# Improving Contaminated Soil Remediation – Using Plants to Promote Beneficial Bacteria

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## Abstract

Earthmaster Environmental Strategies has successfully developed plant growth promoting rhizobacteria (PGPR)-enhanced phytoremediation systems (PEPSystems®) for the remediation of petroleum hydrocarbons (PHC) and salt in contaminated soil. PEPSystems utilize PGPR seed treatment to improve plant growth and increase production of root biomass in impacted soils. This increases amounts of endogenous rhizobacteria in soil to enable degradation of PHCs, and uptake of salt into plant foliage. Oil and gas related contaminants in soil can harm the resident microbes, leading to poor quality soil and inhibited plant growth, resulting in a further reduction in soil quality. PEPSystems can be used to restore soil health to facilitate accelerated degradation of organic contaminants, salt sequestration and improved plant growth. Growth chamber studies were conducted with poor quality subsoil with very low bacteria numbers and salt contaminated agricultural soil. These studies demonstrated that the presence of plants increased the amount of culturable bacteria in the soil and maintained the elevated levels for a longer period of time compared to biostimulated unplanted soil or soil that received the addition of a microbial mix in the absence of plants. Results from full scale phytoremediation studies demonstrated the benefits of having good plant growth when using rhizobacteria to remediate PHCs and showed that bacteria levels are maintained in the soil even when active plant growth is not present, provided some roots and their associated bacteria are present in the soil.

## 1. Introduction

Soil contamination and disruption by industrial activities can harm natural ecosystems and pose a threat to future land use. In Alberta, oil and gas exploration, production, and shipping has led to large amounts of soil contamination by petroleum hydrocarbons (PHCs) and produced water (salt). The Alberta Energy Regulator (AER) estimates that there are approximately 170,000 abandoned wells and well sites (a common source of contaminated soil) in the province (AER, 2020). Estimates by Earthmaster Environmental Strategies has found that approximately 25% of these sites contain salt and/or PHC contaminants and will require remediation (Murray et al., 2019). Soil contamination also arises from pipeline spills and leaks. In Alberta, there is an average of 600 reported pipeline releases per year into the surrounding environment (Nikiforuk, 2017). Between 2006 and 2010, pipelines leaked roughly ~27,700 $m^3$ of oil (Kheraj, 2012).

There are a number of methods available and routinely utilized for the remediation of PHC but there are fewer for salt in soil. These include physical, chemical, and biological methods (reviewed in Ossai et al., 2020; Litalien and Zeeb, 2020). In Alberta, the most common

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method used to remediate PHC and salt contaminated soil is off-site landfill disposal. This method can be cost effective in developed regions where land is cheap and landfills are nearby but quickly becomes prohibitive with remoteness of sites and lack of nearby disposal locations. Furthermore, landfilling is not an environmentally sustainable option as it does not allow for remediation and reuse of the disposed soil, transportation of the soil creates a large carbon footprint, and liability and risk associated with the contamination is simply transferred from the site of origin to the landfill.

There are numerous bioremediation strategies to treat PHCs in soil (reviewed in Koshlaf and Ball, 2017). Natural attenuation is a passive process that relies on the natural degradative capabilities of the soil to reduce PHC levels. Bioaugmentation involves inoculation of contaminated soil with exogenous or enriched endogenous PHC degrading bacteria (reviewed in Fantroussi and Agathos, 2005). Results with exogenous species are highly varied as the contaminated soil environment can significantly impede the successful establishment of foreign microorganisms. Inoculation with enriched cultures of endogenous bacteria can be more successful as these bacteria are better adapted to the environment and are less likely to be inhibited by soil conditions and other microorganisms (Gkorezis et al., 2016; Koshlaf et al., 2017). In cases where the soil is unable to sustain significant bacteria biomass or hydrocarbon metabolism, biostimulation (the addition of nutrients such as terminal electron acceptors and inorganics) can be used to enhance the activity of endogenous organisms (Adams, et al., 2015). Identification of limiting nutrients and their addition to contaminated soil is an effective method for PHC removal (Koshlaf et al., 2017). There is also evidence that biostimulation can be more effective when used in combination with bioaugmentation (Pandey et al., 2009), demonstrating the additive potential of the technologies to maximize the remediation potential.

Phytoremediation is a form of bioremediation that utilizes the physical and metabolic activities of plants to remove environmental contaminants, including both PHCs and salt (Gerhardt et al., 2009; Gerhardt et al., 2017a and 2017b; Greipsson, 2011; Pilon-Smits, 2005). This makes it an attractive remediation method as PHC and salt contamination frequently occur together. Similar to bacterial technologies, phytoremediation of PHCs is a cost effective method when compared to off-site landfill disposal (which is seen as a cheap remedial option), can be applied in remote locations, and time to remedial endpoint is predictive (Murray et al., 2019).

Plant root structures play an active role in physically reshaping the soil environment by enhancing soil porosity, aeration, nutrient content and microorganism biomass/distribution (Gerhardt et al., 2009; Gkorezis et al., 2016), supporting a complex and dynamic interaction between plant roots and the soil microbial community. The plants support microorganism communities present in the rhizosphere by releasing nutrients into the soil via exudates (Bais et al., 2006; Chaudhry et al., 2005; Zhou et al., 2016). In turn, bacteria and fungi in the soil play an essential role in nutrient cycling, plant growth promotion, and disease resistance (Hayat et al., 2010; Pii et al., 2015). In phytoremediation, these bacterial/plant interactions in the soil can be exploited for the enhanced remediation of organic contaminants, PHC degradation, and the restoration of soil health (Chaudhry et al., 2005; Gkorezis et al., 2016; Glick, 2003). This approach can be especially effective when applied with proper agronomic techniques (Gerhardt et al., 2009 and 2017a).

Earthmaster has developed a full scale phytoremediation system that utilizes plant growth in association with Plant Growth Promoting Rhizobacteria (PGPR). PEPSystems® (PGPR-Enhanced Phytoremediation Systems) is a commercial technology developed by Earthmaster, in collaboration with the University of Waterloo, which has been used to successfully remediate

PHC contaminated sites and re-vegetate salt contaminated soil across Canada. Earthmaster is continuing the development of PEPSystems and is particularity focused on improving how the technology enhances soil bacteria proliferation, persistence and efficacy.

This paper reports on preliminary studies that examine the extent to which PEPSystems enhances bacterial biomass under stressful soil conditions, including restoration of soil bacteria levels in poor quality clay soil which previously contained high concentrations of methanol, and in salt contaminated soil. The use of plants in combination with a commercially available bioaugmentation product was also examined. These results will be discussed and compared with plant and bacteria population studies associated with commercial phytoremediation applications.

## 2. Materials and Methods

### 2.1 Plant Growth Promoting Rhizobacteria (PGPR)

PGPR were isolated previously from naturally occurring and common soil bacteria in three Canadian locations. Strains *Pseudomonas sp.* (UW3, GenBank Accession Number KF145175) (Glick et al., 1995; Chang et al., 2014) and *Pseudomonas sp.* (UW4, GenBank Accession Number CP003880) (Glick et al., 1995; Duan et al., 2013) were isolated as described previously (Gurska et al., 2009) from soil located in southern Ontario. Strain *Pseudomonas corrugata* (CMH3, GenBank Accession Number KF041156) was isolated as previously described (Chang et al., 2014) from soil located in southern Saskatchewan. Strains *Pseudomonas corrugata* (Nota 4) and *Pseudomonas marginalis* (Nota 7) were isolated from soil collected from a remote location in the Northwest Territories. PGPR strains (selected for high levels of 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase and indoleacetic acid (IAA)) were purified, cultured, and coated onto the surface of plant seeds using a Hege 14 commercial liquid seed treater (Wintersteiger, Austria). The bioaugmentation product (microbial mixture) was obtained from BioNorth Solutions (Thunder Bay, Canada).

### 2.2 PGPR-Enhanced Phytoremediation Systems (PEPSystems®)

Plant species were chosen for maximum biomass production, suitability for the growing area, and ease of treating and sowing. Specific species were selected for the sites from a group consisting of annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), slender wheatgrass (*Elymus trachycaulus*), and/or creeping red fescue (*Festuca rubra L.*). All seeds were purchased from commercial seed suppliers and were treated by the University of Waterloo or Earthmaster. Seedbeds were prepared using standard agricultural practices and PGPR-treated seed was sown using standard agricultural techniques. Plants were allowed to grow to maturity prior to end-of-season soil sample collection.

### 2.3 Field Sites

Four full scale phytoremediation sites were located in northern Alberta and the Northwest Territories. Contained and secure soil treatment areas were constructed on each site to ensure no off site movement or leaching of PHC from the contaminated soil related to oil and gas drilling and production related activities. PEPSystems was deployed at each site to remediate the PHC contamination. In general, soil samples were collected at the beginning and end of the growing season, depending on site access, using a hand held Dutch auger at depths down to 0.50 m. PHC analyses were conducted as previously described (Murray et al., 2019).

## 2.4 Growth Chamber Studies

Poor quality clay subsoil was obtained from northern British Columbia. The soil contained an average of 135 mg/kg methanol which was volatilized when the soil was dried and homogenized for laboratory studies. Salt contaminated soil (electrical conductivity (ECe) ~14 dS/m and sodium adsorption ratio (SAR) ~17.5) was obtained from southern Saskatchewan. Laboratory test results for the dried and homogenized soil showed an average ECe of ~14 dS/m, a SAR of ~17.5, a chloride concentration of 2,830 mg/kg and sodium concentration of 1,233 mg/kg. All soil analyses were conducted by a commercial third party analytical laboratory accredited by the Canadian Association for Laboratory Accreditation Inc. and/or the Standard Council of Canada for the individual required assays. Results were analyzed for statistical significance using a Student's t-test.

A 3 g mixture of annual ryegrass, perennial ryegrass and tall fescue was grown in 900 g of soil contained in pots without drainage holes such that water added to the pots could not flow out of the bottom of the pot. Plants were grown using a growth chamber (GCW30, Environmental Growth Chambers) with a cycle of 16 hours at 25°C in the light and eight hours at 15°C in the dark. Each pot was fertilized using 36-18-0 fertilizer at the start of the experiment only. Negative controls for the growth chamber studies consisted of pots containing soil plus fertilizer only (biostimulated).

## 2.5 Culturable Bacteria Quantitation

Culturable bacteria were isolated from soil samples as follows. A total of 2 g of soil was weighed into a sterile conical tube or flask. Twenty mL of sterile 0.85% NaCl was added to the soil and the mixture was placed on an orbital shaker at 1,000 rpm for 30 minutes at room temperature. The bacteria in the supernatant was quantitated using 10 fold serial dilutions. A total of 100 µL of each dilution was spread on separate TSA (Tryptone Soya Agar) plates and the plates were incubated at 25°C for 24 to 72 hours. The colonies on each plate were counted and the amounts of bacteria were calculated as colony forming units (cfu's) per gram of dry soil of culturable bacteria. As these results represent only the culturable bacteria they will be an underestimate of the total amount of bacteria. Although not all soil bacteria are represented in these analyses, the results do give an approximation of microbial changes in soil.

# 3. Results and Discussion

## 3.1. Bacteria Biomass Restoration in Poor Quality Methanol Impacted Subsoil

Poor quality clay subsoil was obtained from a well site located in northern British Columbia. Initial laboratory test results for the soil soon after collection from the field showed an average methanol concentration of 135 mg/kg, with levels as high as 21,700 mg/kg. Methanol is highly soluble in water and can be quickly biodegraded (CCME, 2017); however, the methanol was persistent in the soil at this site. A preliminary quantitation of soil bacteria was completed and showed a culturable bacteria level of $<1x10^2$ cfu/g of soil, significantly less than the expected levels of between $10^5$ and $10^8$ cfu/g (Trevors et al, 2010). It is possible that elevated methanol concentrations inhibited the proliferation of bacteria resulting in the low levels encountered in this soil. In addition, the soil was found to be deficient in nitrogen (data not shown). Upon drying and homogenization of the soil, methanol concentrations decrease substantially (<6 mg/kg) which likely led to a more permissive environment for bacterial growth.

In an effort to restore and enhance the bacterial population of the soil, growth chamber studies were conducted using unplanted soil (biostimulation control, n=3) and soil upon which

PEPSystems was deployed (grass seed mix coated with PGPR UW3/4, n=3). Both sets of pots were fertilized with 36-18-0 at the start of the experiment only, such that 160 mg of total nitrogen was added to each pot. The results for the restoration of bacteria amounts are provided in Figure 1.

The culturable bacteria amounts in both the biostimulated unplanted control soil and the bioaugmented PEPSystems soil increased to normal levels within 3 weeks; however, levels quickly dropped in the biostimulated control. The bacteria amounts were more persistent in the pots containing plants, and remained at a higher level for several more weeks when compared to the biostimulated soil. These results were statistically significant ($P<0.05$) suggesting that the absence of plants resulted in reduced bacteria populations. Although both methods were successfully able to restore bacteria levels to the damaged soil in the short term, the prolonged increased bacteria levels in the PEPSystems pots would provide an advantage for remediation of any organic soil contaminants. It is probable that the bacteria amounts in both sets of pots are decreasing due to decreasing soil nutrients; however, despite the pots not being fertilized beyond the start of the experiment and PEPSystems pots supporting plant biomass and utilizing more soil nutrients, there is an advantage for bacterial populations in challenging soil conditions to be in the presence of plants. Although this analysis only gives a measure of culturable bacteria, it does approximate trends in changes in soil bacteria due to plant growth. Also, because many PGPR and PHC degrading bacteria are culturable (Gerhardt et al 2017), such bacteria are represented in the data.

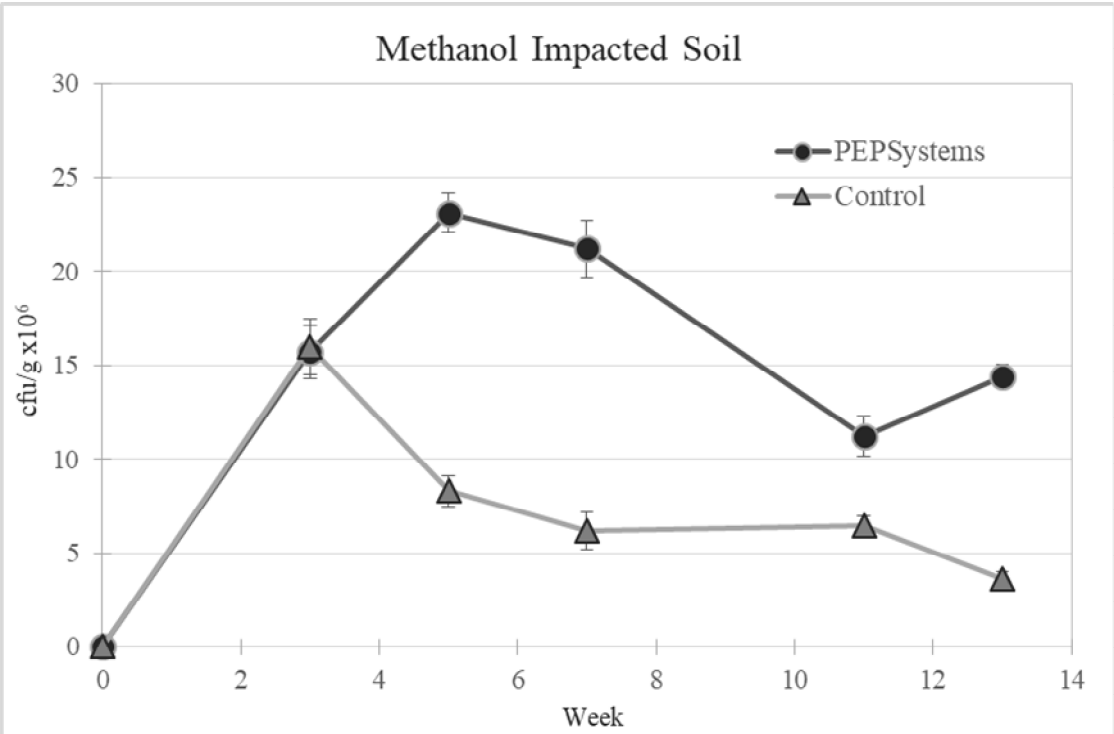


**Figure 1: Culturable bacteria levels in control (biostimulated using fertilizer and water, no plants) and PEPSystems (biostimulated and bioaugmented using plants and bacteria) subsoil impacted by methanol. Bacteria amounts (cfu/g x $10^6$) were plotted over time. Results represent the average bacteria amounts for n=3 pots ± S.E.**

### 3.2. Bacteria Biomass Augmentation in Salt Contaminated Soil

Agricultural soil was obtained from a produced water spill area located in southern Saskatchewan. Laboratory test results showed elevated ECe, SAR, sodium, and chloride levels and the soil was unable to support agricultural crops. A preliminary quantitation of soil bacteria was completed and showed a culturable bacteria level of ~$4.5x10^6$ cfu/g of soil.

In an effort to increase the bacterial amounts in the soil, growth chamber studies were conducted using unplanted soil (biostimulation control, n=3) and soil sown with PEPSystems (grass seed mix coated with PGPR UW3/4, n=3). Both sets of pots were fertilized with 36-18-0 at the start of the experiment only, such that 160 mg of total nitrogen was added to each pot. The bacteria restoration results are provided in Figure 2.

The culturable bacteria amounts in both the biostimulated unplanted control soil and the bioaugmented PEPSystems soil increased during the first 3 weeks with the PEPSystems soil showing higher bacteria counts. This difference was maintained throughout the length of the experiment as the amounts of bacteria remained steady, demonstrating the advantage plants provide to increasing the amounts of bacteria in the soil. Although both methods were successfully able to improve bacteria levels in the salt contaminated soil, PEPSystems was able to provide an advantage for maintaining improved soil bacteria numbers. As with the poor quality subsoil, the pots were not fertilized beyond the start of the experiment. The PEPSystems pots supported plant biomass and likely utilized more of the nutrients than the control unplanted pots; however, despite the drain on nutrients, there was an advantage for bacterial populations to be in the presence of plants. PEPSystems was also able to support plant growth in the soil despite high salt levels.

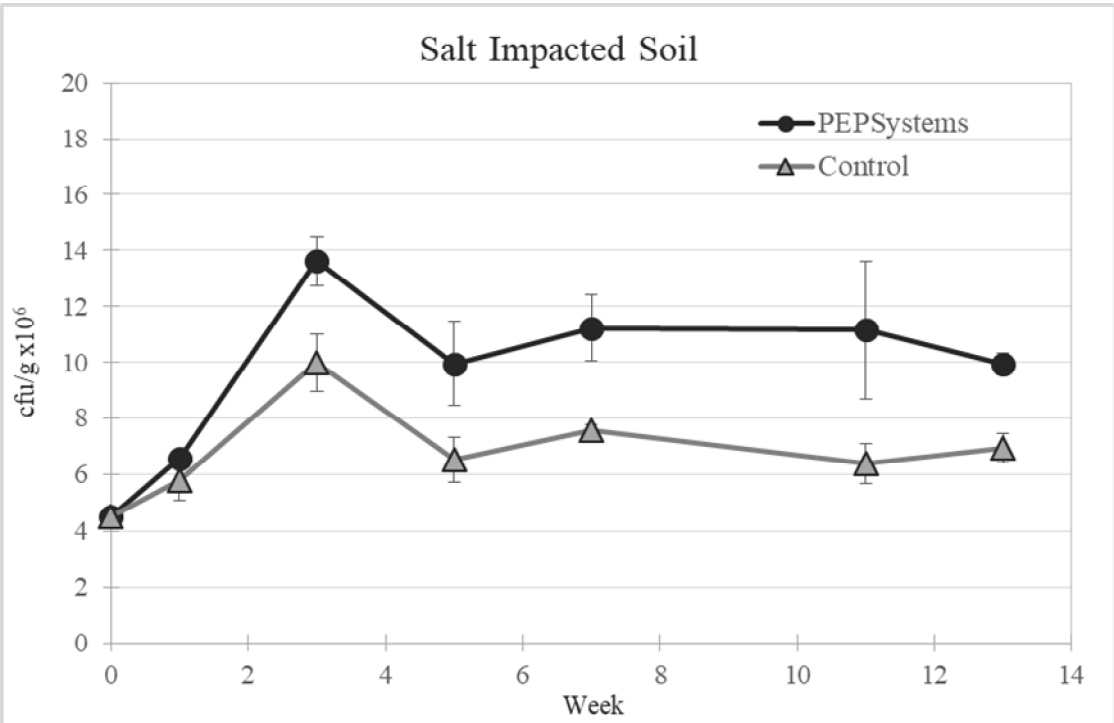


**Figure 2: Culturable bacteria levels in control (biostimulated using fertilizer and water, no plants) and PEPSystems (biostimulated and bioaugmented using plants and bacteria) agricultural soil impacted by salt. Bacteria amounts (cfu/g x $10^6$) were plotted over time. Results represent the average bacteria amounts for n=3 pots ± S.E.**

To further improve the bacteria amounts in salt contaminated soil, growth chamber studies were conducted using unplanted soil (biostimulation control, n=3), soil mixed with a microbial additive (bioaugmentation control, n=3), soil sown with a grass seed mix = (soil+grass, n=3), and soil mixed with a microbial additive and sown with a grass seed mix (soil+microbes+grass, n=3). All sets of pots were fertilized with 36-18-0 at the start of the experiment only, such that 160 mg of total nitrogen was added to each pot. The preliminary results for the quantitation of bacteria are provided in Figure 3.

The culturable bacteria amounts in both the biostimulated control and bioaugmented soils increased during the first week and then started to decrease. Those pots that also contained grass (soil+grass and soil+microbes+grass) showed bacterial amounts that continued to rise above the biostimulated and bioaugmented soil. These results also showed that the effects of bioaugmentation and grass were additive and provided a statistically significant increase in bacteria amounts over the bioaugmentation control (soil+microbes, P=0.01), confirming that there is an advantage for bacterial populations to be in the presence of plants.

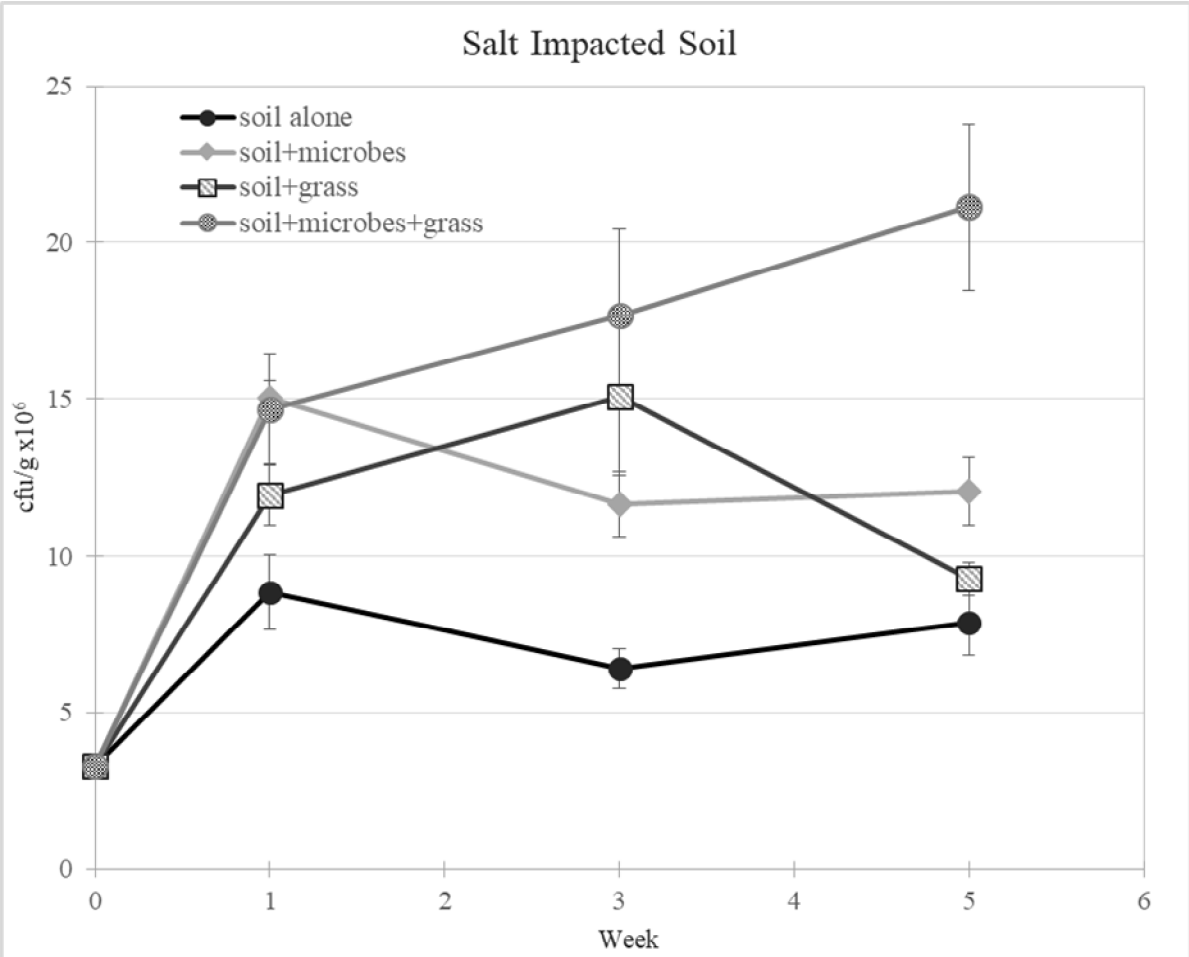


**Figure 3: Culturable bacteria levels in control (biostimulated using fertilizer and water) and PEPSystems (biostimulated and bioaugmented using plants and bacteria) agricultural soil impacted by salt. Bacteria amounts (cfu/g x $10^6$) were plotted over time. Results represent the average bacteria amounts for n=3 pots ± S.E.**

### 3.3. Bacteria–Associated Remediation as a Function of Plant Growth

Full scale commercial deployment of PEPSystems occurred at a site located in the Northwest Territories where PHC fraction F2 (applicable remediation guidelines: CCME, 2008) was the contaminant of concern. PEPSystems remediates PHC through degradation by the bacteria associated with plant roots. Phytoremediation activities had been conducted in 2015 at this site prior to the 2016 growing season shown in Figure 4; therefore, the surface soil layer contained some organic matter (i.e. plant roots and associated PGPR) from the previous successfully remediated overlying soil layer that had been removed. The underlying soil layer was prepared for planting in the spring of 2016; however, very poor weather conditions during spring seeding activities resulted in an area of poor plant growth following germination (Figure 4). Laboratory test results for most soil samples (with the exception of locations 12 and 23) exceeded the remediation guideline value for fraction F2 (260 mg/kg) at the start of the growing season. At the end of the growing season, soil from the sampling locations shown in red continued to exceed the remediation guideline value while samples from the sampling locations depicted in black complied with the guideline value. These two general areas of poor and good remediation corresponded to the areas on the site which supported poor and moderate plant growth, respectively. The treatment area in black showed a decrease in average fraction F2 concentration of 55% over the growing season and the area in red showed a decrease of 40%, demonstrating that remediation was more effective in the areas that had better plant growth. These results also show that remediation continued in underlying soil treatment layers that contain plant roots/biomass and bacteria that originated from the overlying treated soil layer.

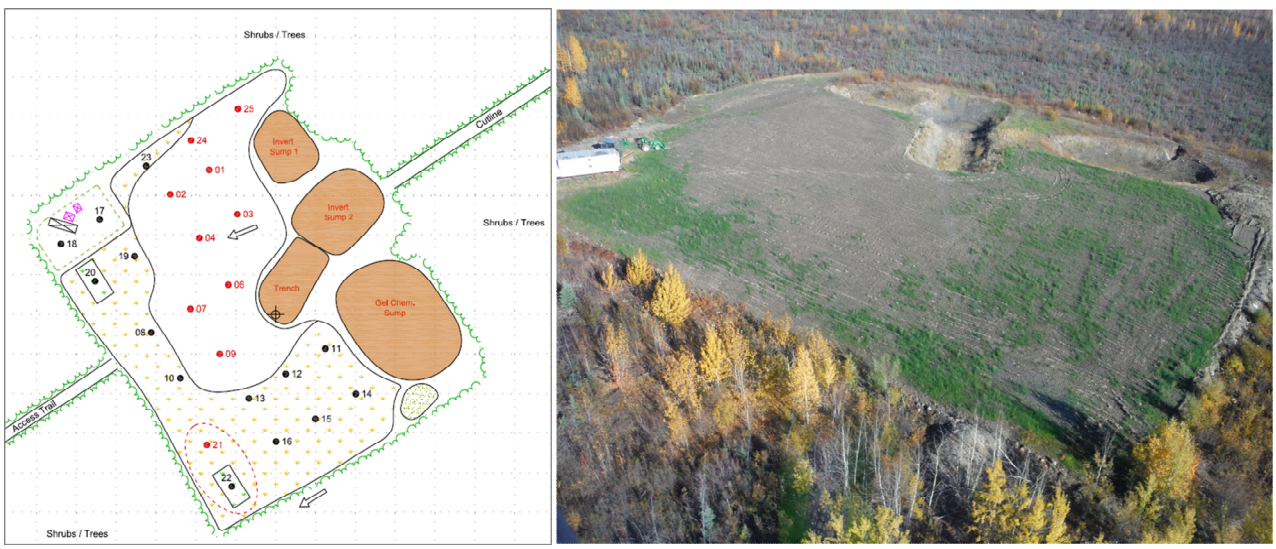


**Figure 4: Northwest Territories phytoremediation site showing the soil sample locations (left) and the plant growth (right). Sampling locations in red had end of growing season laboratory soil test results that exceeded the remediation guideline value for fraction F2 and black sampling locations had results that complied with the guideline value.**

### 3.4. Bacteria Quantitation at Phytoremediation Sites

Full scale commercial deployment of PEPSystems occurred at three sites in northern Alberta where PHC fraction F2 was the contaminant of concern. Treatment area sizes range from 2,700 $m^2$ to 13,000 $m^2$ and contained soil volumes ranging from 6,000 $m^3$ to 11,000 $m^3$. Phytoremediation was conducted on PHC contaminated soil in a series of layers with a depth

equivalent to the rooting depth of the grass species used. Once a surface soil layer was treated it was removed and placed in a stockpile for later reuse and treatment was then initiated on the newly exposed underlying soil layer. Preliminary quantitation of bacteria amounts in soil samples collected from the most recently treated soil layers were compared to bacteria amounts in previously treated soil layers that had been removed from the treatment area and stockpiled.

The results of the culturable bacteria quantitation are presented in Figure 5. There were no statistically significant differences in the bacteria counts between recently treated and more aged treated and stockpiled soil. This suggests that the culturable bacteria amounts in the stockpiles remain at levels comparable to the vegetated and active soil treatment layers, despite not having active plant growth. This is also apparent when non-vegetated older treated soil stockpiles (from 2018) were compared to non-vegetated newer stockpiles (2019, data not shown), suggesting that the treated and stockpiled soil retained their bacteria content for a significant amount of time following the termination of active plant growth, and that remediation of PHC continued in the stockpiles over time. This was expected as the stockpiled soil contained a significant amount of organic matter from the inclusion of above and below ground plant material that was present in the soil layer prior to stockpiling. Figure 5 also suggests that there is no difference in the amount of bacteria in the soil in relation to the amount of PHC.

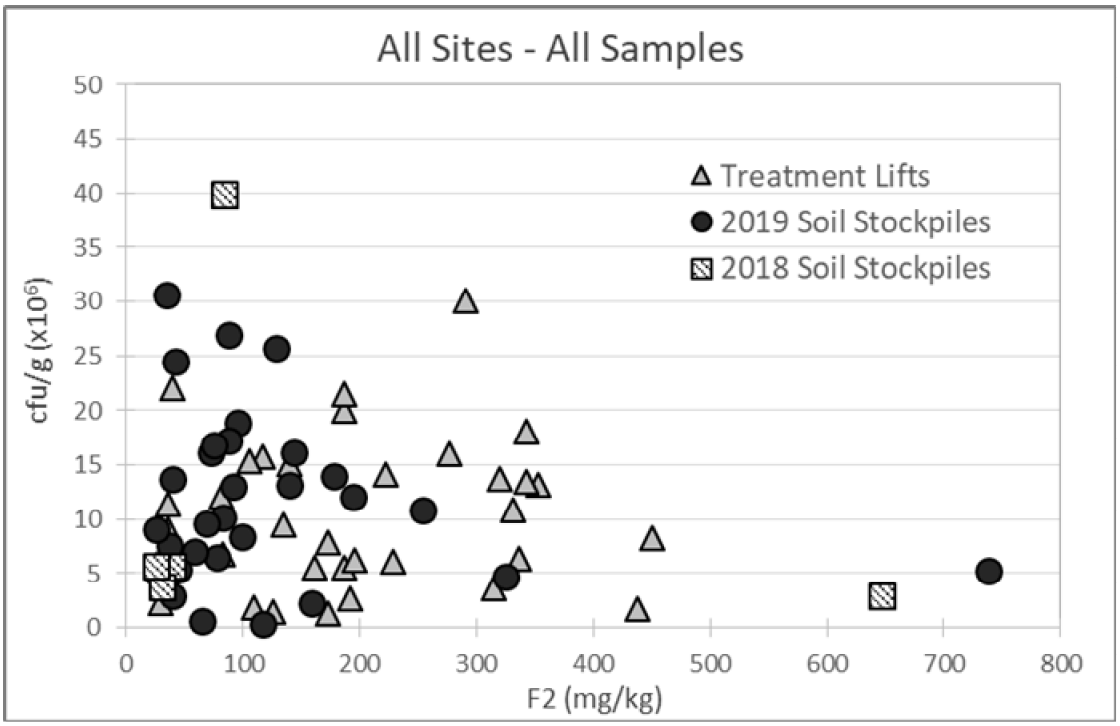


**Figure 5: Soil bacteria levels following remediation by PEPSystems at three sites in Northern Alberta. Bacteria amounts (cfu/g x $10^6$) were plotted against the fraction F2 concentrations (mg/kg) in the soil sample. Results represent the culturable bacteria levels for all samples collected from all three sites.**

## 4. Conclusions

Growth chamber studies conducted with poor quality subsoil with very low culturable bacteria numbers and salt contaminated agricultural soil demonstrated that the presence of plants increased the amount of culturable bacteria in the soil. The soil maintained the elevated levels for

a longer period of time when compared to biostimulated soil and soil containing a microbe mix without plants. Full scale field phytoremediation results demonstrated the benefits of having good plant growth when using rhizobacteria to remediate PHCs. In addition, field results also showed that bacteria levels were maintained in previously treated soil even when active plant growth was not present, provided some residual plant material remained in the soil.

## 5. Acknowledgements

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