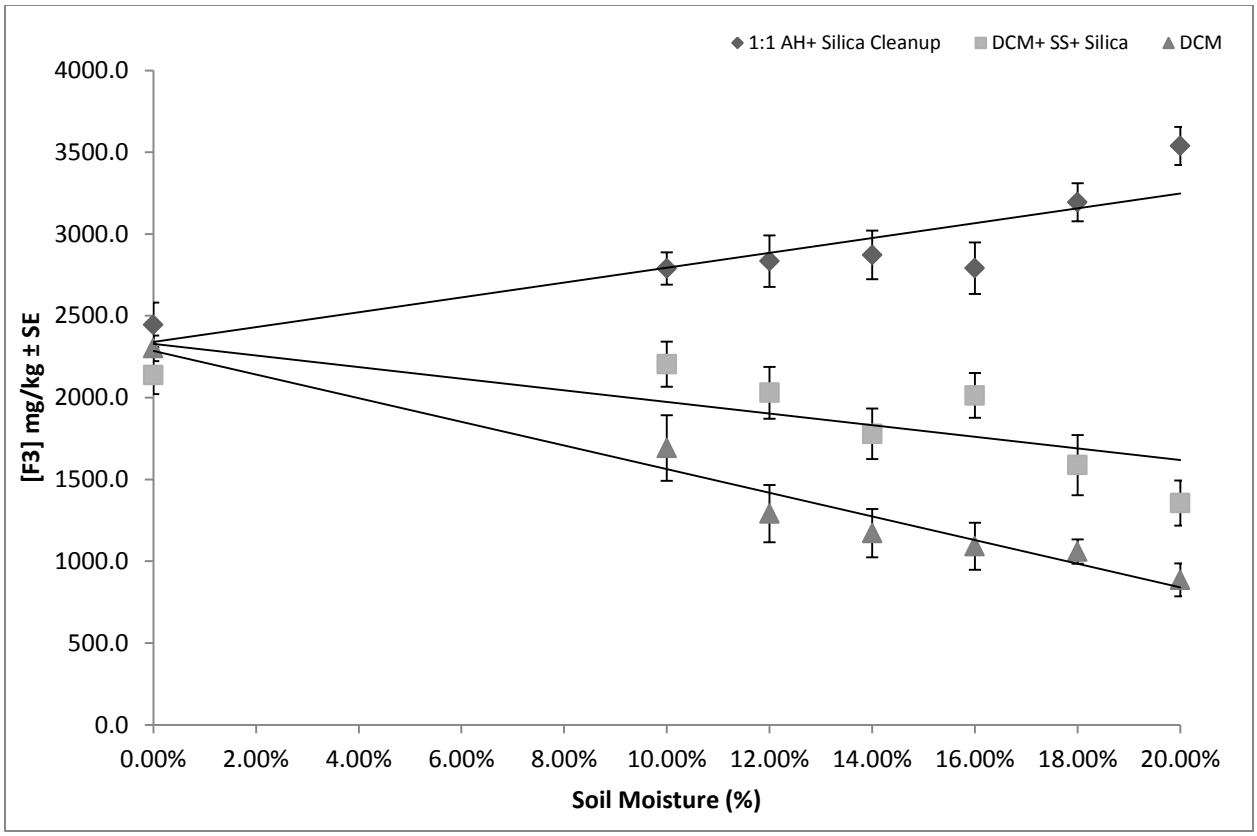


Figure 2.11: PHC Extraction of Soils with Increasing Moisture Content.
Increased amounts of water were added to dried PHC-impacted soil from site in Alberta PHC were extracted from soils using 1:1 AH with silica cleanup, DCM+SS+Silica method and cold shake methods using DCM (n=6).

2.5.0 Discussion

The CCME recommends recovering PHCs from soils using the 1:1 acetone hexane (1:1 AH) extraction solvent with the cold shake method. Unfortunately, due the polarity of acetone, BOC (if present) would be co-extracted along with PHC causing an overestimation of PHC in soils. Ideally, another solvent mixture that extracts PHC as effectively as 1:1 AH, but with less co-extraction of BOC would be preferred. Accordingly, various combinations and ratios of acetone, DCM, and hexane were explored as an alternative PHC extraction solvent. Unfortunately, it appears that polar solvents like acetone are essential for breaking up soil particles, ensuring a complete PHC extraction. Non-polar solvents (DCM and hexane), despite extracting significantly lower amounts of BOC, were not as efficient at extracting PHC from soils relative to 1:1 AH. A strategy for removing BOC from the PHC-extract is the silica cleanup method (1:1 AH with silica cleanup) developed in this chapter. DCM+SS+Silica method (also developed in this chapter) was as effective 1:1 AH silica cleanup method at extracting PHC from soil while limiting BOC co-extraction, as long as soil moisture was below 12.0%.

2.5.1.0 Cold-Shake Extraction of PHC from Soils Using Various Solvent Combination

Acetone, DCM, and hexane were used in various ratios and combinations as a PHC extraction solvent, with varying degrees of success (Figure 2.2). Acetone is a very polar solvent (dielectric constant [ϵ] =20.7; Merck Index) compared to DCM and hexane. The polarity of acetone disperses wet soil into a heterogeneous slurry, increasing the total available surface area of the soils (Semple *et al.*, 2003; Schwab *et al.,* 1999). This promoted the partitioning of the PHC from the soil into the solvent (Semple *et al.*, 2003; Schwab *et al.,* 1999). Thus, solvent mixtures that contained acetone, usually extracted the most PHCs from soils. Among these acetone solvent mixtures, the CCME recommended 1:1 AH for both extraction steps recovered the most PHC. However, 1:1 AH also extracted the most BOC from soils. Interestingly, PHC extraction that used 100% acetone for both extraction steps recovered 12.0% (P=0.059) less PHC than 1:1 AH (Figure 2.2), despite dispersing the soil into a slurry (Figure 2.3). This

indicated that a non-polar solvent in the solvent mixture is important for complete extraction of PHC from the soil.

When acetone levels in an acetone-hexane mixture were below 50%, PHC recovery from soils decreased significantly relative to 1:1 AH extraction (Figure 2.2). This decrease in PHC extraction was likely caused by formation of soil aggregates due to insufficient polarity of the solvent mixture. Images of the soils after the cold-shake extraction were consistent with this, as larger soil aggregates were observed with decreasing acetone concentrations (Figure 2.3). Thus, acetone concentrations of at least 50% are recommended. However, acetone concentrations greater than 50% co-extract significant amounts of BOC from soils, which may interfere with PHC quantification by GC-FID (Figure 2.2; Wang *et al*., 2009).

DCM is a slightly polar solvent (ϵ=9.1; Merck Index), and has limited effectiveness in preventing the formation of soil aggregates when used as an extraction solvent (Figure 2.3). The formation of soil aggregates was likely the reason that, all DCM solvent combinations that were tested extracted less ($P<0.05$) PHC than 1:1 AH (due to PHC being less available; Figure 2.2). Furthermore, the recovery of PHC from soil using DCM as the extraction solvent would likely be less effective at higher soil moistures. Hexane is a less polar solvent (ϵ=1.9; Merck Index) than DCM. Hexane extracted the lowest amount of BOC and PHC from soil (Figure 2.2). The non-polar properties of hexane limit BOC solubilisation but also significantly hindered PHC extraction, notably in high moisture soils. Again soil aggregation was likely the reason for the poor PHC extraction.

Overall, the most effective solvent for PHC extraction was the CCME recommended 1:1 AH, as it extracted the highest amount of PHC from soils, despite co-extracting the most BOCs. Therefore, to accurately quantify PHCs in soils, BOCs should be removed from the PHC-extract prior to analysis by GC-FID. This could be accomplished with the addition of activated silica into the PHC-extract.

2.5.2.0 Silica *In situ* Cleanup of PHC-Extract from Weathered PHC-Impacted Soil

Activated silica can remove polar compounds from organic solvents predominantly through hydrogen bonding and/or dipole-dipole forces (Rimola *et al*., 2013). Activated silica can bind to the polar functional groups of BOCs, removing them from PHC-extract (Wang *et al.*, 2012; Kelly-Hopper *et al*., 2012). However, to maintain silica effectiveness in sequestering BOCs, polar solvents must be removed from the PHC-extract. If polar solvents (i.e. acetone) are not removed they are likely to occupy the majority of binding sites on the silica (fouling) greatly diminishing its effectiveness. Thus, the acetone present in the samples was removed by liquid-liquid extraction with RO-H_2O (Table 2.2). The residual water in the hexane from acetone removal can also foul the silica and must be removed using 0.50 of Na_2SO_4 (Table 2.3).

After the PHC-extract is devoid of both acetone and RO-H_2O the amount of activated silica required for BOC removal was determined. Increasing amounts of activated silica (0.01, 0.10, 0.25, 0.50, 0.75, 1.00 g) were added into PHC-extract effluent. A slight but non-significant decrease in F3 was observed as the amount of silica increased (Figure 2.5a). However, when only the suspected BOC peaks from the GC-FID trace were integrated using equation 2.2 (see section 2.3.6), a significant decrease ($P<0.05$) in BOC was detected with activated silica treatments greater than 0.25 g (Figure 2.5b). The GC-FID traces supported this, as decreases in suspected BOC peaks (in the F3 region) were observed with increases of added silica (Figure 2.6). As well, for concentrations of activated silica above 0.50 g, the GC-FID traces showed a slight decrease in the UCM in areas where no significant amount BOC should be present. This suggested that activated silica at concentration of 0.75 g and 1.00 might remove some PHC from the PHC-extract. This was an unexpected result, because PHC are highly non-polar and as such should not interact with activated silica. However, it is possible that the soils analyzed in this experiment contained polycyclic aromatic hydrocarbons (PAH) which interacted with activated silica due to increased electron density within the aromatic rings (Mair and White, 1935). Alternatively, microbial biodegradation may have partially oxidized some PHC in the soil, which could now interact with activated silica (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014).

Uneven distribution of PHC throughout the soil matrix could also have impacted the results, despite vigorous attempts at mechanically homogenizing the soil in the storage container. As a result, some soil samples taken from the storage container might have had higher PHC concentration then others. This PHC variability among soil samples could account for the decrease in PHC concentration when higher amounts of activated silica (>0.50 g) were added into the PHC-extract. To limit such confounding factors, artificial soils spiked with known amounts of both PHC and BOC prior to PHC extraction and silica cleanup, were assessed to confirm the findings with authentic PHC-impacted soils.

2.5.3.0 Silica *In situ* Cleanup of PHC-Extract from Artificial Soil

Artificial soils were used instead of PHC-impacted soil, to limit variability of BOC and PHC. Known quantities of both BOC (dried peat) and PHC (1:10 Diesel: Motor Oil) were added into silica sand. The cold shake extraction with 1:1 AH was used to extract PHC from soils, followed by silica cleanup protocol (See Section 2.5.2.0) to remove any BOC from the PHC-extract.

The addition of activated silica (0.50 g) into the PHC-extracts of Peat+PHC decreased the F3 fraction by 36.0% ($P<0.001$), relative to the PHC-extracts of Peat+PHC without silica (Figure 2.7). Furthermore, the GC-FID trace of Peat+PHC+Silica PHC-extract showed approximately 40% decrease in suspected BOC peaks (Figure 2.8). Thus, the activated silica likely removed BOC from the PHC-extract. However, the magnitude of the F3 fraction decrease (36.0%) following the addition of activated silica into the PHC-extract of Peat+PHC was unexpected, as BOC only accounted for 5.74% of the total F3 fraction (Figure 2.7). Furthermore, this decrease in PHC was only observed in the F3 (composed mostly motor oil) and not the F2 (composed mostly of diesel) fraction. Likely, the motor oil contained compounds with polar functional groups that were sequestered by activated silica, thus decreasing the PHC concentration. This is supported by Kuparvera *et al*. (2012) who found that 33% of motor oil compounds contained phenol, carbonyl, and amine polar functional groups, and coinciding with the 36.0% ($P<0.001$) decrease in F3 fraction following silica treatment (Figure 2.7). Additionally, motor oil could contain aromatic hydrocarbons. Aromatic hydrocarbons have an electron dense system in the center of the carbon ring,

creating a slight negative charge on either side of the molecule. This slight negative on the molecule could interact with silica decreasing the F3 fraction (Hall *et al.*, 2009).

Overall, the addition of activated silica into PHC-extracts of Peat+PHC removed the majority of BOC, confirming the previous findings (see Section 2.5.3). The decrease in F3 fraction was likely the removal of motor oil (due to polar functional groups) in the PHC-extracts by silica. Unlike motor oil, the overwhelming majority of PHC found in nature are extremely non-polar and should not be removed by activated silica. Therefore, it is the opinion of this author that activated silica should be used to remove BOC from PHC-extracts.

2.5.4.0 DCM PHC Extraction Method with Na_2SO_4 and Activated Silica

The removal of acetone from the PHC-extract is a laborious process and a potential source of error. Ideally, an alternative solvent would be preferred. DCM would be a promising alternative solvent, as it does not foul the silica and co-extracts less BOC than acetone (CCME, 2008). However, due to DCM being hydrophobic, wet soils tend to aggregate in its presence, decreasing PHC extraction efficiency (CCME 2008; Richter 2000; Figure 2.2 and 2.3). Thus, drying agents, additional extraction steps, and activated silica were tested, in an attempt improve PHC extraction with DCM by limiting soil aggregation (Figure 2.9).

To limit the formation of soil aggregates and improve PHC extraction, soils were dried prior to extraction process with the *in situ* addition of dry agents (Na_2SO_4 or $MgSO_4$)(Figure 2.9). It was found that drying agents improved all DCM PHC extraction from soils, compared to DCM PHC extraction without drying agents ($P<0.05$). This was expected as drying agents decreased the moisture content in soils limiting the hydrophobic interactions between DCM and water in soils. As a result aggregation of soil is mitigated and PHC are more available for extraction by DCM. This was supported by the fact that drying agents had no significant effect ($P<0.05$) on PHC extraction from soil with the polar solvent (1:1 AH) where soil moisture is not a factor (Figure 2.9). In addition to drying agents, PHC extractions with DCM were further improved upon with additional extraction steps (3x extractions). This was expected as

increased volume of DCM solvent and more agitation likely aided in the breakup of soil aggregates. Therefore, it is likely that the combined effect of both drying agents and additional extraction steps increased PHC extraction with DCM (Figure 2.9).

Activated silica was added either prior (*in situ*) or after (*ex situ*) the DCM extraction to remove BOC contaminants from samples (Figure 2.9). The silica *in situ* addition extracted more PHC from soil compared to both DCM control and *ex situ* silica treatments. Likely, activated silica acted similar to a drying agent, absorbing soil moisture resulting in increased PHC extraction efficiency. Despite H_2O molecules binding to activated silica, the activated silica was still able to remove BOC from the PHC-soil extract, as shown by the decrease in suspected BOC peaks relative to 1:1 AH GC-FID trace. In comparison to *in situ* silica, the *ex situ* silica DCM extraction recovered significantly less PHC than with DCM extraction alone (Figure 2.9). This was an unexpected result, as PHC are highly non-polar and should not be removed by activated silica. Furthermore, the magnitude of the F3 decrease with *ex situ* silica DCM was not observed with 1:1 AH with silica cleanup. Thus, more research is required, to determine why *ex situ* silica decreases F3 in PHC-extract. Overall, it was determined that DCM with 3x extraction steps, with the addition Na_2SO_4 (0.50±0.01g), followed by 0.50 ± 0.01 g of *in situ* silica (DCM+SS+Silica method) was an effective alternative to 1:1 AH with silica cleanup method, as both methods extracted roughly the amount of PHC ($P=0.462$) from soils (Figure 2.9).

Despite, DCM+SS+Silica method being comparable to 1:1 AH with silica cleanup in respects to PHC extraction, it was only tested on one soil type. Thus, weathered PHC-impacted soils from two additional sites (Site A and B) were used to confirm that DCM+SS+Silica method was as effective at PHC extraction as 1:1 AH with silica cleanup (Figure 2.10). However, in most cases DCM+SS+Silica method extracted less PHC than 1:1 AH with silica cleanup from soils with higher moisture content. Possibly, the amount of Na_2SO_4 was insufficient to decrease soil moisture to a point where aggregation did not occur. This would have resulted in poor PHC extraction from soils when using DCM+SS+Silica. However, in some cases DCM+SS+Silica method extracted less PHC than 1:1 AH with silica cleanup, despite having low moisture content. This could be attributed to different soil types which affected the

PHC extraction process when using DCM+SS+Silica. As well, low PHC concentration (>700 mg/kg) in these soil samples might have skewed DCM+SS+Silica method PHC recovery when compared to 1:1 AH with silica cleanup. Overall, the DCM+SS+Silica method compared to 1:1 AH with silica cleanup method, recovered on average, 25.11% and 15.23% less PHC from site A and B, respectively (Figure 2.10). Overall, the PHC extraction efficiency was significantly higher with Site B compared to Site A when using DCM+SS+silica. It is noteworthy that, site B had lower average soil moisture (10.13%) in comparison to site A (12.47%), likely enhancing PHC recovery when using DCM+SS+Silica method (Figure 2.10).

Thus, it is apparent that soil moisture decreases DCM+SS+Silica method ability to extract PHC from soils. To further investigate this, DCM+SS+Silica, 1:1 AH with silica cleanup and DCM cold-shake extraction were used to extract PHC from soils with increasing soil moisture (Figure 2.11). In dry soils (0% moisture), no differences in PHC extraction were observed between extraction methods, suggesting that all extraction methods tested were equally effective at PHC recovery when soil moisture is not a factor. However, as soil moisture was increased, both DCM cold-shake method and DCM+SS+Silica method extract less PHC from soils. Furthermore, DCM+SS+Silica method extracted more PHC from soil compared to DCM cold-shake. This was expected, as the Na_2SO_4 used in DCM+SS+Silica method dried the soil, improving PHC extraction. In contrast, increasing soil moisture did not decrease the efficiency of the 1:1 AH with silica cleanup ability to extract PHC from soils. This was likely due to the polarity of acetone breaking apart soil aggregates. Interestingly, PHC extraction with 1:1 AH with silica cleanup appeared to increase as moisture increased (Figure 2.11). The reason for this is unknown and may merit further investigation in the future.

The DCM+SS+Silica method compared to 1:1 AH with silica cleanup method had similar PHC extraction efficiencies in soils with moistures below 10% (Figure 2.11). In soils greater than 12% moisture PHC extraction with DCM+SS+Silica method decreased. Possibly, the amount of Na_2SO_4 was insufficient to dry the soil (Figure 2.11). Stoichiometrically, 0.50 g of Na_2SO_4 should be able to remove 0.60 g of water from the soil (equivalent to 12% moisture in 5 g of soil); this would explain the decrease

in DCM+SS+Silica method PHC extraction. Furthermore, this supports data in Figure 2.10 where the DCM+SS+Silica method PHC extraction was lower in site A soil with an average moisture content of 12.47% compared to site B with an average moisture content of 10.13%. Despite DCM+SS+Silica method limitations with PHC extraction in wet soils (>12% H_2O), it is a good alternative to 1:1 AH extraction with silica cleanup as it is less labor intensive by comparison while still minimizing BOC interference (Figure 2.11). Thus, in future experiments the DCM+SS+Silica method with higher amounts of Na_2SO_4 could be used to improve PHC extraction from soils, at a ratio of 1.00 g of Na_2SO_4 per 1.20 g of water in the soil sample. Also the DCM+SS+Silica method could be used on soils from drier sites.

2.5.5 Concluding Statements

In conclusion, it was determined that the absence of soil aggregates during the cold-shake extraction maximizes PHC recovery. Generally, this is accomplished with 1:1 AH ratio of a polar solvent (i.e acetone) to a non-polar solvent (i.e. hexane). Interestingly, if soils were dried prior to PHC extractions, DCM (non-polar solvent) has the same effectiveness as 1:1 AH. Thus in the future, soils could be air dried prior to PHC extraction assuming volatile PHC fractions (F1 and F2) are not present, or if soils originated from drier sites.

The reliance on 1:1 AH extraction solvent can result in the overestimation of PHC in soil, due to the co-extraction of both BOC and PHC. To accurately quantify PHC in soils two extraction methods 1:1 AH with silica cleanup (Figure 2.12) and DCM+SS+Silica method (Figure 2.13) were developed that remove BOC from the PHC-soil extract with activated silica. Of the two aforementioned methods, the 1:1 AH with silica cleanup method is effective at most soil moisture levels. In contrast, DCM+SS+Silica method is faster and less laborious, but in wet soils (>12.0% soil moisture) PHC recovery decreases. Overall, it is the opinion of this author that DCM+SS+Silica method should only be used in place of 1:1 AH with silica cleanup in dry soils (<12.0% moisture).

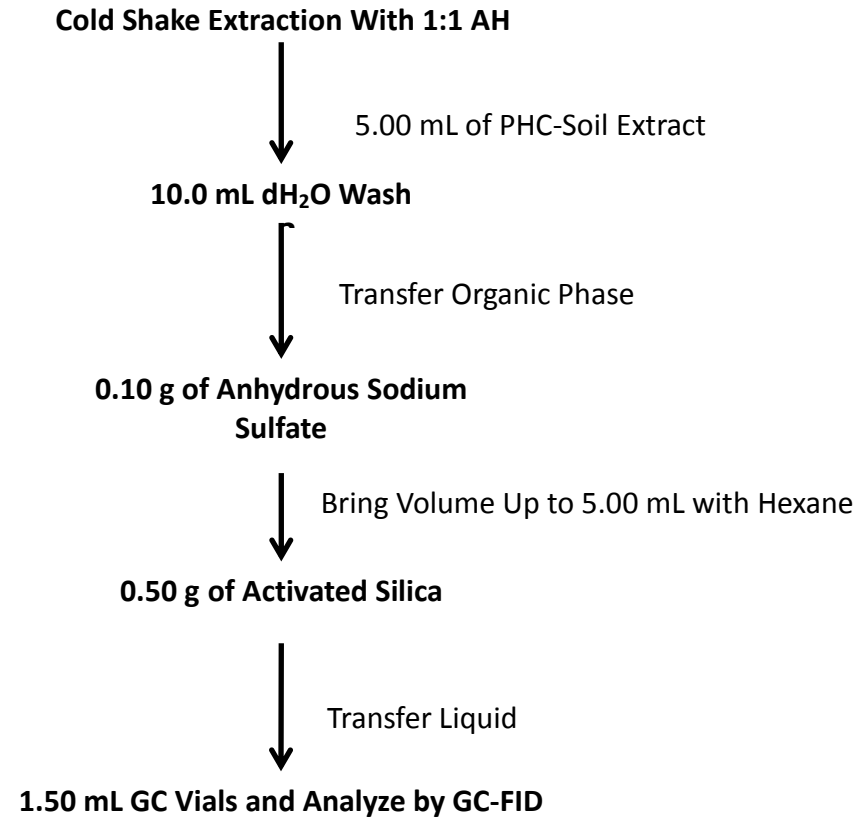


Figure 2.12: Brief Overview of 1:1 AH with Silica Cleanup Method.

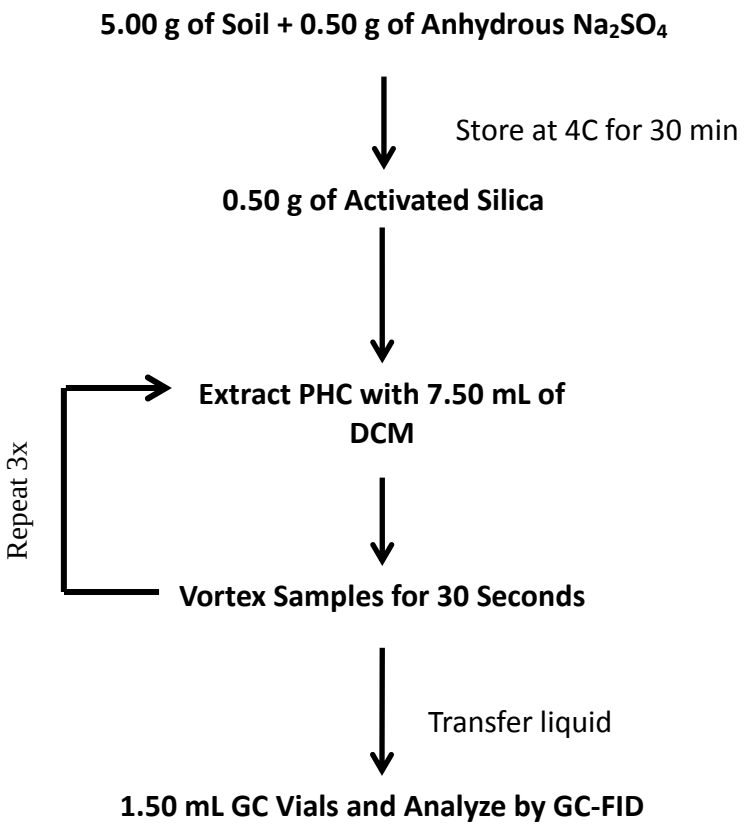


Figure 2.13: Brief overview of the DCM+SS+Silica PHC extraction method.

Chapter 3:

The Effects of Chemical Augmentation with Plant Growth Promoting Rhizobacteria (PGPR) Enhanced Phytoremediation Systems (PEPS) on the Remediation of Weathered-Petroleum Hydrocarbons in Soils.

3.1.0 Overview

Plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation systems (PEPS) use plants and their associated microbiota to remediate petroleum hydrocarbons (PHC) in soils. In addition to PHC, PEPS have also been used to remediate soils impacted with salt, metals, and other organic compounds. However, this process is generally slower compared to physical cleanup methods. PEPS might be able to be used in conjunction with chemical augmentation (CA-PEPS) to improve remediation rates of PHC contaminated soils. The two chemicals classes that could increase PEPS rates are surfactants and oxygen releasing compounds (ORCs). To test this, CA-PEPS greenhouse trials using PGPR-treated seed (*Lollium multiflorum,* annual rye grass), were performed on weathered PHC-impacted soils. Prior to PEPS application, soils were treated with increasing concentrations of surfactants (petroleum sulfonate oil; PSO) and ORCs (calcium peroxide; CaO_2). Higher PSO concentration (12.5 and 100 µL/g) did not increase PHC remediation rates ($P>0.05$), and decreased both *L. multiflorum* root and shoot dry biomass relative to PEPS. Furthermore, 100 µL/g of PSO treatment completely inhibited *L. multiflorum* germination resulting in significant decrease in PHC remediation rate. Conversely, the CaO_2 (3.33 mg/g) treatment increased the PHC remediation rate by 11.0% ($P=0.038$), as well as increasing *L. multiflorum* root and shoot dry biomass by 22.7% ($P=0.016$) and 10.6% ($P=0.086$), respectively, relative to PEPS alone.

The rates of PHC remediation in the CA-PEPS experiments were determined using the PHC extraction methods developed in the first part of this thesis (Chapter 2). The DCM+SS+Silica protocol extracted 7.80% ($p=0.038$) less PHC from soils then 1:1 AH with silica cleanup. Likely, this was due to the moisture content of the soils, preventing complete PHC extraction with DCM+SS+Silica. When soils were treated with PSO, the removal of PSO from the PHC-extract with activated silica is highly recommended. Otherwise, the GC-FID would overestimate the PHC concentration in the soils, as it cannot distinguish PSO from PHC.

3.2.0 Introduction

Phytoremediation involves the use of plants along with their associated microbiota to remove contaminants such as PHC from soils at an affordable cost (Gerdhart *et al.*, 2009; Wiltse *et al.*, 1998). Plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation systems (PEPS), can accelerate phytoremediation in soils by mitigating environmental stress to plants. With respects to petroleum hydrocarbon (PHC), a drawback of phytoremediation and PEPS is the slow rate of remediation compared to physical cleanup methods. This is most notable with weathered and/or high molecular weight (F3 and F4) PHC contaminated soils (Atlas, 1981; McGill *et al*., 1981; Mulligan *et al*., 2000). It might be possible to improve PEPS with chemical augmentation (CA-PEPS), which can accelerate PHC degradation rates in impacted soils, potentially improving its feasibility over slower traditional phytoremediation methods (Mulligan *et al*., 2000).

Surfactants and oxygen releasing compounds (ORCs) are the two most common types of chemical additives for microbial bioremediation and phytoremediation. Surfactants increase the dissolution of organic contaminants (i.e. PHC) into the aqueous phase, thereby increasing their bioavailability, and potentially accelerating phytoremediation (Volkering *et al*., 1999; Mulligan *et al*., 2001). Without surfactants, PHC molecules may remain tightly bound to soil particles limiting their bioavailability (Volkering *et al*., 1999). Surfactants are most effective where PHC bioavailability is low, such as in heavily weathered or high molecular weight PHC contaminated soils (Ron and Rosenberg, 2002; Abbasnezhad *et al.*, 2011). Potential surfactants include compounds such as rhamnolipids, sodium dodecyl sulfate, Triton X-100 (Mulligan, 2005; Volkering *et al.*, 1998; Despande *et al.*, 1999). Petroleum sulfonated oil (PSO) was selected for this study. Unlike some other surfactants, it does not have defined structure, rather it is a complex mixture of sulfonated PHC compounds ranging from F2 to F4 (Sun and Boyd, 1993).

ORCs are chemicals release molecular oxygen as they decompose. The most commonly used ORC is calcium peroxide (CaO_2), which decomposes into molecular oxygen in the presence of water (Northrup and Cassidy, 2008). CaO_2 has a longer half-life in soils compared to most other ORCs (e.g.

H_2O_2), limiting the need for reapplication into soils. With respect to CA-PEPS, ORCs can mitigate the effect of the poor gas exchange common in waterlogged soils, promoting healthy respiration of plant roots (Jackson, 1985). Furthermore, well aerated soils limit the production of phytotoxic compounds such as, hydrogen sulfide, acetic acid, oxalic acid and formic acid (Northrup and Cassidy, 2008; Sharma, 2001). ORCs should also improve the biodegradation of PHCs as the both monooxygenases and cellular respiration of aerobic microbes require molecular oxygen to function (Sierra-Garcia *et al.*, 2013).

The majority of research using chemical additives has been focused on surfactant-augmented microbial bioremediation (Volkering *et al.*, 1999; Mulligan, 2005; Sierra-Garcia *et al.*, 2013). Currently, limited research exists on the effects of ORCs and surfactants on phytoremediation. Furthermore, most of the research on remediation was conducted using bioreactors under ideal conditions, where environmental factors such as sunlight, temperature, and precipitation have minimal impact (Sun and Boyd, 1995; Cassidy and Irvine, 1999). Finally, many chemically augmented remediation experiments use freshly spiked PHC soils, which generally remediate faster than the weathered PHC-impacted soil found at authentic contaminated sites (Robertson *et al.*, 2007; Rahman *et al.*, 2003).

The objective of this investigation was to test whether PSO and CaO_2 augmented PEPS (CA-PEPS) had increased rates of PHC remediation compared to PEPS. To carry this out there were three objectives: (1) Determine effectiveness of CA-PEPS using CaO_2 and PSO, relative to PEPS with respects to accelerating remediation of weathered PHCs in soils in 28 day greenhouse trial. (2) Analyze both the chlorophyll content and dry biomass of plants used in CA-PEPS to determine if PSO or CaO_2 had beneficial or deleterious effects on plants. (3) Apply, the DCM with anhydrous sodium sulfate and silica (DCM+SS+Silica), and 1:1 acetone:hexane extraction with silica cleanup (1:1 AH with silica cleanup) (Chapter 2), in the quantification of PHC in soils from the greenhouse trial.

3.3.0.0 Materials and Methods

3.3.1.0 Weathered PHC Impacted Soil Preparation

Weathered PHC-impacted soil was collected from a site in Alberta Canada and allowed to air dry in a fume hood. Non-volatile F3 composed the majority of the PHC in the soil, thus no significant loss of product was expected. Soils were sieved through a wire mesh (2.00 cm diameter) and vigorously mixed in their respective containers to ensure homogenous distribution of PHC. Soils were then stored at room temperature in sealed containers until required.

3.3.2.0 Water Holding Capacity of Soil (WHC)

The WHC of PHC-impacted soil was determined following the protocol outlined by Environment Canada (2007). Filter paper circle (Fisher Scientific, Ottawa, Ontario) was placed into a glass funnel and primed with 10.0 mL of deionized water. The combined mass of the wet filter paper and glass funnel was weighed on a top loading balance (OHAUS TS400, M&L Testing Equipment (1995) INC., Dundas, Ontario). Soil (100 g) for use in the CA-PEPS experiment was dried at 103°C in an oven. A slurry of 100 g dried soil and 100 mL of deionized water was added to the glass funnel. Excess water was allowed to drain into a 250 mL Erlenmeyer flask. The top of the soil filled glass funnel was covered in aluminum foil to prevent evaporation. After 4 hours the combined mass of wet soil, glass funnel, and the filter paper was weighed on a top loading balance. The WHC was then determined using equation 3.1 as outlined by Environment Canada (2007).

$$WHC = \frac{F-I}{Mass\ of\ Dry\ Soil}\ x\ 100\% \qquad \textbf{Equation 3.1}$$

Where: WHC = Water Holding Capacity of Soil (%); F= Combined mass of filter paper, wet soil and funnel; I = Combined mass (g) of filter Paper, dry soil, and funnel

3.3.3.0 Effective Concentration of PSO in Soil for PHC Solubility

A PHC extraction experiment was performed to determine the concentration of PSO required for increased PHC solubility into the aqueous phase. Increasing amounts of PSO (0.0, 0.2, 12.5, 100.0, 250.0 µL/g) were added to 5.00 g of weathered PHC-impacted soil at 20% soil moisture and vortexed for 30 seconds to ensure full homogenization. Soils were covered with 5.00 mL of reverse osmosis water (RO-H_2O) and vortexed again for 30 seconds. The RO-H_2O layer above the soil (with dissolved PHC and PSO) was then transferred to a test tube where both PHC and PSO were extracted by liquid-liquid extraction with hexane (5.00 mL). PSO blanks were generated (0.0, 0.2, 12.5, 100, 250 µL/mL of hexane) to account for PSO inference with PHC quantification. All samples were analyzed using a gas chromatography (GC) -Flame ionizing detector (FID) (Shizmadu, Model 2014, Guelph, Ontario, Canada).

3.3.4.0 PGPR Seed Preparation

Lolium multiflorum (annual rye grass) seeds were coated with two PGPR strains: *Pseudomonas sp.* UW3 (UW3) and *Pseudomonas sp.* UW4 (UW4). UW3 and UW4 were inoculated into separate flasks containing 100 mL of tryptic soy broth (TSB) and placed on an orbital shaker at room temperature. After an incubation period of 24 hours the bacterial density was quantified by measuring the absorbance of each culture at 600 nm using a UV-2101PC UV-VIS scanning spectrophotometer. When the cultures reached late log phase (absorbance > 2.00) PGPR were isolated and re-suspended with Milli-Q-water to achieve a final absorbance of 2.0. The re-suspended UW3 and UW4 were combined with sterile 10% methylcellulose (200 mL/L of bacterial slurry) (Sigma Aldrich) and Neutral Dye Red (17.5 mL/L of bacterial slurry) (Sigma Aldrich). An aliquot of PGPR slurry (20 mL) was applied onto 2.50 L of *L. multiflorum* seeds (Ontario Seed Co., Waterloo, Ontario, Canada) using a Hege 11 Liquid Seed Treater (Wintersteiger, Saskatoon, Saskatchewan, Canada). PGPR-treated *L. multiflorum* seeds were planted the same day.

3.3.5.0 Chemically Augmented PGPR Enhanced Phytoremediation System (CA-PEPS)

Two, four-week long randomized CA-PEPS experiments (low concentration CA-PEPS and high concentration CA-PEPS) were conducted in a greenhouse using CaO_2 and increasing concentrations of PSO. Both CaO_2 and PSO were vigorously mechanically mixed into soils to achieve full homogenization. Three independent replicates for both CA-PEPS experiments were conducted on weathered PHC-impacted soil to determine PHC remediation rates. The chlorophyll concentration of *L. multiflorum* leaves was measured weekly. Both *L. multiflorum* dry biomass and PHC concentration of the soil were measured at the end of the four-week CA-PEPS experiment.

3.3.5.1 CA-PEPS with Lower Concentrations of PSO and CaO_2

In the first of the CA-PEPS experiments, PHC-impacted soil (150 ± 1.00 g) was added to six-inch plastic pots along with various combinations of reagent grade CaO_2 (CAS# 78403-22-2, Sigma Aldrich, Oakville, Ontario, Canada) and PSO (Frac Rite Environmental Ltd, Calgary, Alberta, Canada)(Table 3.1). The soil was then mixed and brought to water holding capacity (25% soil moisture) with addition of 40.0 mL of de-ionized water. The PGPR-inoculated *L. multiflorum* seeds (5.0 g) were planted into PHC-impacted soils and covered with approximately 0.5 cm of PHC-impacted soil. Pots were arranged in a randomized block design in the greenhouse and plants were allowed to grow for four weeks. Each treatment was repeated in triplicate with three independent replicates (n=9).

Table 3.1: Low Concentrations of PSO and CaO_2 CA-PEPS Treatments.

Plants	CaO_2 (mg/g)	PSO (µL/g)
No Plants	0.00	0.00
		0.07
		0.20
		0.60
		1.80
	0.33	0.00
		0.07
		0.20
		0.60
		1.80
L. multiflorum	0.00	0.00
		0.07
		0.20
		0.60
		1.80
	0.33	0.00
		0.07
		0.20
		0.60
		1.80

During CA-PEPS experiments, pots were watered daily to maintain water holding capacity (25% soil moisture) in soils and were rotated daily to reduce environmental variability within the greenhouse.
In addition 15.0 mL of 20:20:20 (N:P:K) fertilizer dissolved in water (10.00 ±0.01 g of fertilizer per liter of water) was added to all pots.

3.3.5.2 CA-PEPS with Higher Concentrations of PSO and CaO_2

A second 4-week long CA-PEPS experiment was conducted in the greenhouse with higher concentrations of both CaO_2 and PSO compared to the experiment in Section 3.3.5.1. Weathered PHC-impacted soil (100.0 ± 0.5 g) was added to 4-inch plastic pots. Reagent grade calcium peroxide (3.33 mg/g)(CAS# 78403-22-2, Sigma Aldrich, Oakville, Ontario, Canada) and various concentrations of PSO (Frac Rite Environmental Ltd, Calgary, Alberta, Canada) were added to designated soil samples and

vigorously mechanically mixed to ensure homogenization (Table 3.2). The soil was then brought to water holding capacity with the addition of 25 mL of de-ionized water. PGPR-treated *L. multiforum* seeds (5.00g) were added into soils and covered with approximately 0.5 cm of PHC-impacted soil. Pots were arranged in a randomized block design with each block (n=3) containing each treatment (Table 3.2). Each treatment was repeated in triplicate with three independent replicates for the entire experiment (n=9).

Table 3.2: High Concentrations of PSO and CaO_2 CA-PEPS Treatments.

Plants	CaO_2 (mg/g)	PSO (µL/g)
No Plants	0.00	0.00
L. multiflorum	0.00	0.00
		0.20
		12.5
		100
	3.33	0.00
		0.20
		12.5
		100

During CA-PEPS experiments, all pots were watered daily to maintain water holding capacity (25% soil moisture) in soils and were rotated daily to reduce environmental variability within the greenhouse. In addition 15.0 mL of 20:20:20 (N:P:K) fertilizer dissolved in water (10.00 ±0.01 g of fertilizer per liter of water) was added to all pots.

3.3.5.3 Chlorophyll Analysis of *L. multiflorum*

The chlorophyll content of each treatment was measured weekly during the four week CA-PEPS experiment. Two representative whole leaves were collected from each treatment and weighed using an analytical balance. Leaf samples were then submerged in 5.0 mL of dimethylformamide (DMF) and kept in the dark at 4°C for a period of 5 days or until chlorophyll was fully extracted from leaves. After this

period the absorbance of the chlorophyll DMF extract was measured using a spectrophotometer at λ 667 and λ 647 nm (Moran, 1982). If the absorbances were greater than 0.90 samples were diluted with DMF by a factor of five. Chlorophyll content was calculated by equation 2:

Equation 3.2

$$Total\ Chl\ (\frac{g}{mL}) = 7.53\ A_{667} + 30.19\ A_{647}$$

Where: Total Chl is total chlorophyll content, A_{667} is the absorbance at 667 nm; A_{647} is the absorbance at 647 nm.

3.3.5.4 Growth of *L. multiflorum*

At the end of the four week CA-PEPS experiment, shoots and roots were harvested. Roots were washed with water to remove excess soil. Shoots and roots were dried at 50°C for a week and dry biomass was quantified using an analytical balance.

3.3.5.5 Soil Sample Collection & PHC Analysis

At the end of the four week trial, soil samples were collected from each pot from approximately 2.0 cm below the soil surface and were stored at 4°C until analysis. PHC from soil samples were extracted using either the 1:1 acetone:hexane cold-shake method with *in-situ* silica cleanup (1:1 AH with silica cleanup) or the dichloromethane with anhydrous sodium sulfate and activated silica method (DCM+SS+Silica). These methods were developed and described in chapter 2. PHC-extracts were then analyzed using a GC-2014 with FID (Shizmadu, Guelph, Ontario, Canada).

3.3.6.0 Statistical Analysis

To determine if data sets from each replicate could be combined, data were tested for normality prior to statistical testing with Z-values for kurtosis and skewness between -1.96 and +1.96; and Shapiro-Wilk test P-value greater than 0.05. ANOVA along with LSD *post hoc* tests were used to determine statistical significance (α=0.05) for chlorophyll levels, dry biomass, and PHC concentration in soils among CA-PEPS treatments. All statistical tests were conducted using IBM SPSS Statistics software.

3.4.0.0 Results

3.4.1.0 Effective Concentration of PSO in Soil for PHC Solubility

PHC-impacted soils were treated with increasing amounts of PSO (0.0, 0.2, 12.5, 100, 250 µL/g) to determine the effective PSO concentration at solubilizing PHC from wet soils (20% moisture; Figure 3.1). Generally, PHC availability in soils increased as higher amounts of PSO were added into soils. The two highest PSO concentrations (100 and 250 µL/g) had the largest increase in PHC availability (Figure 3.1).

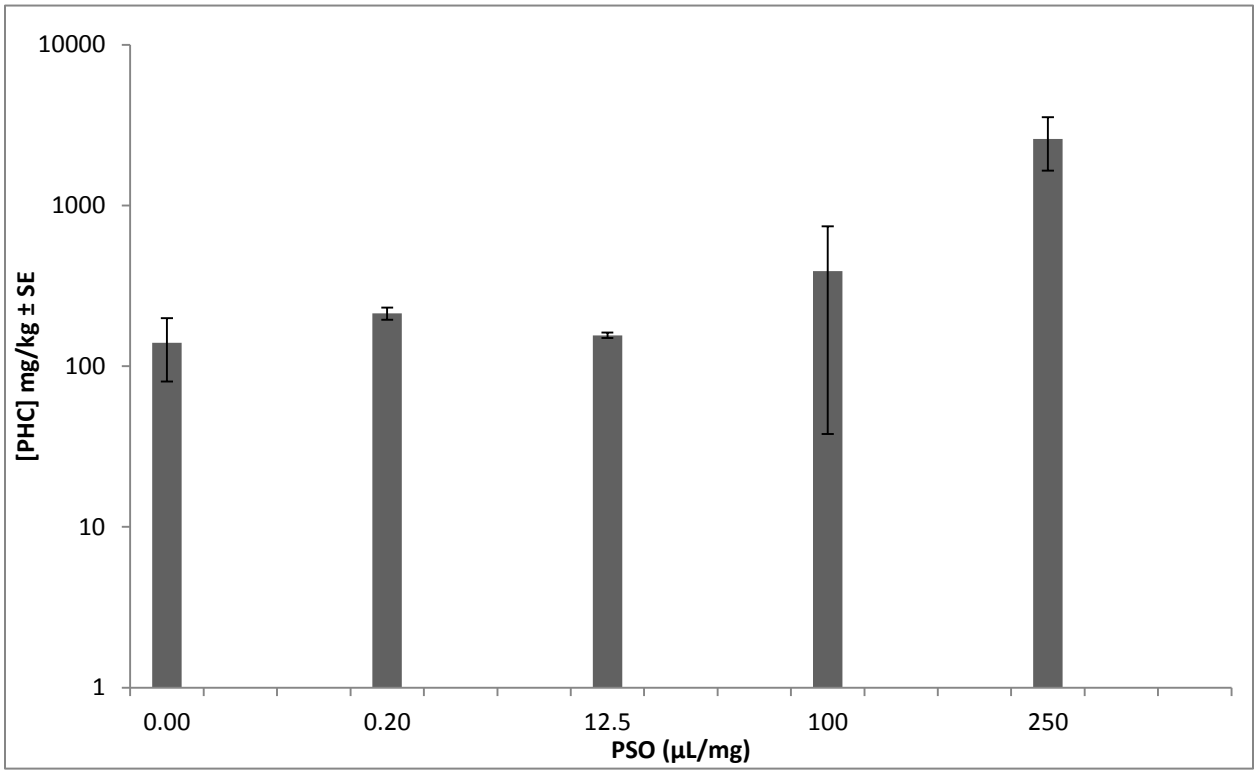
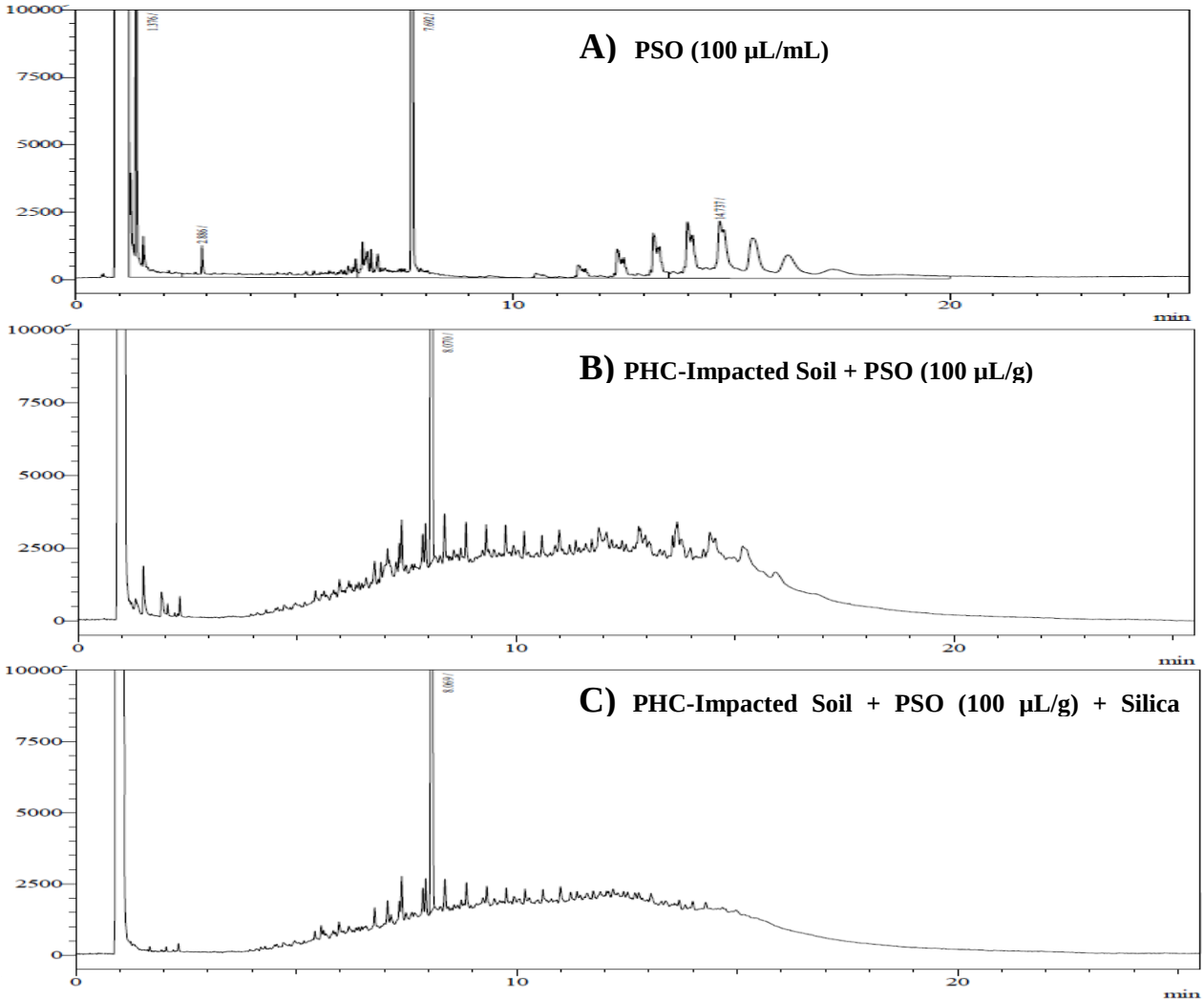


Figure 3.1: Increasing Bioavailability of PHC in Soil with PSO
PHC-impacted soil (5.00 ± 0.01 g) at 20% soil moisture was treated with increasing amounts of PSO (0.00, 0.20, 12.5, 100, 250 µL/mg of soil) in an attempt to liberate PHC adsorbed soil particles. Deionized water (5.00 ± 0.01 mL) was added to solublize any PHC in the aqueous. Hexane was used for liquid-liquid extraction for PHC in residing in the aqueous phase before being analyzed by GC-FID (n=3). Error bars repersent standard error (SE).

3.4.2.0 GC-FID Trace of PSO in PHC-Impacted Soil

PSO is mixture of various carbon compounds, and thus may interfere with the quantification of PHC by GC-FID. The GC-FID trace of PSO dissolved in hexane (100 µL/mL) had several resolved peaks of varying intensity (Figure 3.2a). Similar resolved peaks in the PHC F3 and F4 regions (likely PSO) were observed in GC-FID trace of a CA-PEPS soil sample (100 µL/g PSO treatment), after extraction with 1:1 acetone:hexane (1:1 AH) solvent (Figure 3.2b). When PHCs were extracted from the CA-PEPS soil sample using 1:1 AH with silica cleanup method, suspected PSO peaks were greatly diminished (Figure 3.2c). Thus, PSO should not interfere with PHC analysis if sample silica cleanup was employed.



The GC-FID Trace of: **A)** PSO at 100 µL/mL in 5.0 mL of acetone:hexane. **B)** 100 µL/g PSO CA-PEPS soil samples extracted with acetone:hexane coldshake method. **C)** 100 µL/g PSO CA-PEPS soil samples extracted with 1:1 AH with silica cleanup method. The large peak at 7.62 min is the internal control (o-terphenyl).

3.4.3.0 Comparison between DCM and Acetone: Hexane PHC Extraction Efficiency

PHC-impacted soils from CA-PEPS greenhouse trial were extracted using both DCM with anhydrous sodium sulfate and activated silica (DCM+SS+Silica), and 1:1 AH with silica cleanup methods. The average concentration of PHC (mg/kg) extracted from CA-PEPS soils was measured (Figure 3.3). Overall, the 1:1 AH with silica cleanup method extracted 7.80% (P=0.038) more PHC than DCM+SS+Silica method from CA-PEPS soils (Figure 3.3). Thus, only the PHC samples quantified by the 1:1 AH with silica cleanup method were used to determine the effectiveness of CA-PEPS treatments.

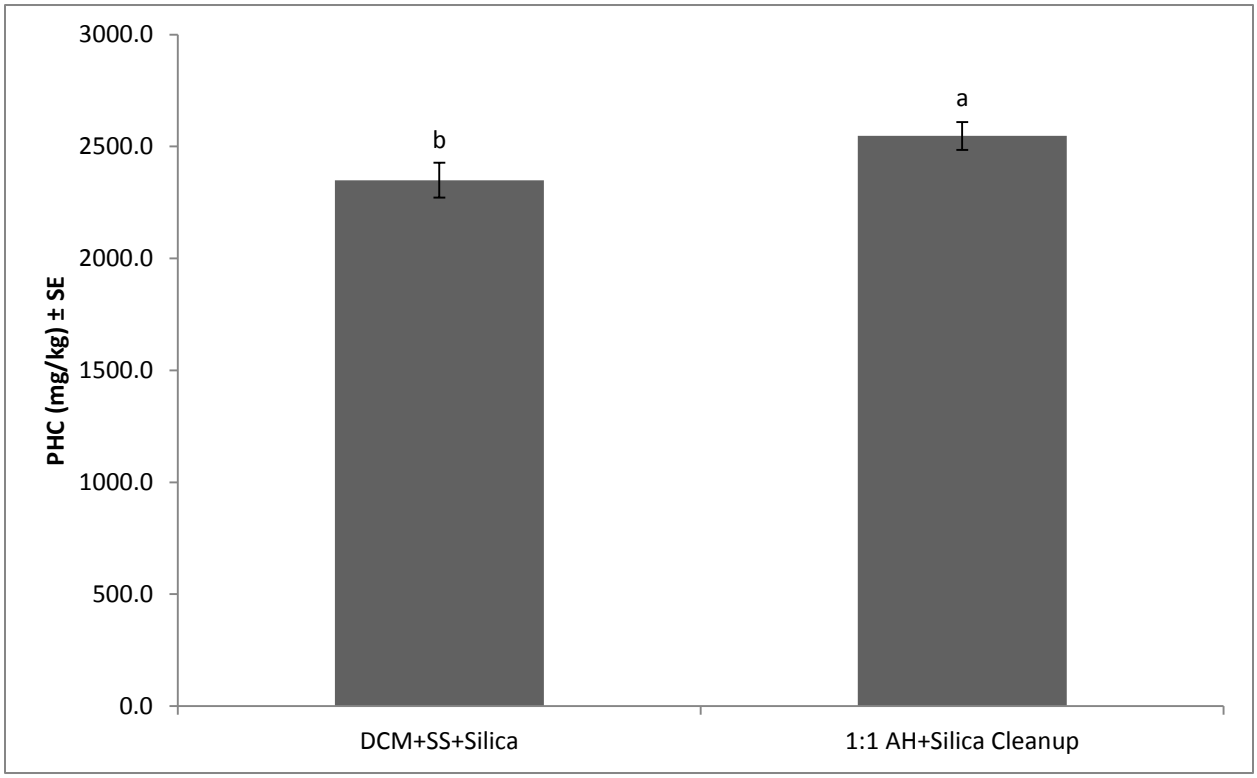


Figure 3.3: Quantification of PHC in CA-PEPS Using both 1:1 AH with Silica Cleanup and DCM+SS+Silica.

A comparison between DCM+SS+Silica and 1:1 AH with silica cleanup method on the amount of PHC extracted from all CA-PEPS soil samples (n=162). Identical letters indicate groups are not significantly different P>0.05 by Welch t-test. Error bars represent standard error.

3.4.4.0 CA-PEPS Low Concentration of PSO and CaO_2

A four week long CA-PEPS greenhouse experiment was conducted on weathered-PHC impacted soils using low concentrations of PSO (0.00, 0.07, 0.20 ,0.60, 1.80 µL/g of soil) and CaO_2 (0.00 and 0.33 mg/g of soil). Lower PSO concentrations would be preferred due to the decreased cost of treating soil in PHC-impacted sites. The amount of CaO_2 treatment (0.33 mg/g) was chosen based on manufacturer's recommendation. Both chlorophyll content and biomass were measured to determine the effects of PSO and CaO_2 on *L. multiflorum.* Upon completion of the experiment, the PHC concentration of soil samples were measured by both 1:1 AH with silica cleanup and DCM+SS+Silica methods to determine PHC phytoremediation rates.

3.4.4.1 Chlorophyll Content of *L. multiflorum*

Weekly chlorophyll measurements of *L. multiflorum* leaves were used to determine if CaO_2 (0.00 and 0.33 mg/g of soil) and/or PSO (0.00, 0.07, 0.20 ,0.60, 1.80 µL/g of soil) resulted in decreased chlorophyll concentrations (Figure 3.4). No significant differences in chlorophyll content were observed for any of the treatments within the same week. PSO did not appear to have a significant impact on chlorophyll content. However, CaO_2 treatment slightly increased ($P>0.05$) the chlorophyll content of *L. multiflorum* for the majority of the samples. Generally, chlorophyll concentration was the lowest in week 1 and then increased significantly in week 2. In week 4 the chlorophyll concentration in samples usually decreased relative to the week 3 chlorophyll concentrations (Figure 3.4). Note that the increase in chlorophyll levels from week 1 to week 2 was likely due to plant maturation; while the decrease in chlorophyll levels from week 3 and week 4 was likely due to plant senescence.

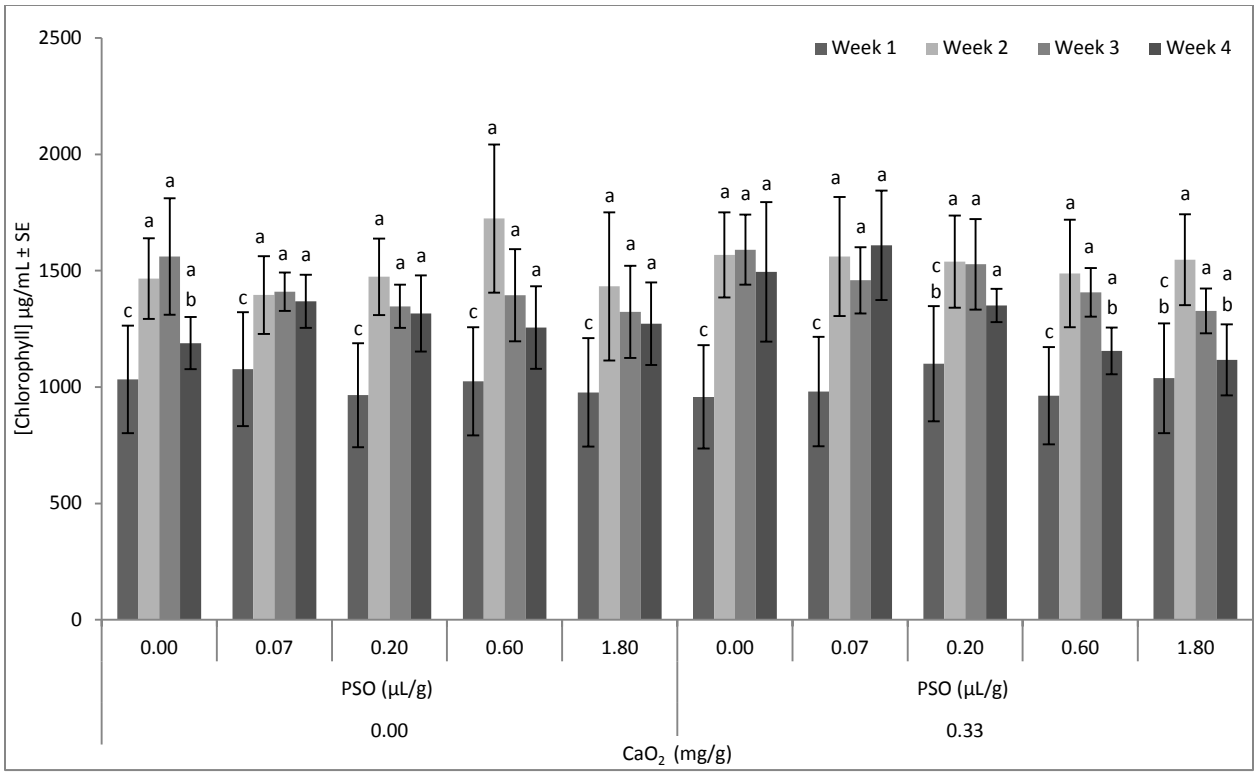


Figure 3.4: Total Chlorophyll Concentration of *L. multiflorum* from the Low Concentration CA-PEPS Experiment.

Total chlorophyll concentration for *L. multiflorum* grown in PHC-impacted soil over a four week period for the CA-PEPS experiment. chlorophyll concentration was measured each week. Each sample was run in triplicate with three independent replicates (n=9). 2 outliers were detected by IQR method (g' = 1.5) and then removed from the data set. Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA. Error bars represent standard error.

3.4.4.2 Effects of Low Concentrations of CaO_2 and PSO on *L. multiflorum* Growth

The dry biomass of *L. multiflorum* was measured at the end of each four week trial to determine if PSO or CaO_2 had any effects on the plant growth (Figure 3.5). PSO treatment had minimal effect ($P>0.05$) on either the root or shoot biomass of *L. multiflorum.* Generally, CaO_2 treatment resulted in a slight increase in *L. multiflorum* root biomass, while having no effect on *L. multiflorum* shoot biomass (Figure 3.5).

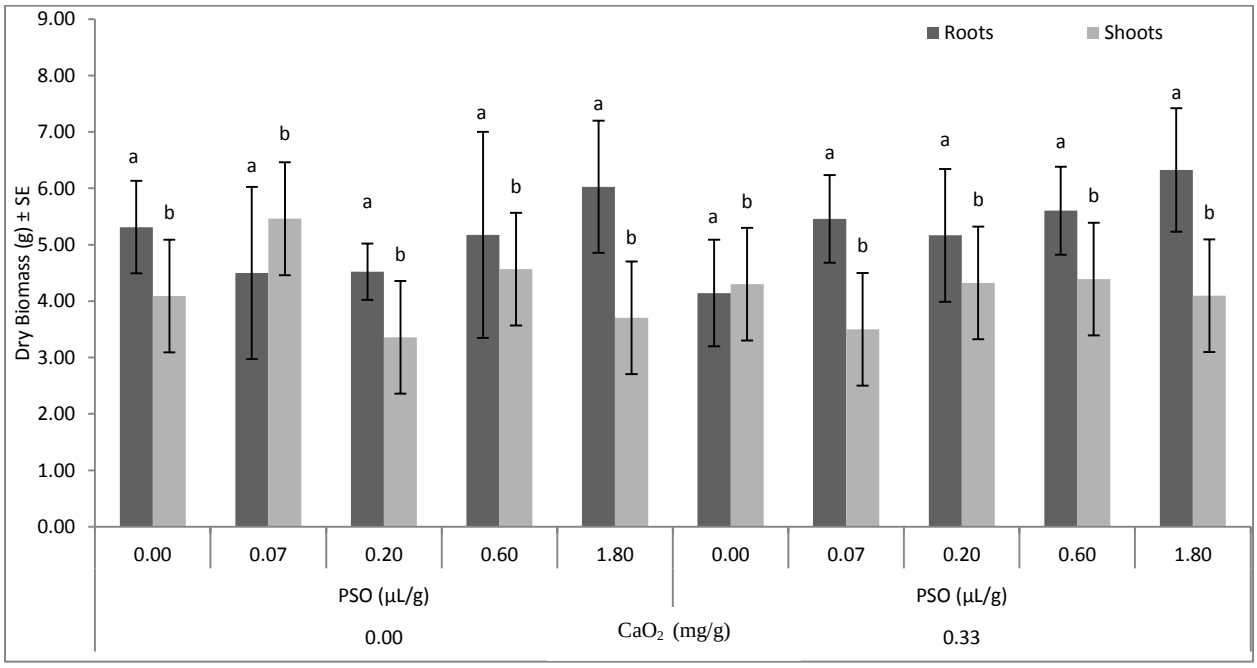


Figure 3.5: Dry Biomass of *L. multiflorum* from the Low Concentration CA-PEPS Experiment.
Dry biomass (g) of *L. multiflorum* after 28 days of growth in PHC-impacted soil with CaO_2 (0.00, 0.33 mg/g) and PSO (0.00, 0.07, 0.20, 0.60, 1.80 µL/g) treatments (n=9). Samples were run in triplicate with three independent replicates (n=9). Identical letters indicate groups are not significantly different $P>0.05$ by one-way ANOVA. Error bars represent standard error.

3.4.4.3 PHC Concentrations in Soils

After 4 weeks of plant growth in the greenhouse, soils were extracted (by 1:1 AH with silica cleanup and DCM+SS+Sillica method) and then PHC was quantified by GC-FID to determine the degree of PHC remediation. DCM+SS+Silica method extracted 1.53-11.2% less PHC among samples compared to 1:1 AH with silica cleanup (Figure 3.6b). Thus, some PHCs may still be left in soil after DCM+SS+Silica extraction, thus only 1:1 AH with silica cleanup was used to determine the degree of PHC remediation.

The initial PHC concentration in soils (t=0) was 3896.80± 219.98 mg/kg, and after undergoing PEPS treatment, soils showed 13.1% (P=0.763) remediation of PHCs (Figure 3.6a). However, none of the CA-PEPS treatments (PSO and CaO_2) showed different levels of PHC remediation. The most effective CA-PEPS treatment was 0.20 µL/g of PSO, remediating 13.8% (P=0.910) of the PHCs in soils. Conversely, the least effective treatment was 0.07 µL/g of PSO with 0.33 mg/g CaO_2, remediating 3.00% (P=0.838) of the PHCs in soils. Finally, no significant differences were detected in the PHC concentration in soils between the samples with *L. multiflorum* and samples without *L. multiflorum* (Figure 3.6a). This might be due to the short time frame of plant growth (4 weeks). As well, the soils used in this study were from a site that has been treated with PEPS for 3 years, and would have elevated levels of microbes capable of degrading PHC.

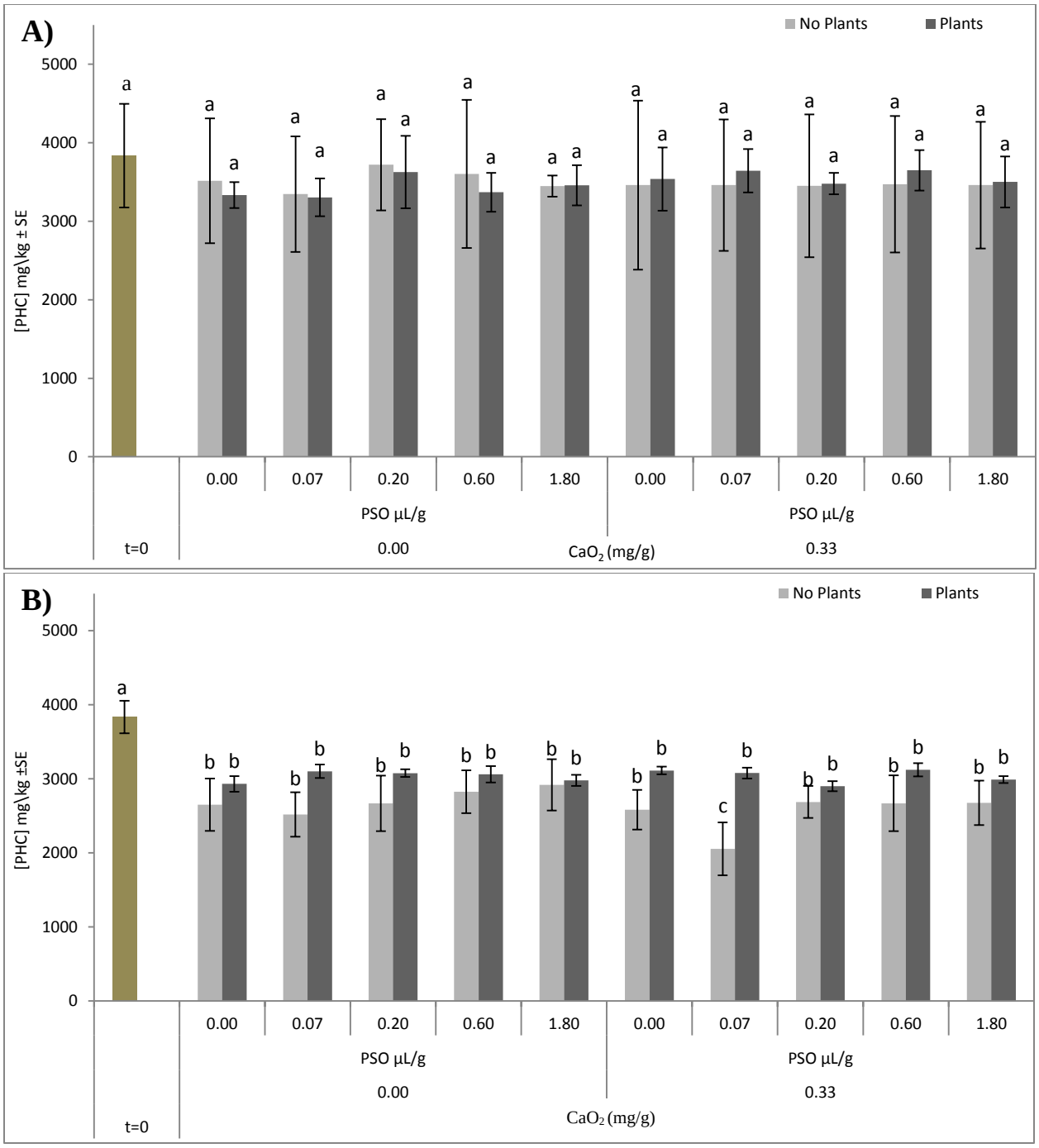


Figure 3.6: PHC Concentration in Soils at the Conclusion of Low Concentration CA-PEPS Experiment.

PHC concentrations in soil (mg/kg) after undergoing CA-PEPS for 4 weeks, treated with increasing concentrations of PSO (0.00, 0.07, 0.20, 0.60, 1.80 µL/g of soil) and CaO_2 (0.00, 0.33 mg/g of soil). t=0 represents the initial PHC concentration in soil. A) CA-PEPS soil samples extracted with 1:1 AH silica cleanup method. B) CA-PEPS soil samples extracted with DCM+SS+Silica method. Samples were run in triplicate with three independent replicates (n=9). Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA. Error bars represent standard error.

3.4.5.0 CA-PEPS with High Concentrations of PSO and CaO_2

The lack of increased PHC remediation in CA-PEPS compared to PEPS suggests that either PSO and/or CaO_2 levels were too low. A second 4-week long CA-PEPS greenhouse experiment was conducted on weathered-PHC impacted soils using higher concentrations of PSO (0.00, 0.20, 12.5, 100 µL/g of soil) and CaO_2 (0.00 and 3.33 mg/g of soil). The chlorophyll content and biomass were measured to determine the effects of PSO and CaO_2 on *L. multiflorum.* Upon completion of the experiment, plant biomass was determined and the PHC concentrations of the soils were measured by both 1:1 AH with silica cleanup and DCM+SS+Silica methods to determine PHC phytoremediation rates.

3.4.5.1 Chlorophyll Content of *L. multiflorum*

Weekly chlorophyll measurements of *L. multiflorum* leaves were used to determine if CaO_2 (0.00 and 3.33 mg/g of soil) or PSO (0.00, 0.20, 12.50, 100 µL/g of soil) resulted in altered chlorophyll levels (Figure 3.7). The soils treated with 100 µL/g of PSO had complete inhibition of seed germination, thus no chlorophyll data was collected. The PSO treatments (excluding PSO 100 µL/g) did not significantly impact chlorophyll levels of *L. multiflorum* within the same week. No significant differences ($P>0.05$) in chlorophyll content were observed between treatments (excluding 100 µL/g PSO) relative to the untreated control soil. Generally, chlorophyll content was highest in weeks 1 and 2, decreasing slightly in concentration in the following 2 weeks (Figure 3.7). Note the decrease in chlorophyll content was likely due to plant senescence.

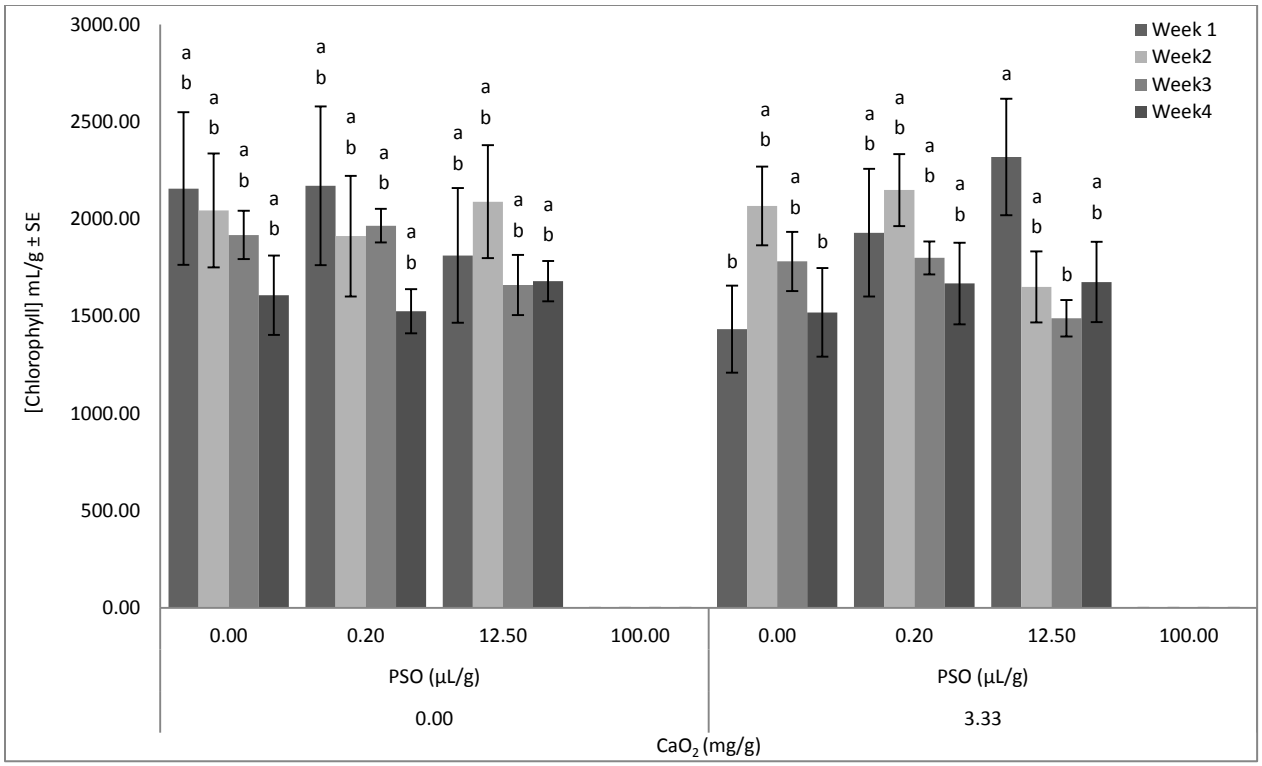


Figure 3.7: Total Chlorophyll Concentration of *L. multiflorum* from the High Concentration CA-PEPS Experiment.

Comparisons between total chlorophyll concentration in annual rye grass grown in PHC-impacted soil over a four week period in the CA-PEPS experiment. Chlorophyll concentration was tested each week. Each sample was run in triplicate with three independent replicates (n=9). 8 outliers were detected by IQR method (g' = 1.5) and then removed from the data set. Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA. Error bars represent standard error.

3.4.3.2 Effects of High Concentrations of CaO_2 and PSO on *L. multiflorum* Growth

Dry biomass measurements were taken for both the roots and shoots of *L. multiflorum* to determine the effects of PSO and CaO_2 CA-PEPS treatments on plant health. Generally, PSO had a negative impact on growth rate of both on *L. multiflorum* root and shoot biomass. Most notably, 100 µL/g PSO treatments completely inhibited *L. multiflorum* germination (Figure 3.8a). When biomass data were combined based on PSO treatment (0.00, 0.2, 12.5, 100 µL/g), a negative trend in biomass was observed with increasing PSO treatment (Figure 3.8c). The largest decrease in growth rate among these PSO treatment (excluding 100 µL/g PSO treatment) was at 12.5 µL/g PSO, which decreased root and shoot biomass by 18.7% (P=0.099) and 25.7% (P=0.003) respectively compared to untreated *L. multiflorum* samples (Figure 3.8b). Conversely, CaO_2 treatment improved both the root and shoot biomass of *L. multiflorum* compared to the respective *L. multiflorum* samples without CaO_2 (Figure 3.8a). When biomass data were combined based on CaO_2 concentration (0.00 and 3.33 mg), the CaO_2 treatment increased both root and shoot biomass by 22.7% (P=0.016) and 10.7% (P=0.086) respectively (Figure 3.8b).

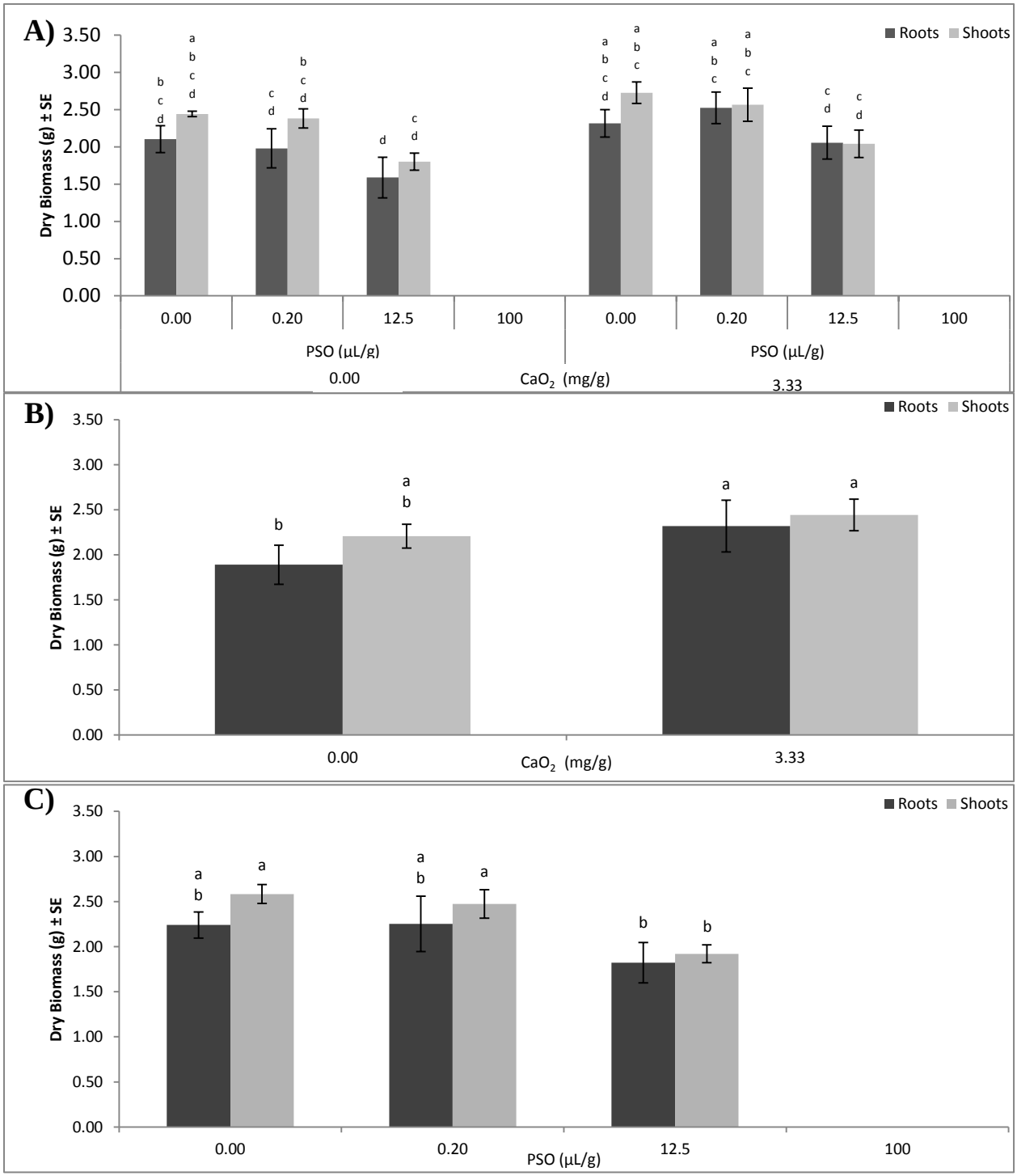


Figure 3.8: Dry Biomass of *L. multiflorum* from the High Concentration CA-PEPS Experiment.
A) Dry biomass (g) of *L. multiflorum* after 28 days of growth in PHC-impacted soil with CaO_2 (0.00, 3.33 mg/g) and PSO (0.00, 0.20, 12.5, 100 µL/g) treatments (n=9). B) Combined dry biomass data (excluding 100 µL/g PSO) of *L. multiflorum* based on CaO_2 treatment (n=27). C) Combined dry biomass data (excluding 100 µL/g PSO) of *L. multiflorum* based on PSO treatment (n=18). Each sample was conducted in triplicate with three independent replicates. Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA. Error bars represent standard error.

3.4.5.3 PHC Concentrations in Soils

After 4 weeks of plant growth, the PHC concentration in soils was quantified by 1:1 AH without silica cleanup, 1:1 AH with silica cleanup and DCM+SS+Silica method to determine the rate of phytoremediation among treatments (Figure 3.9). All samples that underwent a silica cleanup had lower PHC concentrations than samples without a silica treatment. Furthermore, higher concentrations of PSO increased the differences in PHC concentration between with silica cleanup and without silica cleanup samples, indicating that PSO contributed to BOC (Figure 3.9a). Thus, the 1:1 AH silica cleanup method was used in analyzing PHC remediation rates in CA-PEPS experiment as it likely extracted the vast majority PHCs from soils, with little BOC interference (Figure 3.9a). In comparison, DCM+SS+Silica method extracted 3.24-21.8% less PHC among samples compared to cleanup (Figure 3.9b). Thus, significant amounts of PHCs may still be left in soil after DCM+SS+Silica extraction.

The initial PHC concentration (t=0) in the soil was 3896± 219.98 mg/kg and after undergoing PEPS treatment, soils showed an average of 34.2% ($P<0.001$) remediation of PHCs (Figure 3.9a). The various CA-PEPS treatments (PSO and CaO_2) had varied results in improving PEPS (no PSO or CaO_2), remediating 23.9-40.7% ($P<0.001$) of the PHCs in soils. Generally, the PSO treatment did not significantly improve remediation of PHC, despite a slight trend in decreasing PHC concentration as PSO concentration increased from 0.00 to 12.5 µL/g ($P>0.05$). However, PSO 100 µL/g treatment (with and without CaO_2) had the smallest decrease in PHC remediation of all CA-PEPS treatments, excluding the control soil without plants. Furthermore, samples treated with 100 µL/g PSO had complete inhibition of *L. multiflorum* seeds. The use of CaO_2 resulted in increased PHC remediation in all samples compared to their respective untreated CaO_2 soil. Overall, the most effective CA-PEPS treatment was 3.33 mg/g CaO_2 remediating 40.7% ($P<0.001$) of the PHC in soils, a 10% ($P=0.036$) improvement compared to PEPS (Figure 3.9a).

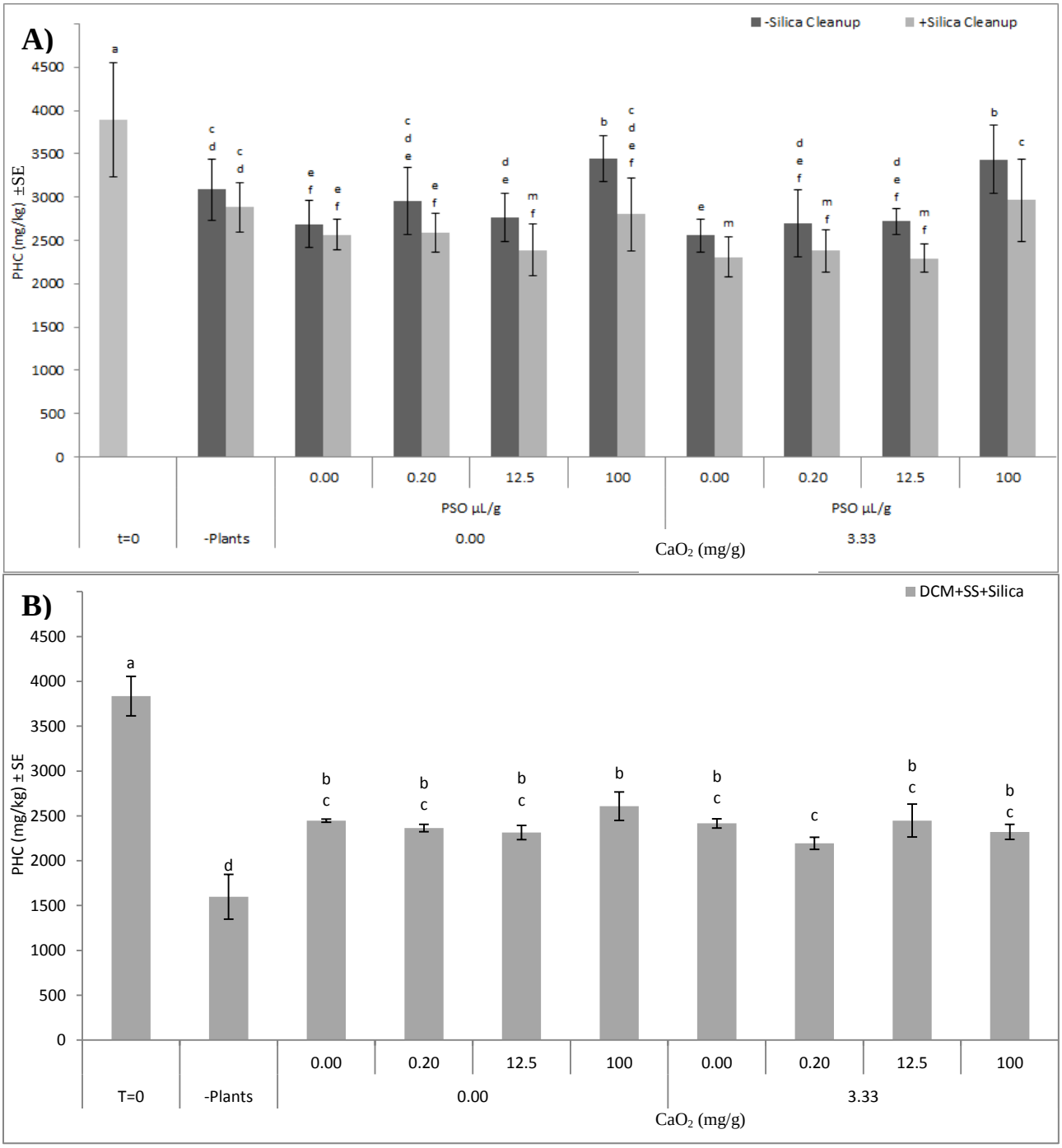


Figure 3.9: PHC Concentration in Soils at the Conclusion of High Concentration CA-PEPS Experiment.

The PHC concentration in soil (mg/kg) after undergoing CA-PEPS for 4 weeks, treated with increasing concentrations of PSO (0.00, 0.20, 12.5, 100 µL/g of soil) and CaO_2 (0 , 3.33 mg/g of soil). t=0 represents the initial PHC concentration in soil. **A)** PHC from soil samples extracted with 1:1 AH. **B)** PHC from soil samples extracted with DCM+SS+Silica. Samples were run in triplicate with three independent replicates (n=9) and analyzed by GC-FID after cold shake extraction. Identical letters indicate groups are not significantly different P>0.05 by one-way ANOVA. Error bars represent standard error.

3.5.0 Discussion

In this study an attempt was made to improve (accelerate) the rate of PHC remediation in soils undergoing PEPS using the chemical amendments PSO and CaO_2 (CA-PEPS). To measure the rates of PHC remediation, the two analytical methods (1:1 AH with silica cleanup and DCM+SS+Silica) developed in chapter 2 were used to accurately determine the PHC concentration in soils. Overall, PSO and CaO_2 chemical treatments had different effects on the chlorophyll levels and *L. multiflorum* growth rate and PHC remediation in soils. PSO CA-PEPS treatments (0.00, 0.07, 0.2, 0.60, 1.80 12.5, 100 µL/g) negatively impacted the both the growth rate of *L. multiflorum* and the rate of PHC remediation relative to PEPS. Conversely, CaO_2 CA-PEPS treatments (0.00, 0.33, 3.33 mg/g) generally improved both the growth of *L. multiflorum* and the rate of PHC remediation in soils. Most notably, the 3.33 mg/g CaO_2 CA-PEPS treatment showed an 11.0% (P=0.036) increase in the PHC remediation compared to PEPS.

3.5.1 Quantification of PHC in CA-PEPS Soils by GC-FID

Generally, soils undergoing phytoremediation or PEPS have elevated levels of biological organic compounds (BOCs) compared to traditional remediation methods. This is due to the high plant and microbial biomass required for phytoremediation and PEPS (Hooper *et al.,* 2003). Thus, BOCs could lead to an overestimation of PHCs in soil (Wang *et al*., 2009). In addition to BOCs, PSO treatments may also lead to an overestimation of PHCs due to their petrogenic origins (Figure 3.2a). Therefore, to accurately quantify PHCs in soils following CA-PEPS, the impact of both of BOCs and PSO must be accounted for (Wang *et al.,* 2009). The two analytical methods for PHC quantification developed in chapter 2 (1:1 AH with silica cleanup and DCM+SS+Silica) used activated silica to remove BOC from the PHC extract. It was hypothesized that silica could also remove PSO from the PHC-extract by binding to PSO sulfonated functional groups. This was supported by the fact that GC-FID traces, which contained PSO with PHC-impacted soil (Figure 3.2b) had significant decreases in suspected PSO peaks when the PHC-extract underwent silica cleanup (Figure 3.2c). By comparison GC-FID traces of the same samples without silica cleanup step had several large resolved peaks similar to size and shape of PSO GC-FID trace (Figure

3.2b). Thus, both 1:1 AH with silica cleanup and DCM+SS+Silica method can mitigate the inference of BOC and PSO on accurately quantify PHCs in soils.

PHC extracted from CA-PEPS soils using the 1:1 AH with silica cleanup consistently had lower PHC concentration compared to PHC extracted from soils using acetone:hexane (1:1 AH) solvent without silica cleanup (Figure 3.9a). Furthermore, the difference in PHC concentration between these two PHC analysis methods increased with higher amounts of PSO CA-PEPS treatments (Figure 3.9a). This increase in PHC when using 1:1 AH without silica cleanup was expected as both PSO and BOC likely caused an overestimation of PHCs in soils. The overestimation of PHC by PSO and BOS was supported by examination of the GC-FID traces of the soil samples. The GC-FID traces of samples that did not undergo silica cleanup showed several resolved peaks (resembling PSO) becoming more prominent with increasing PSO concentration (Figure 3.2b). By comparison GC-FID traces of soils extracted with 1:1 AH with silica cleanup did not show have these suspected PSO peaks (Figure 3.2c). Thus, the 1:1 AH with silica cleanup method could be used to accurately quantify PHC in CA-PEPS soils.

The DCM+SS+silica would be the preferred PHC extraction method as it is faster and less laborious than 1:1 AH with silica cleanup. Like 1:1 AH with silica cleanup, with DCM+SS+silica no suspected PSO or BOC peaks were observed on GC-FID traces, thus both PSO and BOC were likely removed from the PHC-extract. Unfortunately, DCM+SS+Silica extracted 7.8% (P=0.030) less PHC than the 1:1 AH with silica cleanup method (Figure 3.3). The poorer DCM+SS+Silica PHC extraction efficiency could be due to the high soil moisture (14.8%) which prevented DCM from fully extracting PHCs (as shown in chapter 2). Stoichiometrically, the amount of anhydrous sodium sulfate (0.50 g) used in the DCM+SS+Silica could remove a maximum of 0.63 gram of water from soils (12.6% soil moisture). Thus, in the future, higher amounts of anhydrous sodium sulfate could be used to improve DCM+SS+Silica PHC extraction efficiency.

3.5.2 PSO and CaO_2 Effects on Plant Vigour

PEPS is highly dependent on the plant biomass and associated microbes to effectively remediate PHC in soils. Therefore, it was essential that the both PSO and CaO_2 treatments used in CA-PEPS do not negatively impact *L. multiflorum* vigour. Thus, both biomass and chlorophyll of *L. multiflorum* were monitored to determine the effects of the PSO and CaO_2 treatments on plant vigour.

PSO is petrogenic based surfactant, and was used in this study to increase PHC bioavailability in soils. Low concentrations of PSO treatments (0.00-1.80 µL/g) had minimal effect on *L. multiflorum* shoot biomass, while slightly increasing root biomass (Figure 3.5). However, higher PSO concentrations (12.5 and 100.0 µL/g) had negative effects for both root and shoot biomass (Figure 3.8). Furthermore, the 100 µL/g PSO treatment (highest level tested) resulted in complete inhibition of *L. multiflorum* seed germination (Figure 3.8). Thus, it is highly likely that PSO had a toxic effect on *L. multiflorum*.

The composition of the PSO mixture used in this study according to the supplier (Frac Rite Environmental Ltd.) is 30-60% ethylene glycol, 1.0-5.0% isopropanol, and 10-30% petroleum sulfonate. Ethylene glycol at concentrations of 45,000 and 150,000 mg/kg in soils has been found to inhibit perennial rye grass (*Lolium perenne*) seed emergence by 97 and 100%, respectively (Pollard and Dufresne, 1999). In addition, an Environment of Canada (1995) study reported a 25% decrease in seed emergence in both radishes (*Raphanus sativa*) and lettuce (*Lactuca sativa*) when soils were treated with 5,300 to 9,000 mg/kg of ethylene glycol respectively. In this study, the 100 µL/g PSO treatment added roughly 33,300 to 66,600 mg/kg of ethylene glycol to the soil, which was concentrated enough to be phytotoxic (Pollard and Dufresne, 1999; Environment of Canada, 1995). It was also possible that seeds were inhibited by isopropanol, although not as likely as ethylene glycol. Chvapil *et al.* (1962) found total inhibition of barely grain (*Hordeum vulgare*) germination when exposed to 39,420 mg/kg of isopropanol in soils. In contrast, Chadouef-Hannel and Taylorson (1985) found no inhibition of white amaranth (*Amaranthus albus*) seed germination when treated with 36,050 mg/L of isopropanol. Therefore, it was likely that the isopropanol levels in this study (790 to 3,900 mg/kg of isopropanol in soils) were too low to inhibit germination in plants (Chvapil *et al.*, 1962; Taylorson, 1985). Petroleum sulfonates phytotoxic

effects on plants is highly dependent on the composition of the petroleum from which they were from derived, and as such information on its toxicity is limited. However, petroleum sulfonates are classified as anionic surfactants, a group of surfactants which have been shown to be phytotoxic to plants by limiting micronutrient uptake (Ewa and Maceij, 1997; Mohammed and Moheman, 2012). Mohammed and Moheman (2012) showed that when 10 mL/kg of the anionic surfactant sodium dodecyl sulfate (SDS) was added into soils, wheat biomass decreased by 50%. Despite this, it is important to take into consideration that petroleum sulfonates have vastly different chemical structures compared to SDS, thus they may not be as phytotoxic. Therefore, more research is required to determine if the petroleum sulfonates were having an effect on plants in this study. Overall, the ethylene glycol component of PSO was the most likely cause of inhibition of *L. multiflorum* germination.

CaO_2 is an oxygen releasing compound (ORC), decomposing into molecular oxygen, and thus oxygenating the soils after application (Northrup and Cassidy, 2008**;** Figure 3.10). Unlike PSO, CaO_2 treatments generally increased the overall biomass of *L. multiflorum.* The low concentration of CaO_2 (0.33 mg/g) had minimal effect ($P>0.05$) on *L. multiflorum*, only slightly increasing its roots biomass (Figure 3.6). However, the higher concentration of CaO_2 (3.33 mg/g) substantially increased both root and shoot biomass by 22.7% ($P=0.016$) and 10.7% ($P=0.086$), respectively, compared to *L. multiflorum* without CaO_2 treatment (Figure 3.8). It has been shown that CaO_2 improved total plant biomass in waterlogged soils (Sato and Maruyama, 2005; Ollerenshaw, 1984). However, limited research has been conducted on the effects of CaO_2 on plant growth in PHC-impacted soils. The improved growth rate of *L. multiflorum* in this study might have been caused by increased PGPR bacterial density due to the more aerobic environment provided by CaO_2. Furthermore, CaO_2 can increase aerobic PHC biodegradation in soils, decreasing PHC concentration in soils and limiting their adverse effects on plant growth (Shukry *et al*, 2013; Robertson *et al.*, 2007; Baker, 1970).

Alternatively, CaO_2 might be directly stimulating growth of *L. multiflorum* through the production of hydrogen peroxide (H_2O_2; Figure 3.10). H_2O_2 is known to be a plant signaling molecule involved in the mitogen activated protein kinases cascade which has numerous effects on the plant,

including alleviating abiotic stress responses and increasing cell division (Neil *et al.*, 2002; George, 2014; Orozco-Cardenas *et al.*, 1999; Potikha *et al.*, 1999). Several experiments have shown that the addition of H_2O_2 to soil increased overall plant biomass (Liu *et al.*, 2009; Guzel and Terzi, 2012; Espin *et al.*, 2010, Hameed *et al.*, 2004). At this junction, it is unclear if the improved biomass of *L. multiflorum* treated with CaO_2 was a result of increased microbial activity in soils or stimulation of growth by H_2O_2, combination of the two, or a different uncharacterized effect.

$$CaO_2 + 2\ H_2O \rightarrow Ca(OH)_2 + H_2O_2 \qquad (1)$$

$$H_2O_2 \rightarrow H_2O + O_2 \qquad (2)$$

Figure 3.10: The Reactions of CaO_2 in Soils.
Reaction 1 is production of H_2O_2 from CaO_2 and H_2O. Reaction 2 is a decomposition of H_2O_2 from reaction 1 into O_2. (Northrup and Cassidy, 2008).

In the addition to dry biomass, chlorophyll was monitored to assess the PSO and CaO_2 treatments on *L. multiflorum* health (Li *et al.*, 2011; Ramana *et al.*, 2012). The PSO treatments at either low (0.00, 0.07, 0.20, 1.80 µL/g) or high concentrations (0.00, 0.20, 12.5 µL/g) had no significant trend on *L. multiflorum* chlorophyll content (Figure 3.5 and 3.8). However, high concentrations of PSO were shown to be detrimental to *L. multiflorum* biomass, despite not affecting chlorophyll levels (Figure 3.7). This suggests that PSO (0.00-12.50 µL/g) had minimal effect on *L. multiflorum* health following germination. In future experiments, PSO could be applied to soils following seed germination.

The CaO_2 treatments at either low (0.00, 0.07, 0.20, 1.80 µL/g) or high concentrations (0.00, 0.20, 12.5 µL/g) had no impact ($P>0.05$) on *L. multiflorum* chlorophyll content (Figure 3.4 and 3.7). This was suspected as CaO_2 was found to have no toxic effect on plants, and actually resulted in increased *L. multiflorum* biomass relative to untreated control (Figure 3.8).

3.5.3 PSO and CaO_2 Effects on PHC Phytoremediation in Soils

After four weeks of plant growth, the PHC concentrations in soils were quantified (the 1:1 AH with silica cleanup and DCM+SS+Silica methods) to compare the differences in PHC remediation

between CA-PEPS and PEPS. A range of PSO and CaO_2 treatments were explored for CA-PEPS (0.00-100.0 µL/g for PSO and 0.00, 0.33, 3.33 mg/g for CaO_2).

The high concentration CA-PEPS experiment generally showed significantly higher remediation in samples planted with *L. multiflorum* compared to samples without *L. multiflorum* ($P<0.05$; Figure 3.8). However, in the low concentration CA-PEPS experiment, planted samples showed only a minor improvement in remediation ($P>0.05$) relative to unplanted samples (Figure 3.5). The latter result was surprising, as both PEPS and CA-PEPS should have higher rates PHC remediation compared unplanted samples. One explanation is that these soils contained significant amounts of PHC-degrading microbes prior to the CA-PEPS experiment. This was possible as soils used in this study had been previously treated with PEPS for 3 years in the field, and likely contained elevated levels of PHC-degrading microbes (Pilon-Smits, 2005; Dotanyia *et al*., 2015). Therefore, the rhizosphere effect in PEPS would have had minimal effect on increasing remediation rates relative to unplanted soil.

PSO was theorized to improve phytoremediation rates in soils by increasing PHC bioavailability to microbes. Unexpectedly, PSO in low concentrations (0.07-1.80 µL/g) had the opposite effect, slightly decreasing ($p<0.05$) PHC remediation rate compared to PEPS soils (Figure 3.6). The decrease in remediation rate could be the result of microbes using the more bioavailable PSO as preferred carbon source compared to PHC. As well the lack of increased PHC remediation suggests that PSO concentrations (0.07-1.80 µL/g) were insufficient in improving PHC bioavailability (Figure 3.6). This is supported by Sun and Boyd (1995) who found that substantially higher concentrations of PSO (20 – 200 mg/L) were required to increase phenanthrene, naphthene, and PCB solubility within the aqueous phase. Furthermore, it was shown experimentally that only concentrations above 100 µL/g solubilized PHCs in significant quantities (Figure 3.1).

PSO at higher concentrations (12.50 and 100 µL/g in soils) showed more of an effect on PHC concentration in soils compared to low concentrations of PSO (Figure 3.9). The 12.50 µL/g PSO treatment had a 7.2% ($P=0.237$) increase in PHC remediation in soils compared to PEPS. This suggests that the 12.50 µL/g PSO treatment was sufficient for increasing the PHC bioavailability. Unfortunately,

higher PSO concentration (100 µL/g) decreased PHC remediation in soils (P=0.103) compared to the untreated PEPS control. This decrease in remediation was expected, as the PSO treatment (100 µL/g) completely inhibited *L. multiflorum* germination, thus the rhizosphere effect could not occur (Gerdart *et al.*, 2009; Pilon-Smits, 2005; Dotanyia *et al*., 2015). Despite being toxic to plants, it is unlikely PSO had toxic effects on PHC degrading microbes, as both 100 µL/g PSO treated soils and unplanted control soils had comparable rates of PHC remediation. If PSO was indeed toxic towards microbes, a decrease in PHC remediation relative to unplanted control soils would be expected (Figure 3.9). As well, Sun and Boyd (1993) showed even at higher concentrations (20-200 mg/L) PSO had no bactericidal effects in soil. Therefore, the low rate of PHC biodegradation in PSO (100 µL/g) treated CA-PEPS was likely due to the lack of plant growth.

CaO_2 oxygenates soils as it decomposes into molecular oxygen. This could both increase aerobic metabolism of PHC and cellular respiration by aerobic microbes (Sierra-Garcia *et al.*, 2013; Olajire and Essien, 2014). Two concentrations of CaO_2 (0.33, and 3.33 mg/g of soil) were used in CA-PEPS to improve the rate of PHC degradation. The 0.33 mg/g of CaO_2 treatment of planted soils had no significant ($p>0.05$) effect on PHC remediation in soils (Figure 3.6). Likely, the amount of CaO_2 used was too low to have an effect, as CaO_2 has been shown to improve the growth rate of aerobic microbes and stimulate bioremediation (Cassidy and Irvine, 1999; Chapman *et al.*, 1997; Davis *et al*., 1997).

The higher concentration of CaO_2 (3.33 mg/g) without PSO treatment showed 11.0% ($p=0.036$) improvement in PHC remediation rate compared to PEPS soils (Figure 3.9). Furthermore, all soils treated with both CaO_2 (3.33 mg/g) and PSO (0.02, 0.60, 12.50 excluding 100 µL/g PSO treated soils) had lower PHC concentration compared to their respective samples without CaO_2 (Figure 3.9). This is supported by Davis *et al.* (1997) who found that after 28 days, soils treated with CaO_2 (0.28% of soil mass) had a 10% ($p<0.05$) increase in PHC remediation compared to the soils without CaO_2. The increase in PHC remediation was likely a result of CaO_2 supplying oxygen required for aerobic metabolism of PHC by soil microbes (Chapman *et al*., 1997; Northrup and Cassidy *et al.*, 2008). As well, CaO_2 was shown to

significantly increased the root biomass of *L. multiflorum (*Figure 3.8), thus potentially supporting higher density of PHC-degrading microbes within the rhizosphere.

In conclusion, both PSO and CaO_2 treatments did impact the rate of PHC phytoremediation in soils when compared to PEPS. The PSO CA-PEPS is not recommended as it inhibits *L. multiflorum* seed germination and PHC phytoremediation. Furthermore, PSO CA-PEPS treatments may even be detrimental to the environment as solubilized PHC in soils could leach into groundwater. In contrast to PSO, CaO_2 CA-PEPS appears to be valid treatment as it improved both the PHC remediation rate of PEPS and the biomass of *L. multiflorum*. Thus, CaO_2 CA-PEPS might be advantageous for remediating recalcitrant PHC contaminants in soils in a shorter period of time compared to PEPS.

3.5.4 Concluding Statements

Overall, this study shows that PSO and CaO_2 treatments used in CA-PEPS can impact the rate of PHC remediation in soils. The PSO treatment with CA-PEPS is not recommended due to the inhibition of seed germination, resulting in greatly decreased remediation. As well, PSO treatment could add more PHC into soils (increasing length of remediation), as PSO is derived from PHC. However, in future CA-PEPS experiments PSO could be added to soils post seed germination, as PSO might not be toxic to matured plants according to the chlorophyll data (Figure 3.4 and 3.8). The CaO_2 treatment with CA-PEPS in contrast to PSO treatment appears to be a viable for increasing PHC remediation in soils. Furthermore, CaO_2 low cost makes it an attractive option for remediating PHC-impacted soil sites. The CaO_2 treatment (3.33 mg/g) could cost as little as \$1.50 per m^2 of soil assuming depth of 0.50 meter. As well, CaO_2 could easily be applied into soils during fertilization. In future CA-PEPS experiments, higher concentrations (>33.00 mg/g) of CaO_2 could be added into soils in an attempt to further increase PHC phytoremediation and biomass of *L. multiflorum*.

Chapter 4:

Conclusions

The Canadian Council of Ministers of the Environment (CCME) suggests strict guidelines on the allowable levels of petroleum hydrocarbons (PHC) in Canadian soils, which are then enforced on the provincial level (CCME, 2008). The current CCME protocol recommends using 1:1 ratio of acetone to hexane (1:1 AH) to extract PHC from soil followed by quantification using gas chromatography with flame ionization detector (GC-FID). However, due to the polar nature of acetone, biological organic compounds (BOC) produced by plants and microbes in soils are often co-extracted along with PHC. This co-extraction of BOC along with PHC may cause an overestimation of F3 concentration in soils (Wang *et al.*, 2009; CCME, 2008). This BOC interference is even more pronounced in soils undergoing phytoremediation or plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation systems (PEPS) due to the higher amount of biomass required (CCME, 2008; Hooper *et al.*, 2003). This investigation was divided into two sections. First was optimization of PHC quantification methods. Second was the investigation of the efficacy of chemical augmented PEPS (CA-PEPS) with respects to increasing the rate of PHC remediation in soils. The PHC remediation of CA-PEPS soils were tested using the PHC quantification methods optimized in the first part of this study.

In this research two methods were developed to accurately quantify PHC in soils: 1:1 AH with silica cleanup and DCM+SS+Silica. The 1:1 AH with silica cleanup method uses 1:1 AH solvent mixture to extract PHC from soils, followed by the removal of acetone from the PHC-extract, and then addition of activated silica into the PHC-extract to remove any BOC. Activated silica was effective at removing BOC, as the GC-FID trace of PHC-extracts from both artificial and authentic PHC-impacted soils treated with silica showed a decrease in suspected BOC peaks. A drawback of 1:1 AH with silica cleanup method is that involves many steps to remove the acetone from PHC-extract, which could be potential sources of error. However, the 1:1 AH with silica cleanup method works well with most soil moisture levels, as

acetone limits soil aggregation in the PHC extraction solvent. Overall, it is the opinion of this author that 1:1 AH with silica cleanup method should be used in PHC-impacted soils with high levels of BOC to prevent an overestimation of PHC.

The DCM+SS+Silica method uses the extraction solvent dichloromethane (DCM) along with anhydrous Na_2SO_4 to dry soils and activated silica to remove BOC from the PHC-extract. Like the 1:1 AH with silica cleanup method, DCM+SS+Silica method was effective at removing BOC from the PHC-extract. But unlike 1:1 AH with silica cleanup method, the DCM+SS+Silica method uses DCM (instead of acetone) which does not need to be removed prior to addition of activated silica (does not foul silica). Thus fewer transfer steps are needed, decreasing both time and labour required for PHC extraction. However, DCM+SS+Silica PHC extraction efficiency decreases in soil moistures greater than 12.0%, as the amount of Na_2SO_4 was likely insufficient at drying the soils. In future experiments, larger amounts of Na_2SO_4 could be used to in the DCM+SS+Silica to improve PHC extraction in wet soils (> 12.0% moisture). Furthermore, soils could potentially be dried in the future (assuming soils contain mostly F3 and F4 as no significant product loss due to volatilization is expected) prior to DCM+SS+Silica extraction to improve PHC recovery. As of now, it is the opinion of this author that DCM+SS+Silica method should only be used in place of 1:1 AH with silica cleanup in dry soils (<12.0% moisture).

Soils remediated by either PEPS or phytoremediation are generally slower than traditional physical cleanup methods (e.g. removal of soils to land fill), especially in weathered soil. In this research two chemical classes, surfactants and oxygen releasing compounds (ORCs) were investigated used in CA-PEPS to increase PHC remediation. The surfactant (petroleum sulfonate oil; PSO) CA-PEPS treatment was generally ineffective at increasing PHC remediation relative to PEPS. At high concentrations PSO had a negative impact on PHC remediation, as it completely inhibited seed germination of *L. multiflorum*. This phytotoxic effect was likely due to the concentration of ethylene glycol contained in the PSO (Environment of Canada, 1995; Pollard and Dufresne, 1999). In addition to being phytotoxic, PSO treated soils could solubilize otherwise immobile PHC in soils which could potentially reach the water table

causing health concerns. Thus, it is the opinion of this author that PSO should not be used in conjunction with PEPS or phytoremediation technologies.

Calcium peroxide (CaO_2) is a commonly used ORC, which releases molecular oxygen in the presence of water (Northrup and Cassidy, 2011). Unlike PSO, the CaO_2 CA-PEPS treatment increased both the rate of PHC remediation and plant biomass when compared to PEPS. Likely, the increase in available oxygen in the soils improved the aerobic biodegradation of PHC by soil microbes. However, the exact of mechanism of CaO_2 on increasing plant biomass is not as clear. Possibly, the more aerobic environment provided by CaO_2 increased PGPR bacterial density in soils, thus improving plant biomass (Shukry *et al*, 2013; Robertson *et al.*, 2007). Alternatively, H_2O_2 formed as a byproduct of CaO_2 decomposition, can act as signaling molecule in plants stimulating growth (Neil *et al.*, 2002; George, 2014; Orozco-Cardenas *et al*., 1999; Potikha *et al.*, 1999). Currently, it is unclear if the increased microbial activity in soils or stimulation of growth by H_2O_2, combination of the two, or a different uncharacterized effect improved the plants biomass. Thus, more research is required to determine the mechanism of CaO_2 on increasing plant biomass. Overall, it is the opinion of this author that CaO_2 treatments (3.33 mg/g) are viable in increasing PHC phytoremediation. Furthermore, the CaO_2 treatment (3.33 mg/g) could cost as little as \$1.50 per m^2 of soil assuming depth of 0.50 meter. This low cost makes it an attractive option for remediating PHC-impacted soil sites. In future CA-PEPS experiments, higher concentrations (>33.00 mg/g) of CaO_2 could be added into soils in an attempt to further increase PHC phytoremediation and biomass of *L. multiflorum*.

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