EFFECTS OF FERTILIZER AND PLANT-GROWTH PROMOTING RHIZOBACTERIA ON CLOROPHYLL LEVELS AND PETROLEUM HYDROCARBON DEGRADATION DURING PHYTOREMEDIATION

By

DARCY LODOEN

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE in
ENVIRONMENT AND MANAGEMENT

We accept this thesis as conforming to the required standard

Dr. Judith Weiler, Thesis Supervisor
Independent Consultant

Dr. Matt Dodd, Thesis Coordinator
School of Environment and Sustainability

Dr. Chris Ling, Director
School of Environment and Sustainability

ROYAL ROADS UNIVERSITY
January 2017

© Darcy Lodoen, 2017
Abstract

The objective of this research was to investigate the efficiency of phytoremediation of petroleum hydrocarbon (PHC) contaminated soils in field remediation sites. Two previously isolated strains of plant-growth promoting bacteria UW3 (*Pseudomonas* spp.) and UW4 (*Pseudomonas putida*) were used in field trials in areas supplemented with fertilizer and areas without fertilizer. Chlorophyll values and PHC Fraction 2, 3 and 4 were analyzed to determine relationships in decreasing overall PHC degradation. Overall the application of Plant Growth Promoting Rhizobacteria (PGPR) a positive and measurable effect on the degradation of PHC’s during the field tests. Statistical analysis showed that the addition of fertilizer had a positive and measurable effect on chlorophyll levels. The methodology used was cost effective and practical for use in this field trial. Since sampling data was limited due to time and budgeting constraints, further research is needed to study the effects of chlorophyll within the plant biomass and its relationship to degrading heavy end PHC’s.
# Table of Contents

Abstract ............................................................................................................................................2

List of Figures ....................................................................................................................................5

List of Tables .....................................................................................................................................6

Acknowledgements ............................................................................................................................7

Introduction ......................................................................................................................................8

  Soil Salinity Impacts on Plant Establishment and Phytoremediation ...........................................9

  Petroleum Hydrocarbon (PHC) Impacts Associated with Upstream Oil and Gas in Alberta .......9

  PHC Guidelines for Soil Protection in Alberta ..............................................................................11

  Impacts of PHC Contaminants on Plant Growth .........................................................................15

  Remediation Techniques for PHC Impacted Soils ......................................................................16

  Plant-Growth-Promoting-Rhizobacteria effects on Plant Establishment ......................................17

  Research Objectives .....................................................................................................................19

Material and Methods ......................................................................................................................21

  Growth of PGPR Cultures ............................................................................................................21

  Seed Treatment with PGPR .........................................................................................................22

  H2O2 Imbibing of Seeds ..............................................................................................................23

  Research Field Site Information .................................................................................................23

      Sundre, Alberta Field Site .......................................................................................................24

  Determination of Soil PHC Concentrations .............................................................................27

  Determination of Plant Material Chlorophyll Values .................................................................28

  Plant Root Biomass Measurement ............................................................................................30
List of Figures

Figure 1: Generic land use scenarios and their associated facilities .............................................14

Figure 2: Plant Growth Promoting Rhizobacteria schematic ........................................................20

Figure 3: Aerial photograph of the Sundre field site .....................................................................25

Figure 4: Sundre field site 2013 planting map ..............................................................................26

Figure 5: Photographs of chlorophyll extraction ...........................................................................29

Figure 6: Sundre 2013 field site sample locations ..........................................................................32

Figure 7: Above-ground dry biomass (g/m²) ..................................................................................34

Figure 8: July through October 2013 precipitation data for Sundre, Alberta ................................35

Figure 9: Sundre 2013 plant growth photographs .........................................................................38

Figure 10: Sundre 2013 plant growth photographs +Fertilizer and -Fertilizer .................................39

Figure 11: PHC Fraction 2 and 3 concentrations for July and October 2013 ...............................43

Figure 12: Linear regression graphs for samples collected from the fertilized area and unfertilized area. ..........................................................................................................................45

Figure 13: Representative GC-FID chromatograms from the land treatment area soil samples...46

Figure 14: Total chlorophyll values from each sample point collected in October 2013 within the +fertilizer and –fertilizer areas ........................................................................................................48
List of Tables

Table 1: Alberta Tier 1 Soil and Groundwater Remediation Guidelines for PHC’s in a natural land use scenario ...........................................................................................................................................................................15

Table 2: Measured PHC Fraction 2 through Fraction 4 concentrations pre-treatment with PGPR seeds and fertilizer .................................................................................................................................................................................33

Table 3: Vegetation sampling completed for each assessment point within the +fertilizer and – fertilizer areas during the mid-season assessment for plant height, plant cover and overall health rating ........................................................................................................................................................................................................36

Table 4: Vegetation sampling completed for each assessment point within the +fertilizer and – fertilizer areas at the end of season assessment for plant cover and rooting depth ..................41

Table 5: PHC Fraction 2, Fraction 3 and Fraction 4 concentration within the +fertilizer and – fertilizer supplemented sections of the treatment area .................................................................................................................................42
Acknowledgements

There are numerous people who have assisted in this thesis process. Without the help and encouragement of others, this research trial would not have been a success. Thank you to my supervisor Dr. Judith Weiler who provided immeasurable support and direction. Thank you to Kelsey Taylor and the Apache Corporation for letting me use one of their historic projects for research field trials. Thank you to Darren Cherniak and North Shore Environmental who provided me with funding and continued support. Finally, a personal thank you to my family, especially my mother, for the constant support and encouragement over the course of this program.
Introduction

Extensive contamination of groundwater and soils is occurring, and we have constructed an extensive network of commercial facilities for storage and production of contaminants of concern (Bonnieux, Carpentier, & Weaver, 1998). Anthropogenic activities such as mining and production of natural resources, modern agricultural methods, manufacturing industries, and energy production have led to negative long-term impacts on a wide variety of natural environments (Bonnieux et al., 1998). Numerous contaminants exist at these agricultural, natural and industrial sites, including organics, metals and salts (Gerhardt, Greenberg, & Glick, 2006). Examples of common and persistent organic contaminants include polychlorinated biphenyls (PCBs); explosives such as trinitrotoluene (TNT); organochlorine pesticides such as dichlorodiphenyl-trichloroethane (DDT); and petroleum compounds such as benzene, ethylbenzene, toluene and xylenes (BTEX), long-chain hydrocarbons (Fractions 1 through 4), and polycyclic aromatic hydrocarbons (PAHs).

Within Alberta, soils with excessively high levels of salt (specifically sodium chloride from produced water), BTEX, and petroleum hydrocarbons (PHCs) are often a by-product of the oil and gas industry (Gerhardt et al., 2006). Various methods of soil remediation and land reclamation have been used at sites impacted with PHC’s. Typically this is done by physical removal and sequestration of contaminated soils. Other methods for remediating PHC contaminated soil include ex-situ methods such as thermal desorption, anaerobic and aerobic bioremediation by constructing land farms, biopiles and turning the soil using alu screening buckets. Also in-situ methods are used such as chemical oxidation through groundwater monitoring systems and percolation trenches. These methods are sometimes only partially
effective and release contamination into the surrounding atmosphere or move them to another area of perceived lower risk (landfills). Phytoremediation has potential to permanently remove these contaminants efficiently and inexpensively.

Soil Salinity Impacts on Plant Establishment and Phytoremediation

Soil salinity is an issue within the environment due to its impacts on plant establishment and health. Produced water affected soils, which can occur in areas of upstream oil and gas production in Alberta, typically contain high levels of sodium and chloride. They also have higher than normal levels of calcium, magnesium, potassium and sulfate (Howes Keiffer, & Ungar, 2002). These individual ions are essential for plant growth but at high concentrations, salinity is one of the most severe environmental stresses on plants. Sodium is a concern for plant establishment because of its impacts on packing of charged soil particles. Excess sodium results in soil particles packing more tightly, disrupting the ability for air, water and root penetration into the soil (Bohn, McNeal, & O’Connor, 1985). In the case of this study, soil salinity and metals concentrations were not contaminants of concern and were therefore, not considered.

Petroleum Hydrocarbon (PHC) Impacts Associated with Upstream Oil and Gas in Alberta

In Alberta, since the discovery of oil in 1947, oil and gas exploration and production have grown steadily. It is estimated that only 12.7 percent of Alberta’s total area does not contain oil and gas potential (Sneider, 1992). More than 300,000 wells have been drilled and more than 350,000 km of pipelines have been constructed to distribute petroleum products from source to refineries and markets (Alberta Environment and Parks (AEP), 2014). Access roads, off-site sumps, pits, and batteries need to be constructed as part of wellsite development and facility function. The total land area associated with past and current upstream oil and gas development
is approximately 10,000 square kilometers (km²) or 1,000,000 hectares (ha) (2,470,000 acres (ac)). Each year, 10,000 to 15,000 new wells are drilled in Alberta and are currently being developed for oil and gas production in Alberta at a rate of approximately 120 ha (300 ac) a day (AEP, 2014).

The oil and gas industry includes several “upstream” processes where a range of site contamination can happen and significant concentrations of contaminants may be released into the environment. Upstream oil and gas industries are involved in the earlier stages of production: exploration, seismic exploration, surveys, oil and gas drilling, distribution through pipelines and some processing of unrefined oil and gas products at batteries (AEP, 2014). There are several types of other up-stream oil and gas facilities in Alberta, such as compressor stations, as well as oilfield injection and treating facilities, though battery sites are the most numerous. A battery is an upstream facility in an oil or natural gas field that collects raw oil or natural gas from one or more wells. Oil, gas and water are separated at this facility, impurities removed and the purified liquids are piped for further processing or distribution (AEP, 2015). Water may be piped to an injection well where it is disposed of into a geologic formation deep beneath the earth's surface (AEP, 2015). All these upstream processes and facilities have large potential for releasing hydrocarbon related contaminants of concern into the environment.

Elevated levels of hydrocarbon pose serious risks to the sensitive ecological environment and human health. Oil and gas site investigation, remediation, risk management and restoration is the responsibility of well operators by law; however, there is a potential risk of some operators not fulfilling their responsibilities due to their financial viability (Auditor General of British Columbia Report, 2010). Oil and gas companies have the ability to investigate and
independently remediate their contaminated sites, but costs and convenience plays a major role in the development and implementation sustainable remediation methods.

Currently a significant number of contaminated sites exist in the oil and gas industry which requires effective remediation of hydrocarbon contamination. The ongoing oil and gas exploration, development, production, and facility decommissioning will also result in additional sites requiring remediation in the future. Such hydrocarbon-contaminated soil represents a significant environmental liability at the oil and gas industrial sites. These liabilities are often passively managed at the source site due to a lack of reliable on-site remedial options and/or the high cost of transporting the contaminated soil to centralized treatment/disposal facilities. The on-site passive management does not address the ongoing environmental risks, such as contaminant exposure to terrestrial plants and animals or the potential migration of pollutants to surface and/or groundwater. The centralized treatment and disposal facilities may reduce the potential exposures, but they do not eliminate the environmental risks, particularly when no active soil remediation is completed prior to soil disposal in the landfill; therefore, the environmental risk is only being moved from an area of higher risk to the location of perceived lower risk (Auditor General of British Columbia Report, 2010).

**PHC Guidelines for Soil Protection in Alberta**

Soil is a resource that supports important ecosystem functions. Soil is relied upon as a resource to supply food and fibre, water purification, degrading waste materials, healthy forest and grassland development and a structural foundation for infrastructure (AEP, 2016). At sufficient concentrations, PHC contaminations can impair the ability of soil to perform its
imported ecosystem functions as well as pose a significant risk to ecological and human health (AEP, 2016).

PHC concentrations in soil are a cause for concern due to their impact on soil biomass and plant growth. PHC describes a mixture of organic compounds found in or derived from geological organic substances such as oil, bitumen and coal. Petroleum products released to the environment typically contain hundreds to thousands of compounds in varying proportions (Canadian Council of Ministers of the Environment (CCME), 2008). PHC contamination in the environment is a concern for a number of reasons. PHC’s pose a fire/explosion hazard due to volatility, they are toxic to some degree, lighter hydrocarbons can be highly mobile and can cause problems a great distance from their release point due to transport in groundwater or air. Also larger and branched chain hydrocarbons are persistent in the environment. PHC’s can create aesthetic problem and can degrade soil quality by interfering with water retention and nutrient transmission (CCME, 2008).

When petroleum compounds such as crude oil are released into the environment, the compounds undergo physical, chemical, and biological changes collectively referred to as weathering. The degree to which various types of petroleum hydrocarbons degrade under these changes depends on the physical and chemical properties of the hydrocarbons. Monoaromatic hydrocarbons, such as BTEX, have relatively high water solubility and low partition coefficient ($K_{ow}$). These compounds are very volatile and will tend to be dissolved in the water phase or evaporated in the air spaces of soil. Because of their relative hydrophilic nature, they are not attenuated very much by the soil particles or constituents and are very mobile and more easily degradable in the environment (Edwards, Wills, Reinhard, & Grbic-Galic, 1992). For the
purpose of this study, BTEX was not analyzed and a focus on non-soluble and less mobile hydrocarbons was chosen.

In Alberta, PHC’s are considered in four broad physio-chemical fractions. The fractions are defined in equivalent carbon numbers as follow: F1 – C6 to C10, F2 - >C10 to C16, F3 - >C16 to C34, and F4 – >C34 (CCME, 2008). Since soils containing high organic carbon content may give rise to false positives during laboratory analysis, it is important to acknowledge the differences between phytogenic (derived from plants) and petrogenic (petroleum based) hydrocarbons and their biomarkers. 100% activated silica gel is used to clean up soil samples within the F1-F4 hydrocarbon range by means of either an in-situ or ex-situ treatment method. This method removes phytogenic hydrocarbon from the solution so only petrogenic hydrocarbons remain to be analyzed. A benchmark method for determination of PHC’s in soil is presented that addresses major sources of variability and uncertainty related to the extraction, purification, quantification and reporting. Faction 1 PHC’s are isolated through purge and trap procedures followed by gas chromatography with a flame ionization detector (GC-FID). F2 – F4 PHC up to C50 are extracted by a Soxhlet procedure, “cleaned up” on silica gel and determined by GC-FID. PHC C50+, if present, may be determined gravimetrically or through extended chromatography. Specific chromatographic calibration standards are required (CCME, 2008).

In Alberta, PHC guidelines in soils have been developed for five generic land uses; agricultural, natural, residential/parkland, commercial and industrial for both fine and coarse-grained soil textures. Figure 1 illustrates four of the land uses and their specific sensitivities. A generic land use scenario has been applied to each category based on ‘normal’ activities on these lands with specific exposure pathways and receptors. For each land use there are ecological and
human health receptors. Table 1 illustrates the human health and ecological protection pathways for a natural land use area. For human health receptors, group toxicological and physio-chemical properties are used to estimate what concentrations of PHC in soils that would be hazardous along four pathways; inhalation of vapours, direct contact with contaminated soil, and ingestion of cross-contaminated groundwater. For ecological receptors, the focus is on the effects of PHC on the biotic component of a terrestrial ecosystem. Risk is based on the evaluation of the potential for adverse effects to occur from exposures to soil-based PHC at point-of-contact or by indirect means such as soil to groundwater pathways and food chain transfer (CCME, 2008).

Table 1: Alberta Tier 1 Soil and Remediation Guidelines for PHC’s in a natural land use scenario (AEP, 2016).

<table>
<thead>
<tr>
<th>Receptor Pathway</th>
<th>Protection of Domestic Use</th>
<th>Direct Soil Contact</th>
<th>Nutrient Cycling Check</th>
<th>Livestock Soil and Food Ingestion</th>
<th>Wildlife Soil and Food Ingestion</th>
<th>Protection of Freshwater Aquatic</th>
<th>Protection of Wildlife Water</th>
<th>Management Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil Type</td>
<td>Fine</td>
<td>Coarse</td>
<td>Fine</td>
<td>Coarse</td>
<td>Fine</td>
<td>Coarse</td>
<td>Fine</td>
<td>Coarse</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.046 (mg/kg)</td>
<td>0.078 (mg/kg)</td>
<td>60 (mg/kg)</td>
<td>31 (mg/kg)</td>
<td>44 (mg/kg)</td>
<td>18 (mg/kg)</td>
<td>7.9 (mg/kg)</td>
<td>0.17 (mg/kg)</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.52 (mg/kg)</td>
<td>0.95 (mg/kg)</td>
<td>110 (mg/kg)</td>
<td>75 (mg/kg)</td>
<td>2,500 (mg/kg)</td>
<td>980 (mg/kg)</td>
<td>63,000 (mg/kg)</td>
<td>0.12 (mg/kg)</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.073 (mg/kg)</td>
<td>0.14 (mg/kg)</td>
<td>120 (mg/kg)</td>
<td>55 (mg/kg)</td>
<td>1,600 (mg/kg)</td>
<td>640 (mg/kg)</td>
<td>- (mg/kg)</td>
<td>17,000 (mg/kg)</td>
</tr>
<tr>
<td>Xylenes</td>
<td>0.99 (mg/kg)</td>
<td>1.9 (mg/kg)</td>
<td>65 (mg/kg)</td>
<td>95 (mg/kg)</td>
<td>6,600 (mg/kg)</td>
<td>2,600 (mg/kg)</td>
<td>- (mg/kg)</td>
<td>16,000 (mg/kg)</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>1,100 (mg/kg)</td>
<td>2,200 (mg/kg)</td>
<td>210 (mg/kg)</td>
<td>210 (mg/kg)</td>
<td>27,000 (mg/kg)</td>
<td>11,000 (mg/kg)</td>
<td>30,000 (mg/kg)</td>
<td>1,000 (mg/kg)</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>1,500 (mg/kg)</td>
<td>2,900 (mg/kg)</td>
<td>150 (mg/kg)</td>
<td>150 (mg/kg)</td>
<td>25,000 (mg/kg)</td>
<td>9,800 (mg/kg)</td>
<td>30,000 (mg/kg)</td>
<td>520 (mg/kg)</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>-</td>
<td>1,300 (mg/kg)</td>
<td>300 (mg/kg)</td>
<td>- (mg/kg)</td>
<td>30,000 (mg/kg)</td>
<td>16,000 (mg/kg)</td>
<td>- (mg/kg)</td>
<td>1,000 (mg/kg)</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>-</td>
<td>5,600 (mg/kg)</td>
<td>2,800 (mg/kg)</td>
<td>- (mg/kg)</td>
<td>21,000 (mg/kg)</td>
<td>8,400 (mg/kg)</td>
<td>- (mg/kg)</td>
<td>10,000 (mg/kg)</td>
</tr>
</tbody>
</table>

Impacts of PHC Contaminants on Plant Growth

Soils ability to serve as a habitat for plants, microorganisms and soil living animals is an important part of the function of agricultural and forested land use. The importance of biological processes for ecosystem function, specifically for microorganisms are essential for plant growth and the creation of complex structure of the soil fertility (Van de Leemkule, Van Hesteren & Pruksma, 1998). Anthropogenic activities leading to the introduction of contaminants may be harmful to the overall soil environmental and may affect the population and activities of soil microflora/fauna at different functional levels, and reduce the growth and yield of plants (Maliszewska-Kordybach & Smreczak, 2003). Soil biological activity, including soil microbial biomass and enzymatic activity, is influenced by a range of physiochemical, environmental parameters and perturbations (Labud, Garcia, & Hernandez, 2007). Plants growing in PHC contaminated soils are often subjected to combined stress and nutritional deficiency and chemical toxicity. This results in the production of stress ethylene, which leads to plant growth inhibition and decrease in plant function, therefor hindering the effective of a phytoremediation application. Another issue that challenges the full-scale application of phytoremediation is that
high concentrations of chemical contaminants often prevent plants from producing sufficient biomass to achieve acceptable rates of remediation (Harvey et al., 2002).

### Remediation Techniques for PHC Impacted Soils

Various strategies have been employed for remediation and reclamation of toxic environment and industrial sites (Saleh, Huang, Greenberg, & Glick, 2004). Most traditional remediation strategies of terrestrial sites involve physical removal of the contaminated soil to landfill, and processes such as chemical soil washing. These methods tend to be very costly, leave unsightly blemishes on the landscape, and often have limited success. In-situ biologically-based techniques can provide an alternative to “dig and dump” methods. Large amounts of petroleum hydrocarbon contaminated soils are dealt with as dig and dump by excavating and hauling it to a Class 2 landfill facility. The high disposal and trucking cost warrant the introduction of sustainable and innovative technologies for remediation of contaminated sites (Loick, Hobbs, Hale & Jones, 2009).

Many organic compounds can be metabolized by microbes found in, or added to, bulk soil (Stapleton et al., 1998). Alternatively, land farming, a process that involves tilling the soil to bring contaminants closer to the surface, can be used to promote oxidation and microbial degradation of organic contaminants (Huang et al., 2005). These strategies can rarely be exploited successfully under field conditions because microbial populations in the contaminated soil do not achieve sufficient biomass for acceptable rates of bioremediation. In addition to the time factor involved, the effectiveness of this method tends to be limited to small and/or volatile organics (Huang et al., 2004). Addition of nitrogen and phosphorous to soils can enhance the rate of natural microbial degradation of some contaminants, but this method is still slow and does
not lead to complete degradation of larger organic compounds. In this case, organics can be bio-
stabilized in-situ or assimilated into a biological receptor and removed from the site
(Romantschuk et al., 2000).

Phytoremediation uses the ability of plants to either take up ions into their biomass or use
a plant growth promoting rhizobacteria to metabolize the contaminant within the soil. The
accumulation of contaminants into biomass is called phytoextraction which can be used for
organic and inorganic compounds that aren’t able to be metabolized or degraded (MacNeill,
2011). Phytoextraction is dependent on the plants ability to produce an extensive rooting system
to reach contaminants in the soil and uptake those contaminants into the plant biomass or
degrade them within the soil (Glick, 2003). Since non-degradable contaminants are sequestered
into the plant tissue, a high amount of plant biomass is required to attain acceptable rates of
remediation. This is often difficult to achieve in impacted soils (Chang, Gerhardt, Huang, Yu,
Glick, Gerwing, & Greenberg, 2014). When salt contaminants have been sequestered into the
plant material, they can be harvested or grazed safely for animal consumption. If
phytoremediation was used in the process of treating soils impacted with heavy metals, the plant
biomass can be harvested and disposed of safely or if the target metal is of sufficient economic
value, smelted and recovered in a process known as phytomining (Sheoran, Sheoran, & Poonia,
2009).

**Plant-Growth-Promoting-Rhizobacteria effects on Plant Establishment**

Many microorganisms are attracted by nutrients exuded from plant and roots. This
activity by microorganisms in the vicinity of plant roots is known as the “rhizosphere effect”
(Niranjan, Shetty, & Reddy, 2005). Rhizobacteria can directly promote plant growth by the
production of hormones. These rhizobacteria that positively influence plant health and establishment are referred to as plant growth promoting rhizobacteria (PGPR).

Phytoremediation is greatly dependent on the ability of plants to establish growth in contaminated soils. However, contaminants can decrease rates of germination and biomass production, lowering the overall efficiency of the remediation process. One way to accelerate plant growth is to limit the amount of ethylene, a stress hormone, in plant tissue. This can be done by the action of PGPR (Glick, 1995). The PGPR mitigate production of deleterious stress ethylene in the plants, and they synthesize auxin, which is transferred to the plant. Together, these processes promote vigorous root and shoot growth under environmental stress conditions.

PGPR are naturally occurring bacteria which can improve plant establishment through a symbiotic relationship with the plants whose rhizosphere they inhabit (Glick, 2003). These microbes can assist plant growth through indirect or direct methods. Direct methods include the production of siderophores which improve plant nutrient uptake, the production of auxins to stimulate plant root growth, the fixation of nitrogen to improve nutrient availability, and the lowering of the precursor of stress ethylene concentrations through the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Penrose & Glick, 2003). PGPR strains that produce the auxin indole-3-acetic acid (IAA) and consume ACC through high ACC deaminase activity to lower stress response in plants have been used to remediate petroleum and salt impacted soils (Huang et al., 2005).

ACC deaminase activity improves plant growth by lowering the available ACC in the ethylene biosynthesis pathway (Glick, Penrose, & Li, 1998). ACC produced by this pathway is secreted by the plant into the rhizosphere, where ACC deaminase producing PGPR can consume ACC as a source of fixed nitrogen. This promotes the growth of PGPR in the rhizosphere, causing
more ACC to be consumed, and therefore, more ACC to be secreted into the rhizosphere. As more ACC is secreted by the plant, less is available for ethylene biosynthesis and stress signalling, and less plant growth inhibition is observed (Glick et al., 1998). PGPR that produce IAA secrete the auxin into the rhizosphere, where the plant is able to take up the hormone, resulting in improved cell growth. This increase in IAA concentration leads to an upregulation of ACC synthase production and activity, and results in higher levels of ACC formation (Kende, 1993). The proposed pathway of IAA and ACC deaminase activity in plant growth promotion is shown in Figure 2.

Species of PGPR in the genus Pseudomonas present a high potential for hydrocarbon degradation due to their metabolic diversity, abundance in the microbial community and their resistance to chemical remediation agents present at contaminated sites. Pseudomonas has shown resilience to oil contamination by increasing in abundance and changing its community structure. Pseudomonas metabolizes petroleum hydrocarbons by dioxygenation followed by degradation through a meta-cleavage pathway (Palleroni, Pieper, & Moore, 2010).

**Research Objectives**

Phytoremediation enhanced with PGPR has been shown to increase the rate of remediation when used in petroleum contaminated soils (Chang, 2008). Improved biomass production and increased soil infiltration of root systems in PGPR treated plants has been credited with improved remediation rates in phytoremediation trials. This is done by increasing plant biomass to sequester non-biodegradable contaminants and making biodegradable contaminants available to rhizosphere bacteria for consumption.

The research question for this thesis is whether or not chlorophyll concentrations in plant biomass have an effect on levels of petroleum hydrocarbon degradation in soil when supplemented with PGPR and fertilizer.
Figure 2: PGPR schematic. PGPR consumes the ethylene precursor ACC, lowering the stress response within plants by lowering the stress signal. IAA is produced within the plant, stimulating both plant cell growth and upregulation of ACC synthase. This upregulation of AA synthase causes more ACC to be produced, and increases the available fixed nitrogen for PGPR to consume in the rhizosphere (Glick et al., 1998).
For this research, a PHC impacted pre-existing land treatment area (LTA) in central Alberta was used. The area was used in a phytoremediation field trial over one growing season from July to October 2013. The concentrations of soil petroleum hydrocarbon Fraction 1 through Fraction 4 were analyzed in all soil samples collected from the field trial area.

The objectives for this research are:

1. Investigate various plant species for their ability to produce biomass on PHC impacted soils, leading to improved phytoremediation
2. Monitor PHC concentration levels after phytoremediation with PGPR treated seeds.
3. Determine the effects of fertilizer on plant chlorophyll production and overall plant health on PHC impacted soils.

**Material and Methods**

**Growth of PGPR Cultures**

Bacterial stocks of previously isolated ACC deaminase producing strains of Pseudomonas spp. UW3 and Pseudomonas putida UW4 (Glick, 1995) were selected for use in this study. These two strains were isolated from farm soil in Ontario and have been tested for its growth promotion effect on selected plants in several laboratory experiments and field trials (Huang et al., 2004). Prior to use, the bacteria were grown on plates of selective media where ACC was the sole nitrogen source. Both UW3 and UW4 produce ACC deaminase, the plant growth hormone IAA, and iron-sequestering siderophores (Glick, 1995). These bacteria were then inoculated into 100 mL sterile Tryptic Soy Broth (TSB) (BD Biosciences, Mississauga, ON) in a 250 mL Erlenmeyer flask, and grown at 23 ± 1°C on a rotary shaker at 80 rpm. After 24 hours, cultures were transferred aseptically to Falcon tubes, which were centrifuged at 2000 rpm
EFFECTS OF FERTILIZER ON PHYTOREMEDIATION

for 15 minutes. The supernatant was removed, and autoclave sterilized reverse osmosis filtered water (18Ω) (ddH2O) was used to resuspend the pellet of cultured bacteria. The resuspended pellets were then centrifuged again, and resuspended in ddH2O (MacNeil, 2011).

Seed Treatment with PGPR

Bacterial cultures grown as per the previous section were grown in 1 litre of TSB for 24 hours on a rotary shaker at 80 rpm. The bacterial culture was transferred into centrifuge tubes, and centrifuged at 2000 rpm for 10 minutes (MacNeil, 2011). The supernatant was then discarded, and the pellet was washed with ddH2O, and centrifuged again. The supernatant was discarded, the pellet was resuspended in ddH2O and the optical density at 600 nm (OD600) was measured using a spectrophotometer (MacNeil, 2011). The OD600 was then adjusted to 2.0 using ddH2O. Methylcellulose polymer (Sigma Aldrich, Oakville, ON) was prepared at 1.5% w/v by mixing on a stir plate for 1 hour until all clumps had broken apart, and then autoclaved for 40 minutes. The polymer forms a white gel, which undergoes reverse gelatinization upon cooling and becomes clear. This clear polymer was combined with bacterial solution at a ratio of 200 mL methylcellulose to 1 L bacterial solution. A commercial seed colourant (Color Coat Blue, Becker Underwood, Saskatchewan) was added in a ratio of 17.5 mL to 1 L bacteria suspension and methylcellulose polymer slurry (MacNeil, 2011). This coloured slurry was stirred by magnetic stirrer for the duration of seed treatment. Seeds were treated in a HEGE 11 seed treater (Wintersteiger Inc., Austria) for 2 minutes in batches of 2.5 L of seed. Coloured slurry was added at a ratio of 10 mL per 2.5 L of cereal seeds and 20 mL per 2.5 L of grass seed. Seeds were planted within 1 month of bacterial application (MacNeil, 2011).
H$_2$O$_2$ Imbibing of Seeds

Seeds were soaked in a sodium hypochlorite (bleach) solution (1% v/v) in a 1.5:1 bleach solution to seed volume ratio for 10 minutes to surface sterilize the seed, followed by three washings with reverse osmosis (RO) water (18 $\Omega$) (MacNeil, 2011). Seeds were soaked in solutions of H$_2$O$_2$ solution (H$_2$O$_2$ treatment) for 3 hours or in sterile RO water (control treatment) for 3 hours, after which seeds were washed 3 times with sterile RO water and allowed to dry in a laminar flow hood. Seeds were used within 3 days of H$_2$O$_2$ imbibing (MacNeil, 2011).

Research Field Site Information

For this research, a PHC impacted site in West-Central Alberta was used. The site was used in a phytoremediation field trial over one growing season from July to October 2013. The exact location (permanent) of each assessment point was accurately recorded to ensure that all subsequent sampling would be conducted in the same locations to reduce sampling and ultimately analytical test result variability that would affect temporal comparisons of the data sets (Earthmaster Environmental Strategies Inc. (Earthmaster), 2013). Surface soil samples were taken by Dutch auger (7.5 cm diameter) to 30 cm depth, and were made as a composite of three soil cores taken from within a 1.0 m radius of the permanent assessment point. Soils were placed into plastic bags for field screening analysis of volatile organic compounds with a portable photoionization detector. Soil samples collected for organic analysis were tightly packed into sterile 125 ml glass jars to minimize loss of organic vapours into the headspace and immediately stored on ice in an insulated cooler until delivery to the laboratory (Earthmaster, 2013).

Within the treatment areas, a general visual assessment of plant cover was taken during the mid-season and end of year site assessments. The plant height and percent cover were
measured at mid-season within a 1.0 m radius of each permanent assessment point. Plant height was measured using a ruler on a subset of plants within a 1.0 m² area and the average was recorded to the nearest 1 cm. Percent plant cover was based on a visual inspection of a 1.0 m² area. Root depth was measured by digging a small trench and using a ruler to measure depth of observable roots.

Plant tissue samples were collected by Waterloo Environmental Biotechnology Inc. (WEBi) and were submitted to a third party laboratory for chlorophyll analyses. Plant tissue samples were collected by Earthmaster Environmental Strategies Inc. (Earthmaster) and submitted to a third party laboratory for micronutrient, protein, and fibre analyses to assess the overall plant health and quality (Earthmaster, 2013).

**Sundre, Alberta Field Site.** The Sundre field site is located approximately 45 km west of Sundre, Alberta. The reported cause of PHC impacts was the attempted on-site treatment of approximately 245 m³ of calcium nitrate invert drilling mud by way of constructing a land treatment area. The site was mulched, fertilized and or cultivated periodically from 1996 to 2002 (Figure 3). Planting maps from 2013 are shown in Figure 4. The Sundre field site was approximately 5,700 m². The PHC Fraction 2 and 3 were the main contaminant of concern and the focus of the field research due to their abundance within the soil of the land treatment area. The average F2 concentration at the start of the field season was 59 mg/kg, with a range of 21 mg/kg to 160 mg/kg. The average F3 concentration at the start of the field season was 948 mg/kg, with a range of 260 mg/kg to 1900 mg/kg. The pre-existing land treatment area was divided into four treatment areas, two which were fertilized with inorganic fertilizer (+fertilizer,
Figure 3: Aerial photograph of Sundrie field site. The site boundaries are outlined in red while the pre-existing LTA area that has been present since 1996 is shown in purple (Abacus Datographics, 2017).
Figure 4: Sundrie field site 2013 planting map. The entire land treatment area was tilled to 15 cm prior to sampling and planting. All four assessment areas were planted with annual ryegrass, perennial ryegrass, common oats and tall fescue. Areas that were fertilized were treated with 19-18-19 fertilizer at a rate of 378.49 lbs/acre.
sample points 01-05 and 16-20), and two which were not fertilized (-fertilizer, sample points 06-15). To prepare a suitable seed bed, rocks were removed and the treatment area was cultivated to a depth of 15 cm and left to dry overnight as the treatment area soils were very wet and difficult to homogenize for good seed bed preparation. The following day the treatment area was roto-spiked to approximately 5 cm depth. Seeds were planted using a no-drill seeder at a rate of 283.89 lbs/acre for annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), oats (*Avena sativa*) and Tall fescue (*Festuca arundinacea*) in equal seed ratios. These grasses were chosen due to their effective growth promotion with the use of UW3 and UW4. After planting, 19-18-19 fertilizer was applied to 50% of the treatment area at a rate of 378.49 lbs/acre. The treatment area was finished by levelling soils and adding seed cover with chain harrows.

**Determination of Soil PHC Concentrations**

Soil samples were analyzed for extractable PHC (F2-F4). Depending on the amount of F4 material in the sample and user/analyst preferences, extractable PHC may be further subdivided on the basis of detection method (chromatographic/gravimetric). Extractable PHC were recovered by Soxhlet extraction in 50:50 hexane-acetone. The extract was dried over sodium sulphate and treated with silica gel to remove polar material (fats, plant waxes, etc.). A sample of the extract was then injected into a Gas Chromatogram – Flame Ionization Detector (GC-FID) equipped with a poly(dimethylsiloxane) column. Area counts were integrated and then quantified in the following ranges: (1) nC10 to nC16 – “F2”, (2) nC16 to nC34 – “F3”, and (3) nC34 to nC50 - “F4”. This determination of F4 was adequate provided the GC-FID chromatogram returned to the baseline at nC50. If this was not the case, or other evidence
suggests that PHC greater than nC50 are present in appreciable quantities, residual PHC was determined gravimetrically or through extended, high temperature chromatography. If determinations of target PAH (e.g., naphthalene, 6-83phenanthrene, chrysene, benzo(a)pyrene) were made, these were subtracted from the appropriate PHC CWS fractions (generally F3, except F2 for naphthalene) (CCME, 2008).

**Determination of Plant Material Chlorophyll Values**

Chlorophyll A and B values were analyzed from each assessment point to understand the effects of fertilizer on chlorophyll concentrations A and B and to determine if there was a relationship between chlorophyll concentrations and hydrocarbon degradation. Chlorophyll concentrations were determined by N, N-dimethylformamide (DMF) extraction and analyzed by spectrophotometer. For each permanent assessment point sample, 0.5 g of fresh biomass was placed into 13 x 54 mm scintillation tubes with 5.0 ml of DMF. All samples had ocular densities over 1.0 so a 3x dilution was used to increase spectrometer precision. Figure 5 shows fresh biomass both with and without DMF solution. All sample tubes were covered with aluminum foil and the samples were kept in darkness at 4°C for 48 hours for chlorophyll extraction. Absorption at 664 nm at 647 nm was determined by spectrophotometer. Chlorophyll values were calculated using Morans formula below (Moran, 1982).

\[
\text{Chlorophyll A} = 12.64 \times A_{664} - 2.99 \times A_{647}
\]

\[
\text{Chlorophyll B} = -5.69 \times A_{664} - 23.26 \times A_{647}
\]

\[
\text{Total Chlorophyll} = 7.04 \times A_{664} - 20.27 \times A_{647}
\]
Figure 5: A) 0.5 g of fresh biomass B) Fresh biomass within the DMF solution
Plant Root Biomass Measurement

A 100 cm² by 10 cm in depth sample of soil was collected and plant material was to be washed and dried in a 55°C hotbox for 3 days to remove moisture. The dry weight was to be determined for samples to follow plant growth by weighing on a top loading balance. However, during transport, several samples were destroyed and a decision was made to not include this measurement in the study due to the insufficiency in the data set.

Statistical Analysis

All statistical analysis was performed using the software GraphPad Prism 6 (GraphPad Software, Inc.). This program was used to calculate general statistics about the data as well as two-way ANOVA’s, correlation analyses such as Boneferonni post-test, and linear regressions where the dataset supported these analyses. The confidence interval was set at 95% with an alpha = 0.05. The Null hypothesis was that fertilizer treatment will not have a statistically significant effect on the degradation of target PHC fractions within the surface soil, plant growth or chlorophyll values.

Results and Discussion

Field Trial of Phytoremediation with PGPR on PHC Impacted Soils

Field trials were undertaken to test the applicability of PGPR enhanced phytoremediation in a fertilizer and no fertilizer supplement scenario. Field soils were sampled for concentrations of PHC Fraction 2 through Fraction 4 at the time of planting and at the end of the field season. In addition to assessment of plant growth and changes to PHC concentrations, the concentrations of chlorophyll in the plant tissue was examined in both the fertilized and unfertilized areas to determine if chlorophyll concentrations had a direct effect on PHC degradation.
Sundre Field Site

**Initial PHC concentrations before phytoremediation.** Soil samples were taken prior to soil working, planting and application of fertilizer on the land treatment area. Initial PHC concentrations from 20 soil samples were measured and Figure 7 illustrates the assessment points within each treatment area. The initial PHC concentrations of the field site were determined to be an average of 59 mg/kg for PHC Fraction 2, 948 mg/kg for PHC Fraction 3, and 189 mg/kg for PHC Fraction 4. The PHC Fraction concentrations ranged from 21 – 160 mg/kg, 260 – 1900 mg/kg, and 0 – 300 mg/kg for PHC Fractions 2, 3, and 4 respectively. Initial PHC concentration results can be viewed in Table 2.

**Biomass production at the Sundre field site.** The entire land treatment area was planted with UW3 and UW4 treated seeds across four total sections. These sections were separated into fertilized (+fertilizer) and unfertilized (-fertilizer) (Figure 6). Above-ground dry plant biomass for samples collected from representative sampling points were collected in July 2013 before the application of PGPR. Samples of plant biomass were taken from 0.25 m² squares from each permanent assessment point and dry mass was measured. The site had an average above-ground dry biomass production of 411.4 g/m² across the entire land treatment area (Figure 7). Due to extensive grazing of the area between the mid-season assessment and end of season assessment, above-ground dry biomass samples could not be collected in October for comparison and statistical analysis.

**Plant cover, height and overall health.** Plant establishment from the planting date of July 30, 2013 to the mid-season field visit and vegetation assessment was fairly extensive in
Figure 6: Sundre 2013 field site. The land treatment area was separated into four sections, two supplemental with fertilizer and two with no fertilizer. Each section includes five assessment points.
Table 2: Measured PHC Fraction 2 through PHC Fraction 4 concentrations pre-treatment with PGPR seeds and fertilizer. Error shown is standard error.

<table>
<thead>
<tr>
<th>Assessment Point</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>1400</td>
<td>230</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>1900</td>
<td>340</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>760</td>
<td>170</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>690</td>
<td>170</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>950</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
<td>850</td>
<td>190</td>
</tr>
<tr>
<td>7</td>
<td>77</td>
<td>1200</td>
<td>240</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>830</td>
<td>170</td>
</tr>
<tr>
<td>9</td>
<td>43</td>
<td>780</td>
<td>180</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>1100</td>
<td>250</td>
</tr>
<tr>
<td>11</td>
<td>37</td>
<td>650</td>
<td>140</td>
</tr>
<tr>
<td>12</td>
<td>46</td>
<td>740</td>
<td>150</td>
</tr>
<tr>
<td>13</td>
<td>50</td>
<td>900</td>
<td>180</td>
</tr>
<tr>
<td>14</td>
<td>81</td>
<td>1000</td>
<td>200</td>
</tr>
<tr>
<td>15</td>
<td>160</td>
<td>1700</td>
<td>300</td>
</tr>
<tr>
<td>16</td>
<td>43</td>
<td>880</td>
<td>200</td>
</tr>
<tr>
<td>17</td>
<td>21</td>
<td>440</td>
<td>97</td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td>720</td>
<td>150</td>
</tr>
<tr>
<td>19</td>
<td>21</td>
<td>260</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>68</td>
<td>1200</td>
<td>220</td>
</tr>
<tr>
<td>Mean</td>
<td>59(8.38)</td>
<td>948(87.43)</td>
<td>189(15.84)</td>
</tr>
</tbody>
</table>

most areas. This is likely due to the amount of precipitation the site received in the days immediately after seeding which aided in seed germination and plant establishment. Within the first week after planting, the site received 26 mm of precipitation and totalled 84 mm over the duration of the projects growing season. The precipitation data from Alberta Agriculture and Forestry for the duration of the project is shown in Figure 8.

In September 2013, maximum plant height (not species dependent) was measured with a ruler on a subset of plants within a 1.0 m² area and the average recorded to the nearest 1.0 cm, plant cover (%) was based on a visual inspection of a 1m² area surrounding each assessment
point location, and an overall health rating was given on a scale of 1 to 4 (low to high) in both the +fertilizer and –fertilizer sections of the land treatment area. Plant health was assessed using four factors including plant cover, height, productivity and chlorosis with each vegetation health indicator having equal weight for a maximum rating of 4. Within the +fertilizer areas, the overall health rating ranged from 3.0 to 4.0 and within the -fertilizer areas the overall health rating ranged from 2.0 to 3.5. Average overall plant health rating for both the –fertilizer and +fertilizer areas were 2.6 and 3.7 respectfully. Vegetation assessment results from September 2013 can be viewed in Table 3. Only the macronutrient nitrogen, phosphorus and potassium

![Figure 7: Above-ground dry biomass (g/m²) taken from random locations within each of the +fertilizer and –fertilizer areas prior to the addition of PGPR treated seeds and fertilizer. Due to extensive grazing of the area between the mid-season assessment and end of season assessment, above-ground dry biomass samples could not be collected in October for comparison and statistical analysis (Earthmaster, 2013). Copyright 2013 by Copyright Holder. Used with permission.](image-url)
Figure 8: July through October 2013 precipitation data for Sundre, Alberta. Rainfall can be seen within the first week after planting. Heavy rainfall events also occurred within August and early September (Alberta Agriculture and Forestry, 2017).
Table 3: Vegetation sampling completed for each assessment point within the +fertilizer and –fertilizer areas during the mid-season assessment for plant height, plant cover and overall health rating. Areas supplemented with fertilizer showed measurable improvement in all three categories. All samples were taken from 0.25 m² sample areas at the permanent assessment points. Standard Error is shown in parenthesis.

<table>
<thead>
<tr>
<th>Assessment Point</th>
<th>Plant Height cm</th>
<th>Plant Cover cm</th>
<th>Health Rating (1-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Fertilizer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01</td>
<td>35</td>
<td>90</td>
<td>4.0</td>
</tr>
<tr>
<td>02</td>
<td>35</td>
<td>80</td>
<td>3.5</td>
</tr>
<tr>
<td>03</td>
<td>30</td>
<td>70</td>
<td>3.5</td>
</tr>
<tr>
<td>04</td>
<td>28</td>
<td>100</td>
<td>4.0</td>
</tr>
<tr>
<td>05</td>
<td>30</td>
<td>70</td>
<td>3.5</td>
</tr>
<tr>
<td>16</td>
<td>33</td>
<td>40</td>
<td>3.0</td>
</tr>
<tr>
<td>17</td>
<td>36</td>
<td>95</td>
<td>4.0</td>
</tr>
<tr>
<td>18</td>
<td>46</td>
<td>100</td>
<td>4.0</td>
</tr>
<tr>
<td>19</td>
<td>30</td>
<td>40</td>
<td>3.0</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>100</td>
<td>4.0</td>
</tr>
<tr>
<td>Mean</td>
<td>34 (1.73)</td>
<td>79 (7.38)</td>
<td>3.7 (0.13)</td>
</tr>
<tr>
<td>-Fertilizer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>40</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>50</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>30</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>65</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td>11</td>
<td>18</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td>12</td>
<td>37</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td>13</td>
<td>30</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td>14</td>
<td>27</td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>15</td>
<td>28</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td>Mean</td>
<td>23 (3.16)</td>
<td>34 (4.60)</td>
<td>2.6 (0.16)</td>
</tr>
</tbody>
</table>

were considered to have an effect on plant health and biomass production, micronutrients were not considered. Overall plant height within the +fertilizer areas measured from 30 cm to 40 cm tall with an average of 34 cm. Within the -fertilizer areas, overall plant height measured from 10 cm to 34 cm with an average height of 23 cm. Plant cover within the +fertilizer areas measured
between 40 and 100 percent with an average of 79 percent cover while the -fertilizer areas measured between 20 and 65 percent with an average of 34 percent. During the October 2013 site assessment, plant samples for biomass and measurements for plant height and health rating could not be obtained due to extensive grazing by livestock. Photographs of plant growth in July 2013 and September 2013 are shown in Figure 9. The difference in growth between July 2013 and September 2013 can be seen, with the September 2013 photographs showing distinct rows of planted species created by the seeder, and July 2013 showing unorganized growth of grasses and weeds. The difference between the sections supplemented with fertilizer and those without fertilizer can also be seen in Figure 10 with lower plant density being apparent in unfertilized areas.

In October 2013, plant cover and below ground (root) biomass and length were measured within each of the +fertilizer and –fertilizer areas. Within the +fertilizer areas and –fertilizer areas, plant cover ranged from 17.5% to 37.5% in both areas. No measurable increase in plant cover was noted between the two areas. Results are however incomplete due to the cattle grazing that occurred before sampling could be completed. Rooting depth and below ground biomass was measured in each of the +fertilizer and –fertilizer areas for depths ranging from 0-10cm, 10-20cm, and 20-30cm. Within both the +fertilizer and –fertilizer areas, 90-96% of the total biomass was measured within the 0-10 cm soil profile. No measurable increase in root
Figure 9: Sundre 2013 plant growth photographs. A) Plant growth before tilling the area and seeding. B) Plant growth mid-season after two months of plant establishment.
Figure 10: Sundre 2013 plant growth photographs. A) September 2013 plant establishment and health in the north fertilizer supplemented section. B) September 2013 plant establishment and health in the south-central non-fertilizer supplemented section. The fertilizer supplemented area has substantially more vegetation density.
biomass was recorded between the two areas. Results of the October 2014 vegetation assessment for plant cover and root biomass can be found in Table 4.

**Remediation of PHC impacts within the Land Treatment Area.** Twenty soil samples were collected in July and 20 soil samples were collected in October. An overview of PHC concentrations for the entire 2013 field season for areas +fertilizer and areas -fertilizer is represented in Table 5. The soil samples collected at the end of July and the middle of October show an average decrease in all PHC Fraction concentrations from the +fertilizer supplemented areas. PHC Fraction 2 concentrations degradation levels varied from an increase of 24 mg/kg to a decrease of 103 mg/kg with an overall PHC F2 decrease of 42.6%. PHC F3 concentration degradation levels varied from an increase of 190 mg/kg to a decrease of 990 mg/kg with an overall PHC F3 decrease of 30.4%. PHC F4 concentration degradation levels varied from an increase of 30 mg/kg to a decrease of 150 mg/kg with an overall PHC F4 decrease of 24.7%.

Soil samples collected from the –fertilizer areas showed an average increase in PHC concentrations. PHC Fraction 2 concentrations varied from an increase of 169 mg/kg to a decrease of 51 mg/kg with an overall PHC F2 increase of 20%. PHC Fraction 3 concentrations varied from an increase of 720 mg/kg to a decrease of 700 mg/kg with an overall PHC F3 increase of 5.6%. PHC Fraction 4 concentrations varied from an increase of 110 mg/kg to a decrease of 140 mg/kg with an overall PHC F4 increase of 2.5%. The decrease of PHC fraction 2 and 3 across the entire land treatment area is shown in Figure 11. The increase in overall PHC remediation within the fertilizer supplemented areas may be due to increased above-ground dry biomass production. Statistical analysis by 2 way ANOVA of the F2, F3, and F4 data
Table 4: Vegetation sampling completed for each assessment point within the +fertilizer and –fertilizer areas at the end of season assessment for plant cover and rooting depth. Extensive grazing occurred before plant cover assessment could be completed leading to biased and incomplete results. Areas supplemented with fertilizer showed no measurable improvement in rooting depth or root biomass. Standard Error is shown in parenthesis.

<table>
<thead>
<tr>
<th>Assessment Point</th>
<th>Plant Cover %</th>
<th>Root Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 – 10 cm</td>
</tr>
<tr>
<td>+Fertilizer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>01</td>
<td>17.5</td>
<td>90</td>
</tr>
<tr>
<td>02</td>
<td>27.5</td>
<td>93</td>
</tr>
<tr>
<td>03</td>
<td>27.5</td>
<td>92</td>
</tr>
<tr>
<td>04</td>
<td>27.5</td>
<td>96</td>
</tr>
<tr>
<td>05</td>
<td>27.5</td>
<td>95</td>
</tr>
<tr>
<td>16</td>
<td>17.5</td>
<td>95</td>
</tr>
<tr>
<td>17</td>
<td>37.5</td>
<td>92</td>
</tr>
<tr>
<td>18</td>
<td>27.5</td>
<td>91</td>
</tr>
<tr>
<td>19</td>
<td>27.5</td>
<td>95</td>
</tr>
<tr>
<td>20</td>
<td>22.5</td>
<td>95</td>
</tr>
<tr>
<td>Average</td>
<td>26 (1.83)</td>
<td>93 (0.65)</td>
</tr>
<tr>
<td>-Fertilizer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>27.5</td>
<td>95</td>
</tr>
<tr>
<td>7</td>
<td>32.5</td>
<td>95</td>
</tr>
<tr>
<td>8</td>
<td>37.5</td>
<td>91</td>
</tr>
<tr>
<td>9</td>
<td>17.5</td>
<td>95</td>
</tr>
<tr>
<td>10</td>
<td>17.5</td>
<td>93</td>
</tr>
<tr>
<td>11</td>
<td>37.5</td>
<td>95</td>
</tr>
<tr>
<td>12</td>
<td>22.5</td>
<td>90</td>
</tr>
<tr>
<td>13</td>
<td>22.5</td>
<td>96</td>
</tr>
<tr>
<td>14</td>
<td>27.5</td>
<td>91</td>
</tr>
<tr>
<td>15</td>
<td>32.5</td>
<td>92</td>
</tr>
<tr>
<td>Average</td>
<td>27.5 (2.36)</td>
<td>93 (0.68)</td>
</tr>
</tbody>
</table>
Table 5: PHC Fraction 2, Fraction 3 and Fraction 4 concentration within the +fertilizer and –fertilizer supplemented sections of the treatment area. Standard error is in parentheses.

<table>
<thead>
<tr>
<th>Assessment Point</th>
<th>F2 - mg/kg</th>
<th>F3 - mg/kg</th>
<th>F4 - mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Fertilizer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01</td>
<td>86</td>
<td>40</td>
<td>1400</td>
</tr>
<tr>
<td>02</td>
<td>150</td>
<td>47</td>
<td>1900</td>
</tr>
<tr>
<td>03</td>
<td>31</td>
<td>34</td>
<td>760</td>
</tr>
<tr>
<td>04</td>
<td>29</td>
<td>53</td>
<td>690</td>
</tr>
<tr>
<td>05</td>
<td>49</td>
<td>28</td>
<td>950</td>
</tr>
<tr>
<td>16</td>
<td>43</td>
<td>46</td>
<td>880</td>
</tr>
<tr>
<td>17</td>
<td>21</td>
<td>21</td>
<td>440</td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td>23</td>
<td>720</td>
</tr>
<tr>
<td>19</td>
<td>21</td>
<td>0</td>
<td>260</td>
</tr>
<tr>
<td>20</td>
<td>68</td>
<td>21</td>
<td>1200</td>
</tr>
<tr>
<td>Average</td>
<td>54 (12.5)</td>
<td>31 (5.0)</td>
<td>920 (151.2)</td>
</tr>
<tr>
<td>Average Change %</td>
<td>-42.6%</td>
<td>-30.4%</td>
<td>-24.7%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assessment Point</th>
<th>F2 - mg/kg</th>
<th>F3 - mg/kg</th>
<th>F4 - mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Fertilizer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06</td>
<td>47</td>
<td>43</td>
<td>850</td>
</tr>
<tr>
<td>07</td>
<td>77</td>
<td>26</td>
<td>1200</td>
</tr>
<tr>
<td>08</td>
<td>48</td>
<td>32</td>
<td>830</td>
</tr>
<tr>
<td>09</td>
<td>43</td>
<td>100</td>
<td>780</td>
</tr>
<tr>
<td>10</td>
<td>56</td>
<td>53</td>
<td>1100</td>
</tr>
<tr>
<td>11</td>
<td>37</td>
<td>56</td>
<td>650</td>
</tr>
<tr>
<td>12</td>
<td>46</td>
<td>42</td>
<td>740</td>
</tr>
<tr>
<td>13</td>
<td>50</td>
<td>37</td>
<td>900</td>
</tr>
<tr>
<td>14</td>
<td>81</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>15</td>
<td>160</td>
<td>140</td>
<td>1700</td>
</tr>
<tr>
<td>Average</td>
<td>65 (11.5)</td>
<td>78 (22.1)</td>
<td>975 (96.2)</td>
</tr>
<tr>
<td>Average Change %</td>
<td>20%</td>
<td>5.6%</td>
<td>2.5%</td>
</tr>
</tbody>
</table>
Figure 11: PHC Fraction 2 and 3 concentrations for July and October 201. The red horizontal lines represent Alberta Tier 1 guideline targets of 150mg/kg for F2 and 1300 mg/kg for F3 (Earthmaster, 2013). Copyright 2013 by Copyright Holder. Used with permission.
determined that there is no statistically significant difference between columns (July vs. October) but statistical significance exists between the rows which represent treatment type (+fertilizer, -fertilizer) and possibly sample location (F2, F=2.229, p=0.044), (F3, F=2.267, p=0.0412), (F4, F=3.534, p=0.0042). The Tukey’s Multiple Comparisons Test determined that a statistically significant difference existed between F2 and F3, and F3 and F4 in July and October. No statistically significant difference was found to exist between F2 and F4 in July and October. The linear regression analysis determined that there is no linear (cause and effect) relationship between the fractions with or without fertilizer. Further, the difference in the slopes of the lines is not significant while the line elevation difference is highly significant (Figure 12). PHC fractions appear to be degrading independent of each other but at a similar rate which also appears to be dependent on treatment type.

A comparison of representative gas chromatograms from the July and October 2013 laboratory analyses is presented in Figure 13. The chromatograms show that the soil samples have biphasic unresolved complex mixtures (UCM) which is fairly typical of those that would be expected at a site that contains PHC Fraction 3 as shown by the biphasic UCM with a dip in the middle of the trace at approximately seven minutes. These chromatograms are consistent with chromatograms for soil samples analyzed from other PHC impacted sites. The chromatogram for the October sample shows a peak at approximately 13.5 minutes which is not apparent in the July samples. This may be due to an addition of organic molecules from the plant rhizosphere and is not expected to impact remediation results. Evaluating the presence of naturally occurring PHC’s against PHC’s that are introduced from previous human activities remains a
significant concern. It is important to understand what the source and characteristics of these background organics may be. Low level residual petroleum hydrocarbons may be present in

Figure 12: Linear regression graphs for samples collected from the fertilized area (A) and unfertilized area (B).

---

**Figure 12**

Linear regression graphs for samples collected from the fertilized area (A) and unfertilized area (B).
Figure 13: Representative GC-FID chromatograms from the land treatment area soil samples. Samples were analyzed by Maxxam Analytics in Calgary, AB. A) Sample 05 (0.0-0.3 m) July 2013 and B) sample 05 (0.0-0.3 m) October 2013 (Earthmaster, 2013). Copyright 2013 by Copyright Holder. Used with permission.
EFFECTS OF FERTILIZER ON PHYTOREMEDICATION

The increases of PHC concentrations within the some sampling points of the land treatment area are potentially due to the heterogeneous nature of the soil and PHC distribution. Sampling locations, although marked for future sampling events, are still approximate and subject to spatial variability. Sample points which experienced increases in PHC concentrations were analyzed for the potential of the PHC increase being due to degradation of heavier end compounds (F3 breaking down into F2). No correlation between hydrocarbon fraction breakdown causing an increase in PHC concentration was determined.

**Effect of fertilizer on chlorophyll concentrations.** Chlorophyll content and chlorophyll a/b ratios of the plant biomass grown within the PHC impacted soil for both the +fertilizer and –fertilizer areas were measured. Within the +fertilizer areas, the total chlorophyll (A and B) values ranged from 348.8 to 796.2 with an overall average of 578.7. Within the –fertilizer areas, the total chlorophyll values ranged from 278.6 to 657.8 with an overall average of 394.5. Chlorophyll A (Ch1A), B (Ch1B) and total (Ch1) values can be viewed in within Table 5 and total chlorophyll values across the site can be viewed in Figure 14. A statistically significant difference in chlorophyll between the +fertilizer and –fertilizer areas was indicated by a Pearson Correlation analysis and was supported by a p value of 0.048. The 2 way ANOVA for chlorophyll also determined high statistical significance based on the month (July vs. October), treatment (+fertilizer, -fertilizer), and possibly location (Row F=68.62, p=<0.0001, Column F=19.91, p=<0.0001). These results show a strong correlation to a positive and measurable
influence of supplementing fertilizer, though small sample size and subject variability may have a factor. Individual chlorophyll A values of the total chlorophyll values ranged from 68% to 75% within the +fertilizer areas and 67% to 77% within the –fertilizer areas. No statistically significant correlation between fertilizer supplementation and individual concentration percentage of A and B chlorophyll was evident.

**Conclusions**

During the course of this work, the efficiency of phytoremediation was tested and similar results were obtained when compared to previous work using PGPR. During the 2013 field season at the Sundre field site, an overall decrease in PHC F2, F3 and F4 within the +Fertilizer
areas was 42.6%, 30.5%, and 24.7% respectfully. This matches single season degradation levels (over 30%) observed in other field tests (Greenberg, 2006) where the use of fertilizer is common. It is possible that a portion of the lighter and more volatile PHC F2 concentrations were lost during the working and preparation of the site, therefore leading to higher levels of successful remediation. The working of the soil prior to seeding could also be responsible for the increased variability in the soil sample concentrations, especially those that increased considerably over the course of the growing season. Due to the limited budget, cattle grazing and timeframe of the research project (three months over one growing season), data that was gathered was limited and sample variability was high due to the smaller data set.

Overall the application of PGPR a positive and measurable effect on the degradation of PHC’s during the field tests. Statistical analysis shows that the addition of fertilizer had a positive and measurable effect on chlorophyll levels. The methodology used was cost effective and practical for use in this field trial. Since sampling data was limited due to time and budgeting constraints, further research is needed to study the effects chlorophyll within the plant biomass and its relationship to degrading heavy end PHC’s. Several key changes to the experimental design could have been completed in order to improve the research is discussed in the following section.

**Recommended Future Work**

Further studies into the effectiveness of Pseudomonas and its correlation to chlorophyll production and hydrocarbon degradation should be conducted. Further studies in the area should include some of the following recommendations for data collection:
- Establishing several years of background natural attenuation rates for PHC F2, F3 and F4 within the research area prior to supplementing the area with PGPR and fertilizer;
- Establish background fertility (nitrogen, phosphorus and potassium ratios, concentrations and utilization rates within the research area.

Research plots should be random, the same size, fenced, and replicated (i.e., four 2.0 m x 2.0 m plots) for each treatment. The research plots should have well-established PHC starting concentrations (enough samples to eliminate within subject variability in each plot). Treatment plots to consider in future studies would be

- background control - no treatment, no vegetation (track PHC natural attenuation and background fertility);
- positive control - pseudomonas addition to soil only (effect of soil pseudomonas on PHC and background fertility - eliminate all vegetation, keep soil bare),
- vegetation only - no fertilizer and no pseudomonas (effect of plants only on PHC).
- Pseudomonas and fertilizer - no vegetation (effect of fertility on pseudomonas and PHC).
- Pseudomonas and vegetation (single plant species selected for high biomass potential and short maturity - possible C4 photosynthesis, possible hybrid so genetic variability is accounted for).
- Pseudomonas + vegetation + fertilizer (determine the effect of fertilizer on the plant, PHC and pseudomonas.

The sampling and analysis should take place over a minimum of two growing seasons to account for annual variability and sampling frequency should occur at two week intervals during the growing season to account for seasonal variability. Growing conditions such as weather
conditions (ambient temp), growing degree days, seed emergence time, maturity time, and precipitation should also be documented and analyzed.
References


Persistent Petroleum and Organic Contaminants in Soils.” In B. Tisch, K. Zimmerman, P.
White, P. Beckett, L. Guenther, A. Macleod, S. Rowsome and C. Black (Eds.), CLRA 2006:
Reclamation and Remediation: Policy and Practice, pp. 124-133. Canadian Land
Reclamation Association, Calgary, AB.
Harvey, P.J., Campanella, B.F., Castro, P.M., Harms, H., Lichtfouse, E., Schaffner, A.R.,
hydrocarbons, anilines and phenols. Environmental Science and Pollution Research
Howes Keiffer, C., & Ungar, I.A. (2002). Germination and establishment of halophytes on brine-
of three grass species to creosote during phytoremediation. Environmental Pollution,
130(3), 453-463
process phytoremediation system for decontamination of persistent total petroleum
hydrocarbons (TPHs) from soil. Microchemical Journal, 81(1), 139-147.
contaminated soil by composting in biopiles. Environmental Pollution, 107(2), 245-254.
Molecular Biology, 44, 283-307.
properties of a sandy and a clay soil. Chemosphere, 66, 1863-1871.


Appendix A: Statistical Analysis Exports

Appendix A-1: Two-way ANOVA analysis exports

Table Analyzed   F2

Two-way ANOVA   Ordinary
Alpha 0.05

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>% of total variation</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Row Factor</td>
<td>68.86</td>
<td>0.044 *</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Column Factor</td>
<td>0.2463</td>
<td>0.701 ns</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

ANOVA table SS  DF  MS  F (DFn, DFd)  P value
Row Factor   57880 19  3046   F (19, 19) = 2.229  P=0.044
Column Factor 207 1  207   F (1, 19) = 0.1515  P=0.701
Residual     25961 19 1366

Table Analyzed   F3

Two-way ANOVA   Ordinary
Alpha 0.05

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>% of total variation</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Row Factor</td>
<td>67.7</td>
<td>0.0412 *</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Column Factor</td>
<td>2.433</td>
<td>0.2285 ns</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

ANOVA table SS  DF  MS  F (DFn, DFd)  P value
Row Factor   3841288 19  202173  F (19, 19) = 2.267  P=0.0412
Column Factor 138063 1 138063  F (1, 19) = 1.548  P=0.2285
Residual     1694288 19  89173

Table Analyzed   F4

Two-way ANOVA   Ordinary
Alpha 0.05

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>% of total variation</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Row Factor</td>
<td>76.54</td>
<td>0.0042 **</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Column Factor</td>
<td>1.796</td>
<td>0.2246 ns</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

ANOVA table SS  DF  MS  F (DFn, DFd)  P value
EFFECTS OF FERTILIZER ON PHYTOREMEDIATION

Row Factor  158710  19  8353  F (19, 19) = 3.534  P=0.0042
Column Factor  3725  1  3725  F (1, 19) = 1.576  P=0.2246
Residual  44913  19  2364

Table Analyzed  Chlorophyll XY

Two-way ANOVA  Ordinary
Alpha  0.05

Source of Variation  % of total variation  P value  P value summary  Significant?
Interaction  1.376  0.2383 ns  No
Row Factor  64.1  <0.0001  ****  Yes
Column Factor  9.298  <0.0001  ****  Yes

ANOVA table SS  DF  MS  F (DFn, DFd)  P value
Interaction  26854  2  13427  F (2, 54) = 1.473  P=0.2383
Row Factor  1250941  2  625470  F (2, 54) = 68.62  P<0.0001
Column Factor  181445  1  181445  F (1, 54) = 19.91  P<0.0001
Residual  492220  54  9115

Appendix A-2: Pearson Correlation Analysis Exports

"Assessment Point + Fertilizer vs. Assessment Point - Fertilizer"
Pearson r
r  0.9972
95% confidence interval
R squared  0.9943

P value
P (two-tailed)  .048
P value summary  *
Significant? (alpha = 0.05)  Yes

Appendix A-3: Linear Regression Analysis Exports

F2 Concentration (mg/kg)  F3 Concentration (mg/kg)  F4 Concentration (mg/kg)
Best-fit values ± SE
Slope  -2.188 ± 1.312  -27.22 ± 16.66  -4.239 ± 3.345
Y-intercept  53.8 ± 9.545  920 ± 121.1  177.7 ± 24.33
X-intercept  24.59  33.8  41.92
1/slope  -0.4571  -0.03673  -0.2359

95% Confidence Intervals
Slope  -4.945 to 0.5696  -62.21 to 7.77  -11.27 to 2.789
EFFECTS OF FERTILIZER ON PHYTOREMEDIATION

Y-intercept 33.75 to 73.85 665.5 to 1175 126.6 to 228.8
X-intercept 12.88 to +infinity 17.08 to +infinity 18.38 to +infinity

Goodness of Fit
R square 0.1337 0.1292 0.0819
Sy.x 30.18 383.1 76.94

Is slope significantly non-zero?
F 2.779 2.671 1.606
DFn, DFd 1, 18 1, 18 1, 18
P value .113 .120 .221
Deviation from zero? Not Significant Not Significant Not Significant

Equation Y = -2.188*X + 53.8 Y = -27.22*X + 920 Y = -4.239*X + 177.7

Data
Number of X values 20 20 20
Maximum number of Y replicates 10 10 10
Total number of values 20 20 20
Number of missing values 0 0 0

F2 Concentration (mg/kg) F3 Concentration (mg/kg) F4 Concentration (mg/kg)
Best-fit values ± SE
Slope 1.303 ± 2.425 4.375 ± 15.05 0.4861 ± 2.701
Y-intercept 64.5 ± 17.64 975 ± 109.5 200 ± 19.64
X-intercept -49.51 -222.9 -411.4
1/slope 0.7676 0.2286 2.057

95% Confidence Intervals
Slope -3.793 to 6.398 -27.25 to 36 -5.188 to 6.16
Y-intercept 27.44 to 101.6 745 to 1205 158.7 to 241.3
X-intercept -infinity to -5.006 -infinity to -22.15 -infinity to -27.36

Goodness of Fit
R square 0.01578 0.004672 0.001797
Sy.x 55.78 346.2 62.12

Is slope significantly non-zero?
F 0.2885 0.08449 0.0324
DFn, DFd 1, 18 1, 18 1, 18
P value .598 .775 .859
Deviation from zero? Not Significant Not Significant Not Significant

Equation Y = 1.303*X + 64.5 Y = 4.375*X + 975 Y = 0.4861*X + 200
EFFECTS OF FERTILIZER ON PHYTOREMEDIATION

Data

<table>
<thead>
<tr>
<th></th>
<th>1st Group</th>
<th>2nd Group</th>
<th>3rd Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of X values</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Maximum number of Y replicates</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total number of values</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Number of missing values</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>