A SURVEY OF THE BACTERIAL ROOT ENDOPHYTES ASSOCIATED WITH THE NATURAL VEGETATION AT THE BITUMOUNT PROVINCIAL HISTORIC SITE, ALBERTA, CANADA

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ABSTRACT

The Bitumount Provincial Historic site is the location of two of the world's first oil extracting and refining operations. Bitumount, located in the Athabasca oil sands of Alberta, is thought to be impacted by hydrocarbons through both natural and human activity. Plants have been able to recolonize the site in spite of varying hydrocarbon levels, through means of natural revegetation. Due to the apparent resilience of these plant species, Bitumount offers a unique opportunity to study the root-associated bacterial communities. This study was designed to achieve a better understanding of the root-associated partnerships occurring within naturally revegetated hydrocarbon contaminated soils. Plant and soil samples were collected in June 2014. Six representative plant species were identified and collected based on abundance on site, including smooth brome (*Bromus inermis*, SB), horsetail species (*Equisetum spp.*, HT), slender wheatgrass (*Agropyron trachycaulum*, SW), Kentucky bluegrass (*Poa pratensis*, KB), an unspecified member of the pea family (*Fabaceae*, PF), and wild strawberry (*Fragaria virginiana*, WS). Population abundance of rhizosphere and root endosphere bacteria was significantly influenced ($p<0.05$) by plant species and sampling location. The vegetation was found to support diverse root endophytic communities despite hydrocarbon contamination. Culture dependent techniques were able to identify some of the more abundant bacteria characterized by high-throughput sequencing. In general, members of the *Actinomycetales*, *Rhizobiales*, *Pseudomonadales*, *Burkholderiales*, and *Sphingomonadales* orders were the most commonly identified via both techniques. Community structure of root-associated bacteria was found to be influenced by both plant species and sampling location. Quantitative real-time polymerase chain reaction was used to determine the potential functional diversity of the root endophytic bacteria. The gene copy numbers of 16S rRNA and two hydrocarbon degrading genes (CYP153 and *alkB*; both of the alkane hydroxylase family) were quantified. The gene copy abundance of 16S rRNA, CYP153, and *alkB* was significantly affected by the interaction of plant species and sampling location. The increased colonization of hydrocarbon degrading bacteria within grass species emphasizes their ability to be used for reclamation efforts.

Overall, it was found that the endosphere was able to support diverse bacterial communities with known plant growth promoting abilities. In addition, the diversity and abundance of the endophytic bacteria was influenced by many different factors instead of one

sole dominant one. The findings of this study provide insight into the root-associated bacterial communities occurring within natural revegetated soils.

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ACC	1-aminocyclopropane-1-carboxylate deaminase
AlkB	Alkane hydroxylase
AlkG	Rubredoxin
AlkT	Rubredoxin reductase
BMO	Butane monooxygenase
FAD	Flavin adenine dinucleotide
HT	Horsetail spp. *(Equisetum spp.*)
KB	Kentucky bluegrass *(Poa pratensis*)
LB	Liquid broth
MDS	Multidimensional scaling
MRPP	Multi-response permutation procedures
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
PF	An unspecified member of the pea family *(Fabaceae)*
pMMO	Particulate methane monooxygenase
QPCR	Quantitative real-time polymerase chain reaction
sMMO	Soluble methane monooxygenase
SB	Smooth brome *(Bromus inermis)*
SOM	Soil organic matter
SW	Slender wheatgrass *(Agropyron trachycaulum)*
TSA	Tryptic soy agar
WS	Wild strawberry *(Fragaria virginiana)*

1.0 INTRODUCTION

Refined petroleum and petroleum-based products have become an integral part of our modern society. Originating from fossil deposits, petroleum is a fuel with a variable composition of compounds, mainly hydrocarbons. Bitumen, an unconventional petroleum, comes from unconsolidated sand deposits known as the oil sands (Masliyah et al., 2004). A viscous and heavy oil, bitumen must be refined to produce fuels that can be used by consumers. As the demand of petroleum products increases, the development and mining of the oil sands remain economically important to Canada. Next to Saudi Arabia and Venezuela, the Alberta oil sands are the third largest crude oil reserves in the world (Alberta Energy, 2016). This industry plays a significant role in the Canadian economy and it is anticipated that from 2010- 2035 investment in oil sands development and operations will contribute 2.28 trillion CAD to the country's gross domestic product (Parajulee and Wania, 2014).

In Canada, hydrocarbons are one of the most dominant soil contaminants (Canadian Council of Ministers of the Environment, 2014). They can enter the environment through transportation, pollutant discharges, mining, and extraction (Lozada et al., 2014), causing risk to any living plant or animal tissue (Das and Chandran, 2011). Current laws require oil sands companies to reclaim all disturbed land and return it to the government in a productive state (Alberta Government, 2014). Reclamation requires that rather than the land be identical to its previous state, that it represent a boreal ecosystem in Alberta's northern areas. Reclamation involves managing a wide range of disturbances that can occur to the soils during exploration and recovery including those to physical, chemical, and biological properties. A major process used in reclamation is to establish plant species native to the area (Canadian Association of Petroleum Producers, 2015). However, newly reclaimed land can be a challenge for plants to recolonize, due in part to residual hydrocarbon contamination causing plant toxicity, or an imbalance of soil conditions.

Bitumount, located within the Athabasca oil sands of northeastern Alberta, is the location of the first oil separation and refining operation. It is now a provincial historic site, noted for pioneering many of the technologies and extraction processes currently used in today's oil industry (Ferguson, 1985). In addition to its historical importance, Bitumount stimulates interest due to its physical characteristics. Bitumount is thought to be impacted by hydrocarbons through both natural and anthropogenic processes. The Athabasca oil sands are characterized by their shallow bitumen deposits which lead to increased hydrocarbon content near the soil surface. In addition, Bitumount has never been reclaimed since its closure in the 1950's, allowing for additional hydrocarbon to persist after decades of oil exploration and mining. These two conditions have resulted in elevated hydrocarbon content throughout the site. Despite this, a variety of vegetation has been able to recolonize the site, including an area of hardened bitumen known as the Quarry, through the process of natural revegetation.

Natural revegetation in an contaminated environment is generally slow or minuscule (de-Bashan et al., 2012), and relies largely on the dispersal of seeds and vegetative propagules by wind or animals from nearby vegetation (Young et al., 2013). However, the potential of natural revegetation of hydrocarbon contaminated soils is associated with several different plant species, for example, sunflower (*Helianthus annuus)* and Saskatoon berry (*Amelanchier alnifolia)* (Rahbar et al., 2012). The ability of plant species to colonize hostile environments can be influenced by plant-microbe interactions that is shown to alleviate the stress caused by hydrocarbon contamination allowing for plant growth (Arslan et al., 2015; Tara et al., 2014).

It is well recognized that plant roots interact extensively with a diverse community of soil biota; however, the nature of this relationship is not well resolved. The microbial community associated with plant roots consists of all the microorganisms within the rhizosphere, the rhizoplane, and the endosphere. Each microorganism is linked to the plant and therefore has the ability to affect the entire system. Soil microbes significantly influence many processes that occur within the soil environment including nitrogen cycling, nutrient acquisition, and soil formation (van der Heijden et al., 2008). Endophytic bacteria are of great interest in several studies due to their close relations with the host plant and their ability to directly act as plant growth promoters (Afzal et al., 2014). Endophytic bacteria are described as microorganisms that

colonize the interior of a plant without exerting any negative effects on the host (Reinhold-Hurek and Hurek, 2011). Awareness of the presence of bacterial endophytes dates back over a century (Hardoim et al., 2008) and their ability to promote plant growth has been extensively studied, as has their involvement in the remediation of hydrocarbons in the environment. The specific nature of their involvement often depends on the contaminant present, however several bacteria including *Pseudomonas*, *Mycobacterium*, *Acinetobacter* sp., and *Serratia marcescens* are noted as contributors to the remediation of hydrocarbons (Oliveira et al., 2012). The plant's ability to grow in hostile environments, such as Bitumount may be the result of this partnership with endophytic bacteria that have aided plant growth. Therefore, by identifying the endophytic bacteria, we can gain a better understanding of the interaction, which in turn, may lead to more efficient reclamation efforts to re-vegetate the land that was used for oil sands mining.

The main objective of this research was to increase our understanding of the plant microbe interactions associated with hydrocarbon impacted soils. The specific aims of this study were: 1) to conduct a survey of the endophytic bacteria associated with the naturally growing vegetation at the Bitumount Provincial Historic site using both culture dependent and independent methods, and 2) to quantify the presence of hydrocarbon degrading genes within the endophytic bacteria of plants growing throughout the site. To accomplish the above, as series of studies were conducted to evaluate the following hypotheses:

1. There will be a variety of endophytic root bacteria associated with the vegetation, and the bacterial populations will differ depending on the plant species and sampling location within the Bitumount site.
2. A portion of endophytic bacteria associated with plants growing at Bitumount will be culturable in a laboratory setting.
3. Endophytic root bacterial populations will contain hydrocarbon-degrading genes.

The following thesis is presented in manuscript-style format. Following this introduction, a literature review is presented in Chapter 2 which is then followed by two research studies (Chapter 3 and 4). Chapter 3 contains information of the specific endophytic root bacteria that were identified using culture dependent and independent methods. Chapter 4 focuses on the

quantification of hydrocarbon-degrading genes present within the root endophytic bacteria. Lastly, Chapter 5 summarizes the major findings of the research and suggests future work. Each study chapter is written to stand alone, therefore some repetition of information may occur between chapters.

2.0 LITERATURE REVIEW

2.1 Oil Sands

Oil sands are commonly referred to as tar sands and bituminous sands. They are composed of a combination of bitumen, quartz grains, spar grains, mica flakes, clay minerals, and other trace minerals (Mossop, 1980). Bitumen, a high molar mass viscous petroleum, consists of a mixture of hydrocarbons and is composed of a higher carbon and sulphur content than conventional crude oils (Ferguson, 1985). Bitumen is a very thick substance that does not flow freely, and must be processed and upgraded to a lighter synthetic crude oil, and then generally refined into gasoline or diesel fuels (Charpentier et al., 2009).

Canada is one of the largest sources of bitumen in the world, with its major deposits located in the western part of the country within the province of Alberta. Alberta has the third-largest crude oil reserve worldwide, next to Saudi Arabia and Venezuela (Alberta Energy, 2016). As of 2014, verified oil sands reserves in Alberta were 166 billion barrels, which underlie 142, 200 km^2 of land surface (Alberta Energy, 2016). The Alberta oil sands are thought to contain around 75-80% inorganic material such as sand, clay and minerals, 3-5% water and 10-18% bitumen (Engelhardt and Todirescu, 2005). Canada's three major bitumen sources located in Alberta are known as the Athabasca, Cold Lake and Peace River deposits.

2.1.1 Athabasca oil sands

The Athabasca oil sands, located in northeastern Alberta, are the largest of the three major oil sand areas. They cover an area of 40,000 km^2 (Oil Sands Discovery Centre, 2014) and account for 75% of the total oil sands reserves in Alberta (Ferguson, 1985). There are several theories on the origin of the Athabasca oil sands, which stem from the significant variability in its oil content. However, many agree the oil has migrated from permeable regions due to pressure, concentration, and gravitational gradients (Czarnecki et al., 2005). One of the defining

characteristics of the Athabasca oil sands is that the deposits are located near the surface of the soil, which allows removal by surface mining (Hein and Cotterill, 2006). The near surface deposits have not only allowed less invasive techniques for bitumen extraction but because of their ease of extraction permitted earlier oil exploration in the area compared to other oil deposits in the province.

2.1.2 Bitumount Historic Site

The Bitumount Provincial Historic site, located within the Athabasca oil sands, is of importance as two of the world's first oil sands extraction plants once operated on site (Fig. 2.1). Various companies including the Alcan Oil Company, International Bitumen Company, Oil Sands Limited, and the Government of Alberta (Ferguson, 1985) first implemented at Bitumount many of the pioneering technologies and extraction processes currently used in today's oil sands industry. Bitumount represents a time of early innovation as activity on the site contributed to the shift towards efficient commercial development of the oil sands via its innovative practices.

2.1.2.1 History

In 1923, Robert C. Fitzsimmons took over the Alcan Oil Company, which was located approximately 90 km north of Fort McMurray, Alberta (Oil Sands Discovery Centre, 2014). Fitzsimmons, a former farmer and businessman, began a drilling exploration at the site which he renamed Bitumount. In 1927, Fitzsimmons formed the International Bitumen Company Ltd. and continued to search for pools of oil (Oil Sands Discovery Centre, 2014). After being unable to adequately locate pools of oil using conventional drilling, Fitzsimmons adopted the method of mining and extracting. In 1930, he built a hot water separation plant on the Bitumount site that was modeled after on Dr. Karl Clark's experimental plant located on the Clearwater River (Oil Sands Discovery Centre, 2014). The process consisted of mixing oil sands with hot water in tanks, which would separate the bitumen froth and sand tailing. Due to lack of funding, the plant passed through many different owners and presidents, including Oil Sands Limited and the Government of Alberta, until 1958 when Royalite Oil Company ceased operations (Oil Sands Discovery Centre, 2014).

Bitumount was declared a Provincial Historic Site in 1974; however, public access is presently prohibited to ensure preservation. To date, there are no active efforts to reclaim the site

or to open the gates for public visitation. Bitumount is currently managed through the Historic Places Stewardship program, under the jurisdiction of Alberta Culture.

Fig. 2.1 Aerial photograph of the Bitumount Provincial Historic site, Alberta, Canada. Photograph taken by Jiri Rezac (2009).

2.1.2.2 Natural revegetation

Bitumount is not only of great interest due to its historical context, but also excites interest as a result of its natural physical characteristics which are thought to be impacted by hydrocarbons through both anthropogenic, and natural processes (Yergeau et al., 2012). Located near the Athabasca River, Bitumount has a natural bitumen outcrop resulting from deposits near the surface of the soil. In addition, since its closure in the late 1950's, Bitumount has never been reclaimed. These two variables have resulted in high levels of hydrocarbon content throughout the site. Despite this, a variety of plant species were able to recolonize the site through means of natural revegetation.

Research has focused on the natural revegetation of contaminated soils for several years. In an earlier study, Robson et al. (2004) conducted a survey of hydrocarbon-tolerant plant species in semi-arid grasslands in Western Canada. Although they had less plant cover than uncontaminated areas, they found a variety of grasses and legumes that were able to naturally revegetate flare-pit soils despite the stressful conditions. Their ability to naturally revegetate the contaminated land was largely related to presence of self-pollinating plant seeds and the proximity of native plants from nearby native prairies (Robson et al., 2004).

It has widely been discovered that a plant's ability to naturally revegetate depends on the plant-microbe interactions occurring on site. Fernet et al. (2015) determined that seed-associated bacteria were partially responsible for several plant species to persist in hydrocarbon contaminated soils due to the presence of hydrocarbon-degrading genes. Phillips et al. (2006) also determined that indigenous plant bacteria have the ability to impact contaminant degradation and therefore can potentially influence a plant's ability to colonize a hostile environments. Although there may be many factors involved, understanding the processes that occur during natural revegetation is important for guiding reclamation efforts.

2.2 Contaminated Soils

2.2.1 Hydrocarbon contaminated soils

One of the major drawbacks of exploiting petroleum as an energy source is the possibility of organic compounds being discharged into the environment as a result of contamination during exploration, transportation, and storage (Tara et al., 2014). The discharge of contaminants, such as hydrocarbons, polyaromatic hydrocarbons, toluene, and benzene can lead to their contamination of soil and water. Due to the toxic nature of the organic compounds, mutagenicity and carcinogenicity in animals and humans may occur if the discharge of the organic compounds enters the food chain (Afzal et al., 2014). Not only is the presence of hydrocarbon contamination potentially harmful to human and animal health, but it can also affect the natural environment. For example, hydrocarbon contamination can affect photosynthesis thus reducing nutrient acquisition for plants, alter microbial community structure, and affect soil structure and cause water stress within affected soils (Nie et al., 2011). Due to the many potential consequences caused by the discharge of organic compounds through the use of petroleum as an

energy source, the removal of these compounds from water and soil is a fundamental problem that needs to be addressed by the field of environmental sciences.

2.2.2 Reclamation

Given the known harmful consequences and impacts associated with the oil exploration and recovery industry, this same industry, under obligation of law is required to reclaim land used in the pursuit of its objectives (Alberta Government, 2014). The goal of reclamation is to bring the disturbed land to an equivalent land capability as it was prior to oil industry presence through the stabilization, contouring, maintenance, conditioning, and reconstruction of the land (Environmental Protection and Enhancement Act, 2014). Reclamation involves managing the physical, chemical, and biological disturbances that occur to soils during the exploration and recovery phases. This can include soil fertility, pH, nutrient cycles, and microbial communities (Sheoran et al., 2010). Currently, one major part of a reclamation effort is to plant vegetation native to the disturbed area. However, potential residual hydrocarbons within reclaimed land can cause toxicity leading to an inhibitory effect on growth and development of plants and lower organisms, including soil microorganisms (Khan et al., 2013). Therefore, as a complement to conventional reclamation techniques, biological approaches within contaminated systems have the potential to return land into an ecologically productive state.

Petroleum contaminated soils are treated using several different physical and chemical technologies, such as photolysis, combustion, and dispersant addition (Xu et al., 2013). However, not only can these methods be high cost and ineffective, but they can also lead to the addition of more toxic compounds within the contaminated system (Shahi et al., 2016). Biological approaches, such as phytoremediation and bioremediation, utilize plants, as well as their associated microorganisms, to degrade hydrocarbons in the soil. They are cost-effective, environmentally sustainable, and offer complete degradation of the pollutants (Fuentes et al., 2014). Although phytoremediation often works as a viable solution, several obstacles occur when the technique is used on a large-scale basis. These difficulties may include toxicity to plants due to the contaminants, low bioavailability of the contaminants, and potential evapotranspiration of volatile pollutants (Weyens et al., 2009). Therefore, the effectiveness of biological approaches

relies greatly on the establishment of a strong interaction between the plants and their associated microorganisms (Abioye, 2011).

Plant roots are able to support a variety of different microorganism which can potentially aid in the promotion of a healthy plant through several mechanisms, including pollutant degradation and phytotoxicity reduction. However, the ability of contaminants to impede these symbiotic relationships may potentially influence the effectiveness of the remediation. Even though bacteria play an important role in phyto- and bioremediation, there is still little known about how hydrocarbons influence plant-microbe interactions within contaminated soils. Therefore, a better understanding of the effects that contaminants have on the plant-microbe interaction is required to improve biological approaches as a technique for reclamation purposes.

2.3 Endophytes

2.3.1 Root microbiome

It is well recognized that plant roots and soil biota interact extensively, but the nature of the relationships with individuals or consortia of microorganisms is not well resolved. The community of microorganisms associated with plant roots are considered to be part of the root microbiome. This microbiome consists of all the microorganisms, including bacteria, within with the rhizosphere (soil surrounding the plant root), the rhizoplane (the root-soil interface) and the interior of the plant root (the endophytes). Each microorganism that is linked with a plant is a part of the plant-microbial system and can affect the functioning and structure of the entire system (Chebotar et al., 2015).

Within the rhizosphere and rhizoplane, bacterial growth can be stimulated by the interaction between bacteria and plant root exudates (Rosenblueth and Martínez-Romero, 2006). In turn, bacteria may be able to help stimulate plant growth through several mechanisms such as nitrogen fixation, solubilisation of phosphate, and production of siderophores and phytohormones (Farina et al., 2012). Certain types of bacteria, known as endophytes, are able to move from the rhizosphere into plant root tissue. Endophytic bacteria are defined as microorganisms that colonize the interior of a plant without exerting any negative effects on the host (Reinhold-Hurek and Hurek, 2011). Endophytes are considered to be a sub-population of the rhizosphere and rhizoplane microbiome (Germida et al., 1998), but they have characteristics

which distinguish them from other bacteria (Turner et al., 2013). As a result of evolution, endophytic bacteria have the ability to use the interior of plants, the endosphere, as an ecological niche to protect themselves from the unfavourable environmental conditions and to gain a stable source of nutrients (Mercado-Blanco, 2015).

The ubiquitous presence of endophytes has enabled their successful isolation from a wide diversity of plants. Likewise, endophytic bacteria are isolated from various plant tissues including the roots, stems, leaf blades, petioles, and leaves (Nair and Padmavathy, 2014). Once inside the plant, the bacteria may remain localized or may transfer throughout the plant. Similar to bacteria found within the rhizosphere and rhizoplane, endophytes have the potential to promote plant growth through several different mechanisms. The ability to help alleviate stress for the plant can help to revegetate hostile environments that would be more difficult without the benefits conferred through these endophytic associations.

2.3.2 Endophyte colonization mechanisms

The high percentage of endophytes within the root tissue are an indication that the roots are the primary site of endophyte entry (Kobayashi and Palumbo, 2000). After primary colonization occurs, a variety of bacterial endophytes can move to other areas of the plant, including stems, leaves, and reproductive organs, by entering the vascular tissues and dispersing systemically (Brader et al., 2014; Mercado-Blanco, 2015). Since endophytic bacteria are able to colonize the interior of plant tissue, they are thought to represent specialized members of the root microbiome. It is assumed that a combination of both environmental and genetic factors give a particular bacteria the ability to colonize the interior of a plant root (Hardoim et al., 2008). Given the presence of required elements (e.g. genetic material), several methods allow bacteria to colonize the interior of plants through both passive and active penetration.

Active penetration occurs when a bacterium has a specific adaptation that allows it to colonize the plant root (Reinhold-Hurek et al., 2015). An example of this is the formation of nodules by *Rhizobia* spp. The plant-rhizobia interaction is a frequently studied area of research. Colonization in some plant-rhizobium interactions can happen in a fashion similar to non-rhizobia bacteria such as entering through undamaged cells and/or through wounds, particularly from where adventitious or lateral roots protrude (Cocking, 2003). In other instances,

colonization can occur in the interior of hairy roots, when rhizobia penetrate roots tissues and the formation of nodules occurs. For this to occur, the curling of root hairs must be stimulated by the rhizobia and the nod factors (lipo-chitin oligosaccharides), leading to the formation of infection threads that allow the rhizobium to enter the host plant (Perret et al., 2000). This mode of active colonization results from the presence of required genes within the rhizobia.

Passive colonizers differ from active colonizers in that they will become endophytes by coincidentally entering plant roots since they lack the cellular machinery that is required to persist within the endosphere (Gaiero et al., 2013). Passive endophytes do not actively seek to colonize the plant as a result of stochastic events. This happens when bacteria are able to enter the root cortex by cracks that are present at the point of lateral root emergence (Ryan et al., 2008), or at root tips (Reinhold-hurek and Hurek, 1998). However, entering through lateral roots may not be considered a solely passive process as many bacteria have cell-wall degrading enzymes (Turner et al., 2013). The lack of genetic capabilities required for bacteria to actively colonize plant roots causes them to be less competitive than their counterparts. This results in them being less likely to act as plant growth promoters.

2.3.3 Diversity

Endophytic bacteria have not only been shown to colonize a variety of plants but also are comprised of a large variety of species. Within the root microbiome, bacteria are able to interact and to reach a stable level of diversity. Some bacterial species are considered dominant depending on their frequency and abundance within the host plant. However, there are a large variety of bacteria that are considered rare species due to their low abundance throughout the host plant. This equilibrium of dominant and rare species within an environment is considered the most important condition in the establishment of any ecosystem (Lodewyckx et al., 2002). Members of the Proteobacteria phyla, including Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria classes, in addition to members of the Firmicutes and Actinobacteria classes are most consistently identified from the endosphere, whereas members of Bacteroidetes, Planctomycetes, Verrucomicrobia and Acidobacteria classes are least commonly identified (Santoyo et al., 2015).

Endophytic diversity however, depends on variety of factors therefore making it difficult for direct comparisons between studies. For example, in a study conducted by Shi et al. (2014), 13 classes from 449 585 sequences of sugar beet plant were identified using pyrosequencing of which *Alphaproteobacteria*, *Acidobacteria*, *Gemmatimonadetes*, and *Actinobacteria* classes were dominant. The diversity of the endophytic bacteria was found to be influenced by both plant genotype and plant growth stage, as diversity was highest in the rosette formation and tuber growth stage compared to seedling growth and sucrose accumulation stage (Shi et al., 2014). Another study by Zarraonaindia et al. (2015) analyzed the endophytic communities of grapevine in New York and found that the diversity differed depending on the plant tissue sampled. Aboveground samples (leaves, grapes, and flowers) were less diverse than roots, which contained higher proportions of *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* spp., *Xanthomonadales*, *Rhizobiales*, *Bradyrhizobium*, *Saprospirales*, *Cytophagales*, and *Actinomycetales* spp. (Zarraonaindia et al., 2015). Other factors, both abiotic and biotic, influence microbial diversity including: soil moisture, organic matter (Brockett et al., 2012), pH, % carbon (Andrew et al., 2012), seasonality, effect of phytoplasma infection (Bulgarelli et al., 2013), and changes in physical environment (temperature, humidity, irradiance, and wind speed) (Ding et al., 2013).

2.3.4 Abundance

Population density of endophytic bacteria associated with plants depends on many factors including plant genotype, age, season of isolation, and soil type (Rosenblueth and Martínez-Romero, 2006). Overall, populations of endophytes present within the roots are most abundant when compared to stems, vascular tissues and flowers (Beattie, 2007). Culturable root endophyte populations generally range from 10^5 - 10^7 CFUs g^{-1} of fresh root weight (Compant et al., 2010) and decrease in abundance with vertical movement up the plant organs. Average densities of endophytes are 10^4 CFUs g^{-1} fresh weight in the stem and 10^3 CFUs g^{-1} fresh weight in the leaves, and in many cases, the number of bacterial endophytes in generative organs, such as fruits, seeds, and flowers are so minimal that they cannot be detected (Hallmann and Berg, 2006).

Since there are generally higher populations of endophytic bacteria within the roots zones compared to the above ground biomass, it is often thought that this is an indicator of more favourable conditions. According to Hallmann (2001), above ground biomass is considered to be less favourable due to large daily fluctuations in nutrient availability, UV radiation, temperature, and water content. In addition to the root providing exudates, the soil surrounding the roots provides a buffer that helps regulate water availability and change in temperature. However, these differences in populations throughout the plant are not constant and can vary based on both biotic and abiotic environmental conditions.

2.3.5 Plant growth promoting abilities

Endophytic bacteria have widely been characterized as having beneficial effects on the plants they colonize. Taghavi et al., (2009) noted that they aid in plant growth and health, which could be caused by phytohormones, enzymes involved in growth regulation metabolism, or from a combination of the two. They have also been shown to accelerate seedling emergence and promote plant establishment under adverse conditions (Araújo et al., 2002). Various studies have demonstrated that endophytic bacteria have the ability to control pathogens, insects, and nematodes. Bacterial endophytes have the ability to promote plant growth by different mechanisms including phytostimulation, biofertilization, and biocontrol.

Through phytostimulation, phytohormones are produced leading to a direct promotion of plant growth. Many endophytes have the ability to release a wide range of phytohormones, such as cytokinins, gibberellic acids, and auxins (Nair and Padmavathy, 2014) which can act to enhance various growth stages of the plant. The production of enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase aids in plant growth by reducing plant ethylene-levels and therefore reducing abiotic stress (Onofre-Lemus et al., 2009). ACC deaminase is known to be released by a variety of endophytes, including phylogenetically distant genera such as *Pseudomonas* spp., *Bacillus pumilus*, and *Variovorax caulinodans* (Fuentes-Ramirez and Caballero-Mellado, 2006).

A second mechanism by which endophytes facilitate plant growth is through biofertilization. In this process, bacterial endophytes are able to increase a plant's accessibility to or supply of a required nutrient. Examples include nitrogen fixation and phosphorus acquisition.

Nitrogen fixation is one of the most widely researched and studied modes of biofertilization. During nitrogen fixation, atmospheric nitrogen is converted to ammonia, which can be used as a nitrogen source for plants (Hayat et al., 2010). Phosphorus availability can also be increased by bacterial endophytes. During phosphorus solubilisation, bacteria release organic acids that dissolve phosphatic minerals and/or chelate cationic partners, which releases phosphorus into the solution (Khan et al., 2009). This leads to the phosphorus being more accessible for plant uptake.

The final mechanism by which endophytes are known to aid in plant growth is through biocontrol whereby endophytes protect the plant from pathogens. Biocontrol can occur through many methods, including: production of antibiotics, signal interference, destruction of fungal cells walls through enzymes, induced systematic resistance, and competition for nutrients and niches (Lugtenberg and Kamilova, 2009). One of the commonly known means by which biocontrol occurs is through the production of low molecular weight compounds, known as siderophores. Bacterial endophytes are able to produce siderophores, which increase the plant acquisition of iron. The ability for bacterial siderophores to acquire iron differs depending on the bacteria, but this increased competition helps to suppress phytopathogens by depriving them of the essential nutrient (Compant et al., 2005).

A bacterium has the ability to impact plant growth through one or more of these mechanisms giving it the potential to utilize any of these mechanisms at various times throughout the plant's life. Plant growth promoting bacteria have the biggest impact on plants in stressed environment by providing a competitive advantage or alleviating a strain and can have little or no effect when plants are under optimal conditions (Glick, 2010).

2.3.6 Microbial community structure in contaminated soils

Similar to pristine soils, microbial community structure in hydrocarbon contaminated soils is influenced by a wide array of factors including soil type, nutrient content, temperature, oxygen content, and pH. However, it is widely suggested that within polluted environments, microbial community structure is also largely influenced by the concentration and bioavailability of the contaminant (Zhang et al., 2012) as well as time of exposure (Muckian et al., 2007).

A study conducted by Mills et al. (2003), using different molecular profiling techniques found that the microbial community structure shifted after hydrocarbon contamination. Distinct groups of bacteria, such as *Betaproteobacteria,* were found to be dominant before hydrocarbon contamination. However, as levels of the contaminant increased, *Betaproteobacteria* decreased. There was also an increase in other distinct groups of bacteria, notably *Alphaproteobacteria* and *Gammaproteobacteria.* Bell et al. (2014) also found that hydrocarbon contaminant concentration had a significant effect of bacterial community structure. Communities from the rhizosphere of planted willows (*Salix* spp.) from highly contaminated soils (>2000 kg of hydrocarbons $g^{-1}$of soil) were distinct from all others, whereas communities in soils with low contamination (709 kg $g^{-1}$of soil) were distinct with only slight overlap with non-contaminated soils.

The shift in microbial community structure could be a result of different bacterial genera favoring the types and concentrations of hydrocarbons over others. It is documented that hydrocarbon contamination leads to a decrease in the microbial diversity within a given soil environment (Muckian et al., 2007; Richardson et al., 2015; Vázquez et al., 2009). However, several studies have found a higher bacterial diversity in soil as the contamination levels increase compared to less contaminated or pristine soils. In a study conducted by Abed et al. (2014), it was revealed that the operational taxonomic units (OTUs) of microbial communities within a desert soil increased with increasing levels of oil contamination. The same trend was observed by Jung et al. (2010), who saw higher bacterial diversity when hydrocarbon concentrations were increased during a short-term *in-situ* microcosm experiment.

Hydrocarbon content can also influence microbial composition by its effect on soil properties. Contamination of hydrocarbons are found to influence several soil properties including bulk density, soil organic matter (SOM) content, porosity, permeability, and soil respiration (Liang et al., 2012). In a study conducted by Mikkonen et al. (2012), different hydrocarbon gradients were studied in an old farming field that was used for oil refinery waste. Results demonstrated that there was an overall reduction of soil hydraulic properties as seen by the reduction of water holding capacity and an increase of salts (Mikkonen et al., 2012). Suboptimal pH levels and an increase of cemented soil aggregates were also associated with presence of hydrocarbon contamination. The effect hydrocarbon content has on soil properties

has the potential to indirectly affect microbial communities as they are often reported to influence the physical, chemical, and biological properties of soil. Therefore, to better understand the plant-microbe interactions occurring in contaminated areas, the influence of hydrocarbons on soil properties, and not only bacteria, must be considered.

2.3.7 Hydrocarbon degradation

The success of plant-bacterial partnerships within hydrocarbon-contaminated soils depends largely on the presence of bacteria carrying hydrocarbon-degrading genes. Petroleum hydrocarbons include various types of compounds, such as normal alkanes (n-alkanes), and cyclic alkanes (c-alkanes) (Fukuhara et al., 2013). Constituting around 20-50% of crude oil (Yousaf et al., 2010a), the alkane degradative pathway is widely studied in different environments.

Alkanes are saturated hydrocarbons that are formed by carbon and hydrocarbon atoms. They can be categorized as linear (*n*-alkanes), cyclic (cyclo-alkanes) or branched (iso-alkanes) and are found in three states: gaseous (C_{1-4}), liquid (C_{5-16}), and solid ($>C_{17}$) (Singh et al., 2012). Considered relatively chemically inert (Labinger and Bercaw, 2002), alkane metabolism by bacteria is constrained by its accumulation in cell membranes, and its low water solubility (Rojo, 2009). However, bacteria have developed strategies involving specific enzymes and metabolic pathways to use *n*- alkanes as a carbon source (Zampolli et al., 2014), which allows them to degrade alkanes and convert them to easily metabolizable substrates. The enzymes that are used by bacteria to degrade alkanes are dependent on the carbon chain length. Short chain alkanes (C_{1-4}) are broken down by the soluble and particulate methane monooxygenases (sMMO and pMMO) and related propane monooxygenase and butane monooxygenase (BMO) (van Beilen and Funhoff, 2007). However, medium (C_{5-11}) and some long ($>C_{12}$) chain alkanes are dominantly degraded by the ALKB and CYP153 enzyme families (Rozhkova-Novosad et al., 2007).

2.3.7.1 AlkB of alkane hydroxylases

The degradative pathway involving the *alk* systems was first described in *Pseudomonas putida* GPo1, formerly identified as *Pseudomonas oleovorans* GPo1 (Baptist et al., 1963). The initial degradative step involves the oxidation of alkanes which is catalyzed by an alkane

monooxygenase complex that includes alkane hydroxylase (AlkB) (Smith et al., 2013), followed by an electron transfer chain containing rubredoxin (AlkG) and a rubredoxin reductase (AlkT) (Heiss-Blanquet et al., 2005). Through its cofactor flavin adenine dinucleotide (FAD), AlkT transfers the elections from nicotinamide adenine dinucleotide (NADH) to AlkG, this in turn transfers the electrons to the AlkB (Rojo, 2010).

To date, the alkane degradation of the *Pseudomonas putida* GPo1, is the most extensively characterized pathway of alkane degradation (Ji et al., 2013). The presence of the *alkB* gene sequence is studied in a variety of different molecular ecology studies that assess its diversity and distribution within hydrocarbon-contaminated soils.

2.3.7.2 Cytochrome P450 of alkane hydroxylases

The cytochrome P450 monooxygenase is a heme-thiolate protein (Van Bogaert et al., 2011) that is considered ubiquitous as it is found within all kingdoms of life (Das and Chandran, 2011). As it is involved in the initial oxidation of n-alkane, many bacteria are able to degrade medium chain alkanes due to the presence of the cytochrome P450 alkane monooxygenase systems (Amouric et al., 2010). The CYP153 gene is one of the most recent additions to the known biological alkane-hydroxylating repertoire (Koch et al., 2009). Containing the cytochrome P450 monooxygenase, the CYP153 catalyzes the hydroxylation of n-alkanes (Kubota et al., 2005). These proteins require a ferrodoxin and a ferrodoxin reductase for electron transfer from nicotinamide adenine dinucleotide phosphate (NAD(P)H) to the cytochrome (Liang et al., 2016).

Although it been over 30 years since their activity was detected, the first CYP enzyme, CYP153A1 in *Acinetobacter* sp. EB104 (Mojib et al., 2011), was characterized only in 2001 (Maier et al., 2001). Since then, the CYP monooxygenase was the topic of many microbial studies with CYP153 being one of the most studied genes in hydrocarbon contaminated soils (Ivanova et al., 2014; Nie et al., 2014; Tsuboi et al., 2015; Yousaf et al., 2010b).

2.3.8 Diversity of hydrocarbon degrading bacteria

Hydrocarbon degrading bacteria are ubiquitous and their populations are phylogenetically diverse; they are associated with most, if not all, branches of the microbial family tree. Generally

it is found that hydrocarbon degrading bacteria are dominated by members of the *Gammaproteobacteria* class and particularly *Pseudomomas* (Oliveira et al., 2014). However, other commonly isolated bacteria with hydrocarbon degrading genes have included *Flavobacterium, Arthrobacter, Rhodococcus, Ralstonia, Stenotrophomonas, Sphingomonas, Burkholdeira , Acinetobacter, Mycobacterium, Micrococcus, Nocardioides, and Alcaligens* (Phillips, 2008).

Similar to any microbial community, the diversity of hydrocarbon degrading bacteria in a given environment is highly variable and depends on several different factors. In a study conducted by Yousaf et al. (2010a), it was found that diversity of bacteria possessing hydrocarbon degrading gene, *alkb,* was dependent on plant species as there were fewer strains obtained from Birdsfoot Trefoil (dominated by *Betaproteobacteria* and *Gammaproteobacteria*) compared to Italian ryegrass (dominated by *Gammaproteobacteria*). Many studies have indicated that the diversity of endophytic bacteria possessing hydrocarbon-degrading genes in a given soil depends largely on the concentration of hydrocarbons present within the soil environment. Pérez-de-Mora et al. (2011) discovered a negative correlation between diversity and both total hydrocarbon and total inorganic elements when studying the diversity within a co-contaminated forest soil. However, there was a positive correlation between diversity and soil pH. This could suggest both contaminants and other soil properties are potentially influencing the diversity of hydrocarbon-degrading bacteria found within the soil environment.

2.4 Scope of Work

This project was created to better understand the plant-microbe interactions occurring within naturally revegetated Bitumount Provincial Historic site. It focuses on the characterization of the root endophytic bacterial community as an initial step toward determining if they are aiding in plant recolonization by alleviating plant stress. It also investigates the functional potential of the endophytic root bacterial community by quantifying the presence of hydrocarbon degrading genes. The identification of potentially plant growth promoting bacteria can be used to facilitate reclamation of disturbed soils.

3.0 ENDOPHYTIC ROOT BACTERIA ASSOCIATED WITH THE VEGETATION GROWING ON THE BITUMOUNT HISTORIC SITE

3.1 Preface

The Bitumount Provincial Historic site is the location of two of the world's first oil extracting and refining operations. Bitumount, located with the Athabasca oil sands, is thought to be contaminated with hydrocarbons through both natural and anthropogenic sources. This has led to heterogeneity of soil properties, including hydrocarbon content. Despite the hostile conditions occurring at Bitumount, plants have been able to recolonize the site through means of natural revegetation. Root microbial communities have been found to aid in the plant re-establishment of contaminated sites by alleviating plant stress through several plant growth promoting mechanisms such as hydrocarbon degradation, nutrient acquisition, and biocontrol. In particular, endophytic root bacteria are of interest due to their close association with the plant host. Currently, there is nothing known about the soils or the plant-microbe partnerships occurring at Bitumount. However, since Bitumount is ecologically unique, it offers a distinct opportunity to study the root endophytic bacteria within a naturally revegetated area where hydrocarbon contamination is known to strongly influence soil characteristics. In addition, studying root-associated bacteria in a variety of environments can increase our general understanding of these partnerships and may lead to enhanced reclamation efforts within the oil sands. Therefore, the objective of this study was to conduct a survey of the bacterial root endophyte communities associated with six different naturally occurring plant species throughout the Bitumount Provincial Historic site.

3.2 Abstract

The Bitumount Provincial Historic site, located in Northeastern Alberta, is the location of two of the world's first oil extracting and refining operations. Despite varying hydrocarbon levels throughout the site, plants have been able to recolonize the site. Due to this natural revegetation, Bitumount offers a unique opportunity to the plant-microorganisms interactions that have co-adapted to the site. This experiment was designed to conduct a survey of the root endophytic bacteria present within the natural vegetation growing at the Bitumount Provincial Historic site. Plant and soil samples were collected in June 2014. Six different plant species were identified and collected based on their general abundance throughout the site. These included smooth brome *(Bromus inermis*, SB*)*, horsetail spp. *(Equisetum spp.*, HT*),* slender wheatgrass *(Agropyron trachycaulum*, SW*)*, Kentucky bluegrass *(Poa pratensis*, KB*),* an unspecified member of the pea family *(Fabaceae*, PF*)* and wild strawberry *(Fragaria virginiana*, WS*).* Soil samples were analyzed for available nutrients, pH and hydrocarbon content, which ranged from 330 to 24,700 mg kg^{-1}. Using culture dependent techniques, rhizosphere and root endophytic bacteria were isolated. The abundance of culturable root-associated bacteria was significantly influenced ($p<0.05$) by the interaction of plant species and sampling location. Rhizosphere bacteria abundance was negatively correlated with hydrocarbon content ($p \leq 0.05$); however, there was no significant correlation ($p > 0.05$) between root endophytic bacteria abundance and hydrocarbon levels. Culture dependent techniques and high throughput sequencing were used to identify the root bacterial endophytes, which comprised diverse communities. Members of the *Actinomycetales, Rhizobiales, Pseudomonadales, Burkholderiales,* and *Sphingomonadales* orders were the most commonly identified bacteria using both approaches. Further research is needed to determine if the root endophytic communities are aiding plant growth within the contaminated soil conditions at Bitumount.

3.3 Introduction

Hydrocarbon contamination has become increasingly prevalent worldwide and petroleum hydrocarbons are considered the most widespread soil contaminant in Canada (Canadian Council of Ministers of the Environment, 2014). Hydrocarbon contamination in Canada occurs through both natural and anthropogenic sources. It is either present within or formed from geological sources such as oil, coal, bitumen, raw and refined fuels, and lubricants (Canadian Council of Ministers of the Environment, 2014).

The increase in hydrocarbon contaminated sites is largely due to the global dependence on petroleum as an energy source. The Athabasca oil sands, which are considered North America's secure source of oil, are the third largest oil reserves of the world with an estimated 173.2 billion barrels of potentially recoverable oil (Canadian Association of Petroleum Producers, 2015b). In 2014, oil sands companies produced 2.3 million barrels per day and this is expected to climb to 4 million barrels per day in 2024 (Alberta Energy, 2016). As part of oil sands development, oil sands mining companies are legally obligated to reclaim the land that is mined and drilled. This does not necessarily require that the reclaimed land be identical to its previous state, however, it must be representative of Alberta's northern boreal ecosystem. For reclamation to be successful, management of a wide range of disturbances that may affect soil must be taken into consideration including physical, chemical, and biological properties. The planting of native vegetation is a major part of the reclamation process (Canadian Association of Petroleum Producers, 2015a). However, it may be difficult for plants to grow due to stresses that occur from an imbalance of conditions in newly reclaimed soils. Potential contamination from residual hydrocarbons may lead to plant toxicity, as well as the reduction of nutrient and water absorption by plants and bacteria due to hydrophobic conditions (Griffin, 2014).

There are several approaches to reclaim and remediate hydrocarbon contaminated soils such as physical, chemical, and photodegradation methods. However, many methods have drawbacks and can leave behind other compounds which can be more toxic to the environment than the initial contaminant (Shahi et al., 2016). Consequently, biological approaches can serve as an environmentally favorable alternative. Biological approaches utilize plants, as well as their associated bacteria, to degrade hydrocarbons in the soil. Not only does biological remediation

offer a cost effective technique (Biswas et al., 2015), but it also provides a permanent solution as the contaminants are completely degraded (Perelo, 2010). Although the lone use of a plant for biological remediation can be successful, the combined use with bacteria is even more effective in contaminated soils (Afzal et al., 2011).

The success of plant growth and biological remediation relies heavily on the ability of the bacteria to transform and degrade contaminants from the environment through enzymatic activities (Abioye, 2011). Bacteria that reside in different compartments of the plants, and in the soil, synthesize several compounds that are able to reduce the proportion of toxic pollutants, therefore assisting the plant to survive stress (Das and Chandran, 2011). The effectiveness of this decrease in toxicity depends on the survival and metabolic activities of bacteria carrying hydrocarbon degrading genes (Khan et al., 2013). Bateria also promote plant growth through the enhancement of nutrient accessibility such as phosphorus or through nitrogen fixation. This can offset the negative influence that the hydrophobic hydrocarbon content has on plants, allowing them to increase their nutrient uptake. Endophytes are organisms that live in the interior of plant tissue and do not cause harm to the host (Bacon and Hinton, 2014). They are of great interest due to their potential to promote plant growth through a variety of mechanisms, including those previously mentioned.

Plant root-associated bacteria are analyzed in a variety of environments using both culture dependent and culture independent methods. For culture independent analysis, high-throughput sequencing techniques allow for the characterization of highly diverse microbial communities due to the ability to potentially provide hundreds of thousands to tens of millions of sequencing reads per run (Shokralla et al., 2012). Comparably, culture dependent techniques can only achieve characterization of a small portion of the diversity within a community. Despite this, culture dependent techniques remain an integral part in the research and development for reclamation strategies. Microbial isolation allows for the assessment of plant growth promoting genes, allowing for the identification of organisms that can be used in reclamation efforts of hydrocarbon contaminated soils.

The Bitumount Provincial Historic Site, situated within the Athabasca oil sands, is the location of two of world's first oil extracting and refining enterprises. Operations in Bitumount

began in 1923, ceased completely in 1958, and it became a historic site in 1974. One of Bitumount's unique characteristics is that it has evidence of hydrocarbon contamination through both anthropologic and natural sources. Since its closure, no reclamation efforts have occurred allowing hydrocarbons resulting from decades of oil exploration to persist on site. In addition, the shallow oil deposits that are associated with the Athabasca oil sands region makes it easier to exploit hydrocarbon resources in the region, but also increases the hydrocarbon content near the soil surface. Despite these two potentially adverse site characteristics, natural revegetation has occurred at Bitumount. Knowledge of the soils and vegetation growing at Bitumount is limited; however, it provides a natural location to analyze the plant root-associated microbial communities due to the heterogeneity of the site characteristics, varied levels of hydrocarbon contamination, and apparent resilience of the plant community.

This study was designed to conduct a survey of the root endophytic bacteria present within the natural vegetation growing at the Bitumount Provincial Historic site. The specific study objectives were to: 1) determine and compare the endophytic root bacterial community structure and diversity associated with the natural vegetation on site using both culture dependent and independent methods; and, 2) determine and compare the population abundance of rhizosphere and endophytic bacteria associated with the natural vegetation on site.

3.4 Materials and Methods

3.4.1 Sampling and processing

Sampling of the Bitumount Provincial Historic site (hereon referred to as Bitumount) occurred on June 24th, 2014. A total of 12 points were sampled and grouped into six different sampling locations based on physical characteristics (landscape location, vegetation, soil characteristics) (Figure 3.1). The six different sampling locations are designated as River Bank, Pathway, Processing Area, Quarry, Quarry Border, and Entrance. At each location, plant samples were chosen based on abundance, ensuring that the samples collected were representative of each sampling location (Table 3.1). Because the plants sampled were representative of each sampling location, not every plant species is present within each sampling location. Six different plant species were identified and collected for further study. They included smooth brome *(Bromus inermis,* SB*),* horsetail spp. *(Equisetum spp.*, HT*),* slender

Figure 3.1. Sampling locations and sampling points throughout the Bitumount Provincial Historic site, Alberta, Canada. A, River Bank; B, Pathway; C, Quarry; D, Quarry Border; E, Processing Area; F, Entrance. Satellite image taken from Google Earth (ver. 7.1.1557, image acquired 04 August 2011, accessed 18 January 2016).

wheatgrass *(Agropyron trachycaulum*, SW*)*, Kentucky bluegrass *(Poa pratensis*, KB*)*, an unspecified member of the pea family *(Fabaceae*, PF*)* and wild strawberry *(Fragaria virginiana*, WS*)*.

Three samples of each plant and adhering soil were collected at each sampling point by excavating the roots. Additional soil samples were collected to a 15 cm depth. Plant and soil samples were removed using shovels, placed in plastic bags, and stored in coolers during transport. Bulk soil was sub-sampled following sieving (<4mm), and was stored at -80^{o}C. The aerial portion of each plant was removed using a scalpel and discarded. Each root sample was subdivided; half of the roots were stored in -80^{o}C for molecular analysis and the remaining roots were temporarily placed in -20^{o}C for isolation of culturable bacteria.

3.4.2 Soil property analysis

For hydrocarbon analysis, bulk soil from each sampling location was placed in an airtight glass container and was sent to ALS Environmental Laboratory, Saskatoon, Canada. The analytical methods used to analyse the CCME (Canadian Council of Ministers of the Environment) petroleum hydrocarbons comply with the reference methods for the Canada-wide Standard for Petroleum Hydrocarbons in soil (CWS PHC DEC-2000-PUB1310). The F1 fraction (C_6-C_{10}) was measured by extracting 5 g of soil with methanol. The methanol was then separated from the soil and added to a purge-and-trap unit for release of volatile organics. Gas chromatography was used to separate the volatile organics using a 100% poly (dimethylsiloxane) low bleed column. Toluene was used as the primary calibration standard. The BTEX (benzene, toluene, ethylbenzene, and xylene) components were quantified using MS detector and the F1 fraction was quantified by a flame ionization detector. Similarly C_{10}-C_{50} were measured using gas chromatography with flame ionization and 100% poly (dimethylsiloxane) columns. However, the C_{10} peak was separated from the solvent peak during chromatography instead of the C_6 peak. The primary calibration standard was a mixture of approximately equal amounts of C_{10}, C_{16}, and C_{34} normal hydrocarbons.

To analyse soil nutrients, additional bulk soil was air dried, sieved (<5.0 cm) and sent to ALS Environmental Laboratory. Available nitrate and nitrite were measured using methods described by Alberta Agriculture (Alberta, Soils and Animal Nutrition Laboratory, 1988). In

short, a dilute calcium chloride solution was used to extract available soil nitrate and nitrite. Nitrate was then reduced to nitrite by passing through a copperized cadmium column. The nitrite (reduced nitrate included) was measured by diazotizing with sulfanilamide. This was then followed by coupling with N-(1-naphthyl) ethylenediamine dihydrochloride. The resulting water soluble dye (magenta in colour) was measured colorimetrically at 520 nm. Available sulfate was extracted using a weak calcium chloride solution, an inductively coupled-plasma optical emission spectrometer (ICP-OES) was then used to measure sulfate in the extract (Alberta, Soils and Animal Nutrition Laboratory, 1988). The Modified Kelowna solution was used to extract plant available phosphorus and potassium (Qian et al., 1994). The soil phosphorus within the soil extract was measured colorimetrically at 88 nm, and the potassium was measured by flame emission at 77 nm.

Soil pH was measured at the University of Saskatchewan using a protocol modified from Hendershot et al. (2007). In brief, 1 part dry soil and 2 parts distilled water was placed on a rotary shaker for 20 min at 150 rpm. The slurry was allowed to stand for sediment settlement. After calibration of the pH meter, the electrode was immersed into the clear supernatant and the pH was recorded once the reading was constant.

3.4.3 Determination of root endophytic bacterial structure using high-throughput sequencing

Roots were surface sterilized using a modified protocol from Siciliano and Germida (1999). Roots were placed in a 300 mL Erlenmeyer flask containing 100 mL NaClO (1.05% v/v) in phosphate-buffered saline (PBS; 1.2 Na_2HPO_4; 0.18 NaH_2PO_4; 8.5 NaCl (g L^{-1}). The Erlenmeyer flasks containing the roots were placed on a rotary shaker for 15 min at 150 rpm followed by rinsing roots 10 times with 100 mL of sterile tap water. Then, 0.1mL of each final wash was plated on 1/10 strength trypticase soy agar (1/10 TSA; trypticase soy broth (3g L^{-1}); solidified with agar (15g L^{-1}) to confirm sterility. The 1/10 strength TSA plates contained cycloheximide (0.1g L^{-1}), which was used as a fungistat to reduce the chance of fungal contamination. While waiting for sterility assessment, roots were stored at -20^{o}C for 72h. Surface sterility of the roots was confirmed through visual checks of the plates.

Table 3.1. Global Positioning System (GPS) coordinates for sampling locations (UTM Zone 12) and plant species sampled for each location at the Bitumount Provincial Historic site, Alberta, Canada.

Location	Plant Species	Northing	Easting
Quarry	Pea Family	6360410	461160
Entrance	Slender Wheatgrass; Smooth Brome	6360401	461319
Processing Area	Slender Wheatgrass; Smooth Brome; Horsetail	6360533	461211
Pathway	Smooth Brome; Kentucky Bluegrass	6360465	461111
Quarry Border	Wild Strawberry	6360401	461192
River Bank	Slender Wheatgrass; Smooth Brome; Horsetail	6360465	461069

Following sterility confirmation, total genomic DNA was extracted from surface sterilized roots using standard Mo Bio protocols from the PowerPlant® Pro DNA Isolation kit. A Qubit Fluorometer (Thermo Fisher Scientific Canada) was used to quantify DNA concentration in total root DNA extracts. Genomic DNA was sent to Genome Quebec Innovation Centre (McGill University, Montreal, Canada) for sequencing analysis. Using the PE 250bp Illumina MiSeq platform, the V3-V4 regions of the 16S rRNA gene were targeted using the 520F (AGCAGCCGCGGTAAT) and 799R (CAGGGTATCTAATCCTGTT) primers (Edwards et al., 2008). The PCR was performed using the Access Array PCR tool (Fluidigm, San Franscisco, CA). AMPure XP beads (Beckman Coulter Inc. Mississauga, ON) were then used to clean the amplicon libraries. This was followed by quantifying the concentration of the amplicon libraries using Qubit. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was then used to evaluate the fragment size. The Illumina Miseq platform was then used to sequence the pooled libraries to generate 2x250 bp paired ends reads.

The 16S rRNA gene sequence targets were analyzed using Mothur v.1.34.3 (Schloss et al., 2009) (March 19, 2015) using the MiSeq standard operating procedure created by the same laboratory group (Kozich et al., 2013). The two sets of reads for each sample were combined, followed by the assemblage of each sample into a single group file. Sequences with ambiguous bases or sequences longer than 310 bp were removed. Duplicates were merged to attain unique sequences. To ensure sequence overlap the same region, sequences were aligned based on the primers used. Sequences were then filtered to remove overhangs at either end. To avoid redundancy that may have occurred across sequences by trimming the ends, duplicates were remerged to attain unique sequences. To further denoise the sequences, singletons and doubletons were removed. Chimera sequences were then removed using the UCHIME algorithm (Edgar et al., 2011). Undesirable sequences (Eukaryota, Archaea, chloroplast, mitochondria, and unknown), were classified using the Bayesian classifier and then were removed. The sequences were then clustered into operational taxonomic units (OTUs) at a 0.02 cutoff. The taxonomy for each OTU was attained with a distance level of 0.03 (97% similarity). Library sizes varied among samples and ranged between 8069 and 63612 (951106 total sequences). To limit bias due to uneven sampling, OTUs tables were rarefied so that each sample had equal sampling depth. To determine differences that may occur from rarefying at different levels, both the lowest value

(Table 3.3) and median (Table B.1) were used to rarefy. Similar trends occurred between the two methods, however to ensure that no sample was excluded by eliminating samples that were lower than the median, rarefying to the lowest value were presented (Sinclair et al., 2015). Mothur was used to calculate rarefaction curves, the Chao1 richness estimator and the alpha diversity using Shannon and Inverse Simpson indexes.

3.4.2 Bacterial isolation and identification using culture dependent methods.

3.4.2.1 Isolation of rhizosphere bacteria

To isolate rhizosphere bacteria, the protocol described by Siciliano and Germida (1999) was followed with minor modifications. Briefly, 2 g of roots and rhizosphere soil were placed in a 500 mL Erlenmeyer flask containing 200 mL of PBS. The Erlenmeyer flask, containing the roots and PBS, was placed on a rotary shaker (200 rpm) for 20 minutes after which the solution was serial diluted into dilutions of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in PBS buffer (1.0 mL rhizosphere soil suspension in 9.0 mL PBS). The rhizosphere soil was then collected by centrifuging (2000 × *g* for 5 minutes) the 100 mL of the remaining PBS/soil mixture within 50 mL Falcon ® tubes. The supernatant was decanted and discarded and Falcon® tubes containing the rhizosphere soil were placed in -80^{o}C for storage. To enumerate bacteria in rhizosphere soils, 0.1 mL of each dilution was plated in triplicate onto plates containing 1/10 TSA. Plates were incubated at 28^{o}C and colony forming units (CFUs) counted at 24 and 72 h.

3.4.2.2 Isolation of root endophytic bacteria

Surface sterilized roots (2 g), as discussed in section 3.4.5, were suspended in 1/10 (w/v) PBS. The roots and PBS were combined using a sterile mortar and pestle and were serially diluted in sterile PBS (dilutions of 10^{-3}, 10^{-4}, and 10^{-5}) (1.0 mL root/PBS suspension in 9.0 mL PBS). A 0.1 mL volume of each dilution was plated in triplicate on plates containing 1/10 TSA with cycloheximide. The inoculated plates were incubated at 28^{o}C and CFUs were counted after 24 and 72 h.

For each plant, triplicate plates of one dilution containing 30-300 colonies were chosen for isolate selection and identification. Each colony was individually numbered and a random number generator selected 20 colonies per plant replicate. If the random number generator failed

to pick up all the morphologically distinct colonies, additional colonies were selected based on visual assessment. The endophytic bacterial colonies were streaked on 1/10 TSA plates containing cycloheximide. Every 72 h, the selected colonies were transferred onto new plates to ensure purity of the colonies. After transferring the colonies three times, the pure colonies were grown at 28^{o}C under agitation (150rpm) in ½ strength liquid TSB. Endophytic isolates were then stored at -80^{o}C in a 1:1 mixture of TSB and sterile glycerol (v/v) (Germida et al., 1998).

3.4.2.3 Identification of root endophytic bacteria

To identify the culturable root endosphere bacteria, 20 isolates per plant were selected using a random number generator. A total of 240 isolates of the original 960 glycerol stocks, were chosen to be analyzed for identification. From each chosen glycerol stock, 100µl was placed into a 96-well plate. The plates were then placed in dry ice for transport. Sanger sequencing of the isolates were performed at the Genome Quebec Innovation Centre (McGill University, Montreal, Canada). First, polymerase chain reaction (PCR) was used to amplify the genomic DNA using EUB338 (ACTCCTACGGGAGGCAGCAG) and EUB518 (ATTACCGCGGCTGCTGG) (Fierer et al., 2005). Sanger sequencing was then performed using the EUB 338 primer. The 16S rRNA sequences were compared with sequences in the GenBank database using NCBI nucleotide Basic Local Alignment Search Tool (BLAST) program. The 16S rRNA sequences (Bacteria and Archaea) database was used for comparison, while optimizing for highly similar sequences (Megablast).

3.4.4 Statistical methods

Statistical analyses were performed using the SAS program (version 9.3) with a SAS Macro add in (The SAS system for Windows, SAS institute, 2003). Data normality was analyzed using the Shapiro-Wilk statistic. An analysis of variance (ANOVA) was used to determine significant differences between plant types and locations. Differences between treatment means were determined using a Tukey's test for post hoc analysis at a 5% significance level. Pearson correlation was used to explore relationships between soil parameters and CFU abundances.

Ordination analysis was performed to compare 16S rRNA gene sequences of endophytic bacterial communities associated with the natural vegetation growing throughout Bitumount. A non-parametric statistical method, ordination works by using one or a few axes to arrange data

points so that the strongest pattern within a data set are displayed. A nonmetric multidimensional scaling (NMDS) using the Sørensen (Bray-Curtis) distance measure was used for the ordination of the relative abundance of OTUs. NMDS is a nonparametric ordination method which is best suited for non-normal data and is the most effective ordination methods for ecological data (McCune and Grace, 2002). The ordination was carried out in PCOrd v.6.0 (MjM Software Gleneden Beach. OR) using the Autopilot Slow and Thorough analysis option with a random starting point (McCune and Grace, 2002). The final stress values measured through ordination analysis depict the departure from monotonicity (McCune and Grace, 2002). Low stress values are favoured as they represent good interpretation of the data with high confidence in the interpretation. Stress values equal or less than 10 are favored; values greater than 20 are poor and indicate that the ordination is likely misleading (McCune and Grace, 2002).

Multi-response permutation procedures (MRPP) was performed using the Sørensen distance measure in PCOrd v.6.0 (MjM Software Gleneden Beach. OR). MRPP is used to determine if there are differences present between pre-defined groups (McCune and Grace, 2002). The stated *p*-value demonstrates the likelihood that the observed difference is due to chance, and the A-value represents the within group homogeneity, when compared to random expectation. The highest possible value of A is 1, which represent that all items within a group are identical. A will equal 0 when the heterogeneity within groups equals expectation by chance whereas A will be less than 0 if there is less agreement. An A value of > 0.3 is considered high, whereas A values <0.1 are generally found in ecological studies (McCune and Grace, 2002).

3.5 Results

3.5.1 Soil properties

Table 3.2 describes the soil properties analyzed throughout the sampling locations at Bitumount. Hydrocarbon content was highest in the Quarry and lowest in the River Bank location, with concentrations of 24, 700 mg kg^{-1} and 330 mg kg^{-1}, respectively. The total hydrocarbon levels for the remaining sampling locations differed by only two fold and ranged from 1770 to 4120 mg kg^{-1}. Soil pH ranged from 8.2 to 8.5 throughout the site demonstrating

Table 3.2 General soil properties analyzed from each of six sampling locations at the Bitumount Provincial Historic site, Alberta, Canada.

Location	pH	Nitrate	Sulfate	Phosphate	Potassium	Hydrocarbon Fraction†					Total hydrocarbons
						F1‡	F1-BTEX§	F2¶	F3#	F4††	
	----------	----------	----------	----------	----------	mg kg^{-1}	----------	----------	----------	----------	----------
Quarry	7.0	<1	22.1	<2	<20	17	17	2680	14700	7340	24700
Entrance	8.4	2.2	12.3	5.5	36	<10	<10	850	1340	1930	4120
Processing Area	8.2	1.5	13.4	7.3	109	<10	<10	51	1400	2050	3500
Pathway	8.5	<1	4.9	3.7	31	<10	<10	36	864	1450	2350
Quarry Border	8.5	<1	28.2	<2	58	<10	<10	41	853	878	1770
River Bank	8.5	<1	47.1	<2	90	<10	<10	42	147	141	330

† Based on carbon (C) chain length or molecular weight, classified into fractions according to the methods of the Canadian Council of Ministers and the Environment (CCME).

‡F1= C6 to C10 (hexane to decane range)

§BTEX = benzene, toluene, ethylbenzene, and ortho, meta and para-xylene.

¶F2= C10 to C16 (decane to hexadecane range)

#F3= C16 to C34 (hexadecane to tetratriacontane range)

††F4= C34 to C50 (tetratriacontane to pentacontane range).

little variance, with the exception of the Quarry which had a pH of 7.0. The largest observable difference in available soil nutrients occurred for potassium, where concentrations were the lowest in the Quarry (<20 mg kg^{-1}) and highest in the Processing Area (109 mg kg^{-1}). Sulfate concentrations also varied across sampling locations with content ranging from 4.9 mg kg^{-1} in the Pathway to 47.1 mg kg^{-1}in the River Bank.

3.5.2 Identification of endophytic bacteria from targeted 16S rRNA gene sequencing

Differences in the community structure of root endophytic bacteria throughout the Bitumount site were assessed via high throughout sequencing of the 16S rRNA genes (V4 region). A total of 951,106 sequences (averaging 27,974 per sample) were obtained from the root endosphere of the 34 plants sampled from across the Bitumount site. Sequences were clustered into in 8930 unique OTUs at 97% sequence similarity with an average of 1064 OTUs per sample. The number of different OTUs was highest (1376) within the root endosphere of SB in the Processing Area and lowest (700) within the root endosphere of SW in the River Bank. Rarefaction curves reached a plateau in nearly all libraries, demonstrating sufficient sampling depth (Fig. C.1).

Shannon Diversity Index was significantly different ($p \leq 0.05$) for the bacterial communities in the root endosphere of HT in both the Processing Area and the River Bank locations (Table 3.3). The root endophytic community of HT in the River Bank was found to have higher diversity than in the Processing Area (4.52 compared to 2.23). Similarly, the Inverse Simpson Index of the root endophytic bacterial community of HT in the River Bank was significantly higher ($p \leq 0.05$) than that of the HT root endosphere in the Processing Area (40.44 and 5.03, respectively). In addition, the root endophytic diversity of SB in the River Bank (7.02) was also significantly lower than that of HT in the River Bank. The Chao1 Index ranged from 1055 to 3331; however, there were no significant difference between the root endophytic communities.

Root endophytic bacteria were classified into 17 different phyla and 455 different genera (data not shown). Across each replicate, four of the 17 phyla were common to root endophytic bacterial communities, including Proteobacteria (63.6% of total), Actinobacteria (19.9%),

Table 3.3 Number of unique operational taxonomic units (OTUs) and alpha diversity indices of root endophytic communities associated with the vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada.

Location	Hydrocarbon content (mg kg^{-1})	Plant†	Reps‡	No. of Sequences	Shannon Index (H')§	Inverse Simpson Index (1/D)§	Chao 1 Index
Quarry	24700	PF	3	26077	4.17ab	21.82ab	1411
Entrance	4120	SW	2	24211	4.03ab	25.84ab	1055
		SB	3	27614	3.43ab	12.58ab	1366
Processing Area	3500	SW	3	24720	3.84ab	20.24ab	1350
		SB	3	41246	3.54ab	21.03ab	1893
		HT	3	19735	2.23b	5.03b	1315
Pathway	2350	SB	3	24360	3.86ab	27.99ab	1316
		KB	3	26507	2.95ab	12.68ab	2169
Quarry Border	1770	WS	2	57433	3.08ab	12.70ab	2673
River Bank	330	SW	3	27765	3.25ab	11.94ab	3331
		SB	3	22801	2.84ab	7.02b	1922
		HT	3	21781	4.52a	40.44a	2162

†SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.
‡Number of sequences and alpha diversity are presented as means of each replicated sample.
§Different letters in same column indicated significant difference ($p \leq 0.05$) using Tukey's Post-hoc.

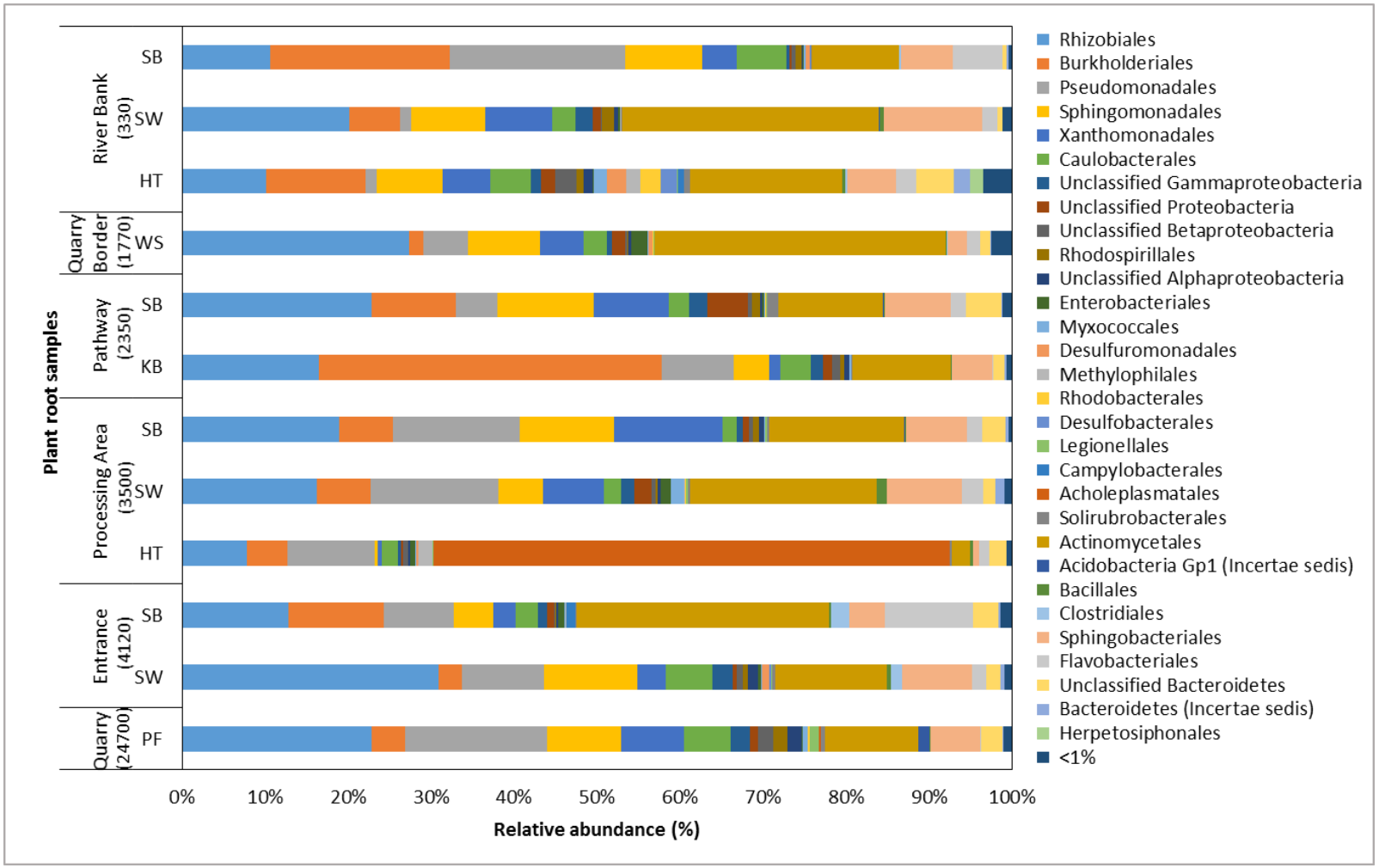

Figure 3.2 Relative abundance (%) of bacterial orders identified based on Illumina MiSeq analysis of 16S rRNA genes from root endophytic bacteria associated with the vegetation growing at the Bitumount Provincial Historic site. Values are presented as means. Values in parentheses on y-axis represent total hydrocarbon content (mg kg^{-1}) for each sampling location. SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.

Bacteroidetes (9.9%), and Firmicutes (0.8%). These phyla were further subdivided into 88 bacterial orders. Figure 3.2 illustrates the orders (n=45) that represent at least 1 % of the bacterial profile. The most common orders identified within the endosphere of plant roots were *Rhizobiales* (18.5%), *Actinomycetales* (19.2%), *Burkholderiales* (10.5%), *Pseudomonadales* (10.0%), and *Shingomonadales* (8.0%). The most dominant genera associated with these orders consisted of *Pseudomonas* (99.3% of *Pseudomonadales*), *Rhizobium* (48.1% of *Rhizobiales*), *Sphingomonas* (68.8% of *Sphingomonadales*), *Kineospora* (28.3% of *Actinomycetales*), and *Burkholdeira* (38.8% of *Burkholderiales*).

Of the most common orders, *Actinomycetales* (range of 10.6 to 36.0% relative abundance), *Pseudomonadales* (range of 1.3 to 21.9% relative abundance), *Burkholderiales* (range of 2.9 to 41.3% relative abundance), and *Rhizobiales* (range of 10.1 to 27.4% relative abundance) were also the most dominant orders found in the root endosphere of each plant sampled. The exception to this occurred within the root endophytic community of HT in the Processing Area, which was largely dominated by bacteria of the *Acholeplasmatales* order (62.2%) and consisted of the sole genus of *Acholeplasma*. *Acholeplamatales* was also present in the root endophytic community of SB in the entrance and WS in the quarry, but only accounted for < 0.1% and 0.4% of each profile, respectively.

Non-metric multidimensional scaling (NMDS) analysis was performed to demonstrate the relationships between root endophytic bacterial communities at different sampling locations and plant species (Figure 3.3). The NMDS yielded a 3 –dimensional solution (final stress= 15.3) that distinguished communities by both sampling location and plant species with Axis 1 and Axis 2 accounting for the majority of variability (35.5% and 25.5%, respectively). The third axis accounted for 15.5% of the total variability, however did not provide any obvious separation between plant species at different sampling locations.

Ordination analysis of each root endophytic community demonstrated that both plant species and sampling location significantly affected the community structure. MRPP analysis confirmed the significant effect of plant species (A=0.163; $p<0.001$), as well as sampling location (A=0.128; $p<0.001$). Root endophytic community structure associated with SB overlapped, suggesting that despite different sampling locations root bacterial endophytes

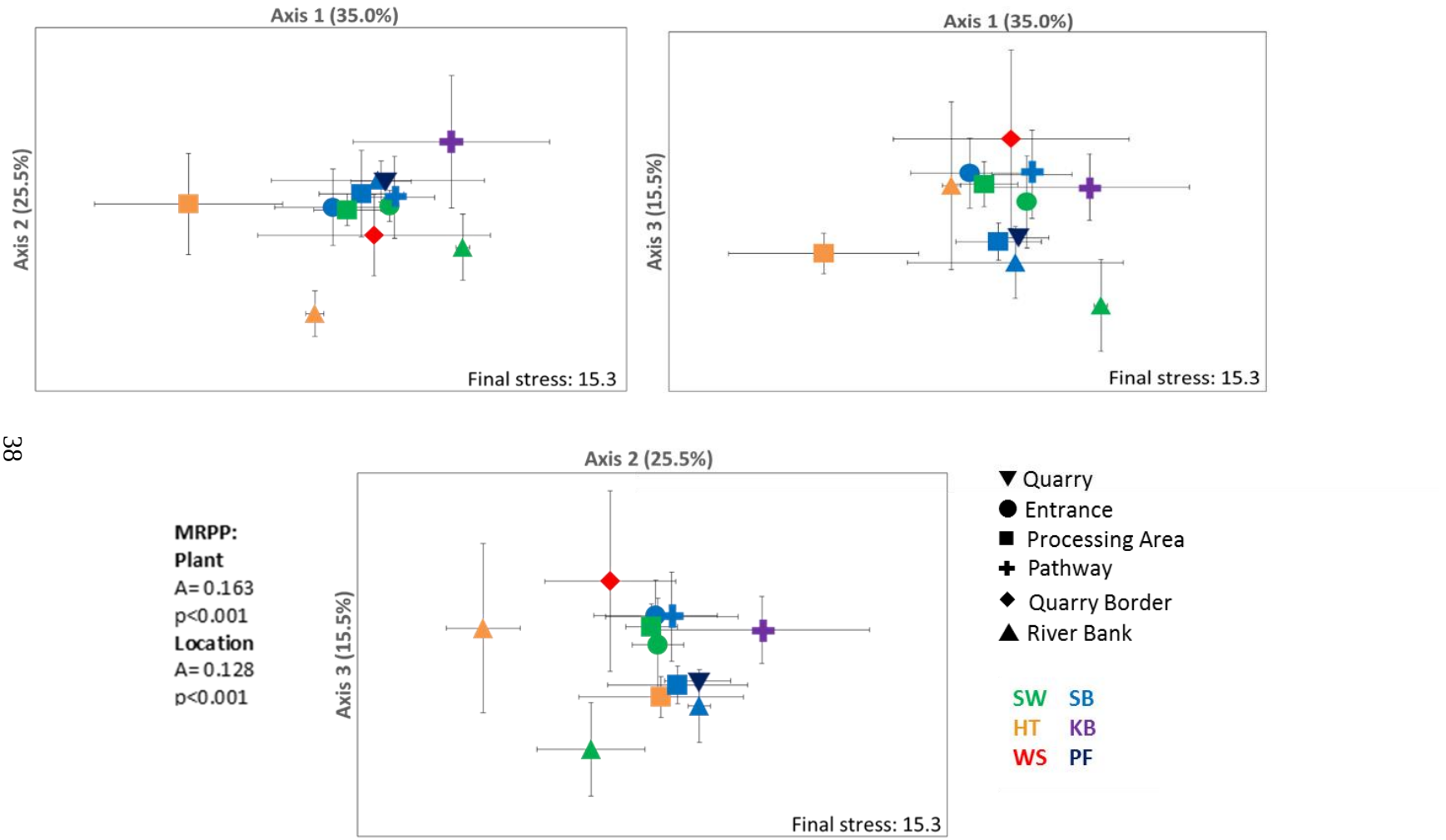

Figure 3.3 Non-metric multidimensional scaling (Bray-Curtis distance matrix) of the operational taxonomic units of endophytic root bacteria throughout the Bitumount Provincial Historic site, Alberta, Canada. SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.

community structure were similar. Endophytic root communities of SW in the Entrance and Processing Area also overlapped, however community structure of SW in the River Bank was positioned separately, indicating that the community structure in the River Bank was less similar to the other two locations. Root endophytic communities of HT were positioned separately from the remaining plant species, indicating that the community structure was unique from the remaining root endosphere bacterial communities. In addition, root endophytic communities of HT in the Processing Area were positioned apart from HT in the Quarry suggesting that the community structure was influenced by sampling location.

An ordination analysis without the inclusion of *Acholeplasmatales*, which was only dominant in the root endosphere of HT in the Processing Area, resulted in a 3-dimentional solution with a final stress of 15.3 (Fig. 3.4). The majority of the variability was accounted for by Axis 1 (38.8%), with Axis 2 and Axis 3 accounting for 21.5% and 16.3%, respectively. Similar results were found in the NMDS with and without the inclusion of *Acholeplasmatales*. Bacterial root endophytic community of SW in the River Bank was more different than the SW in the Processing Area and Entrance. However, the root endophytic communities of HT in the Processing Area and River Bank clustered together, indicating that without the dominance of *Acholeplasmatales* in the Processing area, community structure was more similar.

3.5.3 Culturable bacterial community abundance

Abundance of culturable root-associated bacteria varied throughout Bitumount, where sampling location and plant species interacted significantly (p <0.05) to affect the quantity of rhizosphere and root endophytic bacteria (Table 3.4). Rhizosphere bacteria abundance averaged $1.00x10^7$ CFUs $g^{-1}$of fresh root and root endophytic populations averaged $1.00x10^5$ CFUs $g^{-1}$of fresh root. The rhizosphere of HT in the River Bank harbored the highest abundance of bacteria (8.23 log CFUs g^{-1}), whereas the PF rhizosphere in the Quarry supported the lowest abundance of bacteria (6.17 log CFUs g^{-1}) of fresh root. Bacterial endophyte populations were highest in the roots of SB in the Processing Area (6.27 log CFUs g^{-1} of fresh root weight) and were lowest in the root endosphere of HT from the River Bank (4.31 log CFUs g^{-1} of fresh root).

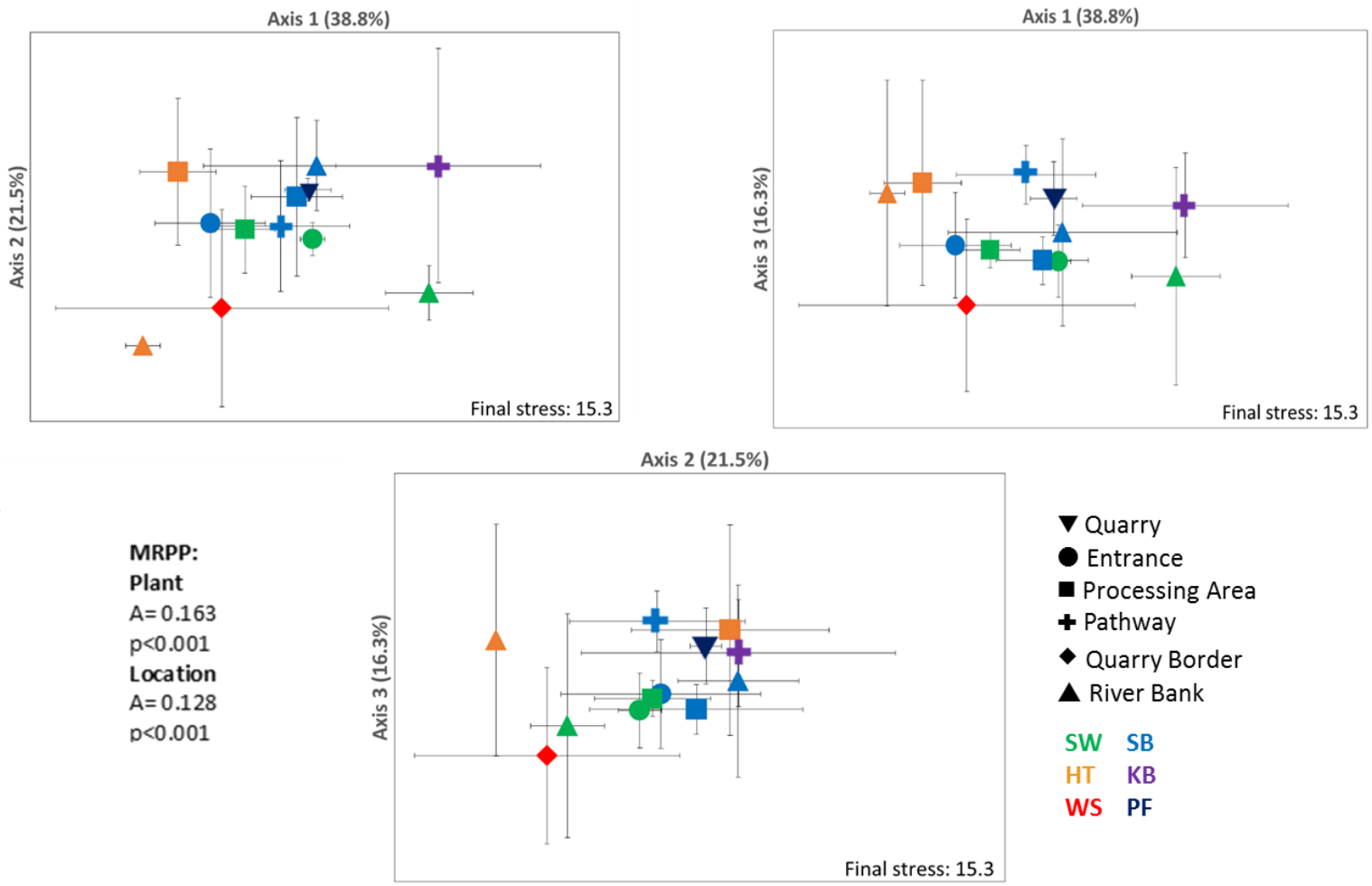

Figure 3.4 Non-metric multidimensional scaling (Bray-Curtis distance matrix) of the operational taxonomic units of endophytic root bacteria throughout the Bitumount Provincial Historic site, Alberta, Canada with the exclusion of the *Acholeplasmatales* order. SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.

Table 3.4 Total colony forming units (CFUs) of rhizosphere and root endosphere bacteria associated with the vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada.

Location	Hydrocarbon content (mg kg^{-1})	Plant†	Log CFUs g^{-1} of fresh root‡	
			Rhizosphere§	Endosphere§
Quarry	24700	PF	6.17±0.17 f	5.78±0.03 ab
Entrance	4120	SW	7.42±0.15 ab	5.17±0.22 abcd
		SB	7.60±0.18 ab	5.21±0.23 abcd
Processing Area	3500	SW	7.63±0.26 abc	5.14±0.19 bcd
		SB	7.99±0.12 ab	6.27±0.36 a
		HT	7.49±0.27 abcd	4.37±0.49 cd
Pathway	2350	SB	6.58±0.4 ef	4.87±0.41 bcd
		KB	6.7±0.16 def	5.20±0.29 abcd
Quarry Border	1770	WS	6.79±0.09 def	5.59±0.34 abc
River Bank	330	SW	6.89±0.21 cdef	5.56±0.44 ab
		SB	7.26±0.27 bcde	5.06±0.71 bcd
		HT	8.23±0.6 a	4.31±0.18 d

†SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.
‡Data presented are means (n=3) of log transformed values with standard deviations.
§Different letters in same column indicated significant difference ($p \leq 0.05$) using Tukey's Post-hoc test.

There was no significant relationship between endosphere bacterial abundance and any of the soil parameters measured (Table 3.5). In contrast, a strong positive correlation ($p<0.001$) of bacterial abundance with nitrate levels was observed within the rhizosphere soils. Likewise, bacterial abundance in the rhizosphere was positively correlated with concentrations of phosphate ($p<0.01$) and potassium ($p<0.01$), but negatively correlated with hydrocarbon content ($p<0.05$)

Table 3.5 Pearson correlation coefficients between general soil properties and bacteria colony forming units (CFUs) culture on 1/10 strength tryptic soy agar from the rhizosphere and endosphere of all plants sampled at the Bitumount Provincial Historic site, Alberta, Canada.

CFUs	pH	Nitrate	Sulfate	Phosphate	Potassium	Total Hydrocarbons
Endophytes	NS	NS	NS	NS	NS	NS
Rhizosphere	NS	0.57***	NS	0.46**	0.44**	-0.35*

*,**,***, Significant at $p \leq 0.05, 0.01, 0.001$

3.5.4 Identification of isolated endophytic bacteria

Randomly selected isolates were identified through Sanger Sequencing to determine the identity of the culturable endophytic bacteria throughout Bitumount. In total, of 240 of the 960 endophytic bacteria isolated using culture dependent methods were sequenced. Figure 3.5 illustrates the relative abundance of bacterial orders identified. Of the sequenced isolates, seven were defined as unclassified. However, they never accounted for more than one isolate (5%) of the isolates from an individual plant sample.

A total of 13 bacterial orders, belonging to four different phyla (Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes) were sequenced. Bacteria belonging to the *Actinomycetales* order were the most commonly identified throughout root endospheres (n=66). *Rhizobiales* (n=41) and *Pseudomonadales* (n=36) were the second and third most common orders isolated. The most common genera associated with *Actinomycetales* was *Microbacterium* (n=16), *Leucobacter* (n=15), and *Leifsonia* (n=12). *Rhizobiales*, were dominated by the *Rhizobium* genera (n=29), and *Pseudomonadales* was solely composed of the *Pseudomonas* genus (n=36). Of all the bacterial orders identified, only *Pseudomonadales* was ubiquitous throughout the site (i.e. present in each plant and from each location). Each of the three most commonly identified orders was also the dominant order identified per sample, with the exception of the root endosphere of SB in the Processing Area which had higher dominance of *Xanthomonadales* (n=5), however had only was only slightly more abundant than *Rhizobiales* (n=4). The rarest of the orders, *Rhodobacterales* (n=2) and *Rhodospirillales* (n=1), were only

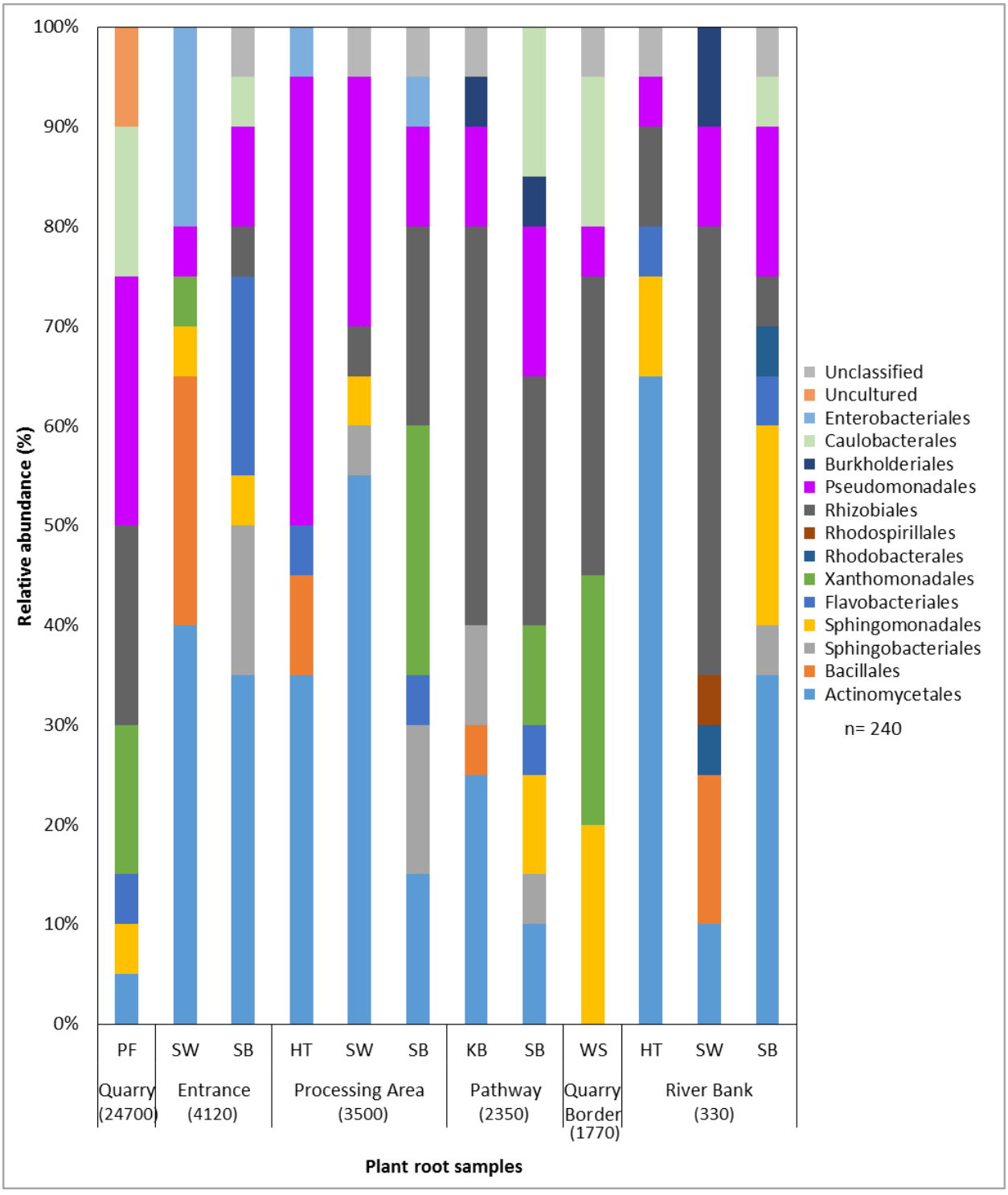

Figure 3.5 Relative abundance (%) of bacterial orders identified by Sanger Sequencing of the root endophytic bacterial isolates associated with the vegetation at the Bitumount Provincial Historic site, Alberta, Canada (n=20 for each sample). Values in parentheses on x-axis represents total hydrocarbon content (mg kg^{-1}) for each sampling location. SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.

isolated from the root endosphere of the grass species sampled in the River Bank. On average, the root endosphere of SB supported the highest number of different bacterial orders, specifically in the Pathway area (n= 9) and in the River Bank (n=8). The remaining root endophytic communities only had between 5 and 7 bacterial orders identified.

3.6 Discussion

The Bitumount Provincial Historic site offers a unique opportunity to study plant-microbe associations that have co-adapted to this hydrocarbon impacted environment. The overall goal of this study was to survey the endophytic root bacteria associated with the natural revegetation growing at Bitumount. By increasing our knowledge of these partnerships, the potential to manipulate plant-bacterial interactions may lead to more efficient reclamation efforts to revegetate land that was disturbed due to oil exploration and mining. As our results demonstrate, even though there are similarities occurring between the results from using both culture dependent techniques and high-throughput sequencing, high-throughput sequencing allows for a more thorough analysis of the bacteria present. Therefore, a combination of using both culture dependent and independent methods is integral to understand the interactions occurring between plants and bacteria.

3.6.1 Targeted 16S rRNA gene sequencing of bacterial root endophytes

Members of the *Actinomycetales, Rhizobiales, Pseudomonadales, Burkholderiales,* and *Sphingomonadales* were the most commonly identified orders identified using high-throughput sequencing. Similar results were documented in other studies that profiled bacterial community structure associated with hydrocarbon contaminated soils. Stefani et al. (2015) analyzed the microbial community within hydrocarbon-contaminated soils from a former petrochemical plant in Quebec, Canada. Community profiling of bulk soil using 454-pyrosequencing of the 16S rRNA gene were comparable to our findings. Members of the *Rhodocylales, Burkholderiales, Actinomycetales, Rhizobiales, Xanthomonadales,* and *Sphingomonadales* were found to be the dominant orders sequenced (Stefani et al., 2015). With the exception of *Rhodocylales,* which only represented a maximum of 0.16% in any of the root endospheres, the remaining five orders from their survey were present within the top seven orders sequenced from the root endosphere of the vegetation at Bitumount. In an additional study conducted by Su et al. (2015) the

endophytic bacterial community of a salt marsh plant (*Spartina alterniflora*) grown within PAH contaminated soils were identified using high-throughput sequencing. Evidence of *Burkholderiales*, *Flavobacteriales*, *Bacilliales*, *Actinomycetales*, *Sphingobacteriales*, and *Pseudomonadales* were all present, all of which are associated with the degradation of hydrocarbons (Su et al., 2015).

Of each of the plants sampled, members of the *Burkholderiales*, *Actinomycetales*, and *Rhizobiales* were the most dominant orders identified in the root endosphere and accounted for 18.4% to 41.3% of the total OTUs sequenced within individual root endophytic communities. The root endophytic community associated with HT within the Processing area was an exception, where over half of the sequences belonged to the *Acholeplasmatales* order, part of the *Mollicutes* class. Although members of the *Mollicutes* class exhibit a variety of different life cycles, *Acholeplasmatales* are saprophytic, free-living bacteria (Zhao et al., 2015), that are commonly considered parasitic or commensal of insects (Ravichandra, 2013). *Acholeplasmatales* members are transmitted by sap-sucking insects allowing them to colonize the interior of vascular plant tissues (Ravichandra, 2013). This suggests that the root endophytic community of HT in the Processing Area was potentially influenced by several factors, including insects, and may not be simply a sub-population of the rhizosphere microbiome, as it is often thought (Gaiero et al., 2013; Turner et al., 2013).

Although common orders were identified throughout the root endosphere of different plant species, the presence and proportion of less dominant orders varied throughout the site. In general, the relative abundance of the identified orders (accounting for at least 1% of one endophytic root community) were found to not be significantly correlated with hydrocarbon content (data not shown). However, *Legionellales* ($r=0.91$, $p<0.001$) and *Rhodospiralles* ($r=0.36$, $p=0.03$) were exceptions. Both of these bacteria are linked to the alkane 1-monooxygenase enzyme, which is involved in the degradation of alkanes (Van Beilen et al., 2003; Nie et al., 2014; Page et al., 2015). Microbial community structures have been observed to undergo a shift towards hydrocarbon degraders in the presence of hydrocarbon contamination (Saul et al., 2005; Vázquez et al., 2009). A study conducted by Siciliano et al. (2001), found that the enrichment of bacterial possessing hydrocarbon degrading genes in the root endosphere were largely in response to a contaminant within the soil. However in our study, the lack of correlation between

hydrocarbon content and additional known hydrocarbon degraders (e.g. *Pseudomonadales*, *Xanthomonadales*, and *Rhodobacteriales*) indicates that hydrocarbon content was not the dominant factor influencing bacterial community composition with the root endospheres.

Environmental factors, such as soil type, are often documented to be the main factor influencing the composition of root-associated bacteria (De Ridder-Duine et al., 2005; Singh et al., 2007; Bulgarelli et al., 2013). However, root-associated bacteria are also influenced by plant specific factors, such as plant species (Haichar et al., 2008; Aleklett et al., 2015;), plant genotype (van Overbeek and van Elsas, 2008; Shi et al., 2014), and plant growth stage (van Overbeek and van Elsas, 2008; Shi et al., 2014). In this study, similar plant species had largely similar root-associated bacterial composition, despite different sampling locations. This was reflected in the NMDS ordination analysis where root endophytic communities of SB were often grouped together across sampling locations. However, community structure was also influenced by sampling location as the root endophytic community of SW in the River Bank was separate from the SW of the Entrance and Processing Area, which overlapped. In addition, the root endophytic community structures of HT were unique from each other. Once the *Acholeplasmatales* order was removed from the ordination, it was apparent that the root endophytic communities between HT from the River Bank and Processing Areas were more similar, suggesting that environmental factors, possibly caused by insects, dominantly influenced the community structure. The apparent influence of both plant species and sampling location in Bitumount, accounts for the variability in the root endophytic community profiles. Each community is affected by separate conditions emphasizing that several factors and their interaction influence community structure.

3.6.2 Enumeration of culturable bacteria

Differences in the abundance of culturable rhizosphere and root endosphere bacteria were the result of the interaction of plant species with sampling location throughout the Bitumount site. Abundance of CFUs were consistently highest in the rhizosphere (10^6 to 10^8 CFUs $g^{-1}$of fresh root) and lowest in the endosphere (10^4 to 10^6 CFUs $g^{-1}$of fresh root). This enhanced bacterial abundance in the rhizosphere is the result of microbial growth, diversity, and abundance being stimulated by plant released substances, commonly referred to as the rhizosphere effect (Marecik et al., 2015). In this study, the bacterial population abundances are consistent with many studies as generally bacterial populations range from 10^7 -10^9 CFUs g^{-1} of fresh weight in

the rhizosphere and from 10^5-10^7 log CFUs g^{-1} of fresh root weight in the endosphere (Compant et al., 2010).

Although root-associated bacterial abundance differed throughout the site, soil hydrocarbon content had less influence on culturable endophytic bacterial abundance than anticipated. Hydrocarbon content is documented to both increase and decrease root-associated bacterial abundance (Kandalepas et al., 2015; Kostka et al., 2011). Peng et al. (2013) studied the abundance of endophytic root bacteria in two different plant species, *Alopecurus aequalis* Sobol and *Oxalis corniculata* L., from polycyclic aromatic hydrocarbon (PAH) contaminated soils. Similar to our findings, CFUs ranged from 10^4 to 10^7, however they found that the number of cultivable endophytic bacteria decreased with increasing PAH concentration, whereas no significant correlation between hydrocarbon content and endophyte abundance was found ($r=0.27$, $p=0.12$) (Peng et al., 2013). Contrary to endophyte bacterial abundance, a negative correlation was found between hydrocarbon content and rhizosphere bacterial abundance ($r=-0.35$, $p=0.04$). In addition, rhizosphere bacterial abundance had a positive correlation with nitrate, phosphate, and potassium. Limited soil fertility can affect rhizosphere communities directly by influencing their nutrition or indirectly by influence root morphology and exudation (Rengel and Marschner, 2005). Generally, bacterial abundance in the rhizosphere will be highest around the root tip zone, where there is more root exudation, and lowest around the mature root zone (Lagos et al., 2015). Root exudates provide a nutrient rich environment where microbial activity is enhanced (de-Bashan et al., 2012). These positive relationships between nutrient availability and bacterial abundance in the rhizosphere indicate that improved environmental conditions (i.e. higher nutrient levels and lower hydrocarbon content) benefit both plants and rhizosphere bacteria.

The lack of correlation between soil properties and root bacterial endophyte abundance may suggest that there were other more dominant factors affecting the abundance of bacteria within plant roots. As previously mentioned, endosphere bacteria are thought to be a subset of the rhizosphere bacteria that are able to colonize the endosphere (Germida et al., 1998). Population of endophytes are highly varied; influenced largely on bacterial species and host genotype, however plants naturally select bacteria that are best adapted for living within roots (Rosenblueth and Martínez-Romero, 2006). Plants are able to actively recruit bacteria through

chemical signals and nutrient sent in root exudates, which allows them to influence the abundance of bacteria that may colonize the plant interior (Gaiero et al., 2013). Plants may be able to use recruitment as a response to a contaminant, to actively recruit bacteria with a specific genotype to defend against the toxin (Siciliano et al., 2001), thus increasing their stress tolerance.

3.6.3 Phylogenetic identification of isolated endophytes

In this study, members of the *Actinomycetales*, *Rhizobiales*, and *Pseudomonadales* orders were the most commonly isolated orders from the root endophytic bacterial communities. Many studies have focused on their dominance within microbial communities associated with hydrocarbon contaminated soils (Balcom and Crowley, 2010; Hayder et al., 2014; Lafortune et al., 2009; Pawlik and Piotrowska-Seget, 2015; Shekhar et al., 2014), indicating that members of these orders can be well-adjusted to hydrocarbon contaminated soils and are amenable to be cultured in a laboratory setting with relative ease.

Similar to this study, the root endophytic communities of salt marsh plants species in contaminated soils were dominated by *Pseudomonas* species and comprised members of the *Sphingobium* (*Sphingomonadales*), *Microbacterium* (*Actinomycetales*), *Ochrobactrum* (*Rhizobiales*), and *Micrococcus* (*Actinomycetales*) genera, all of which are associated with hydrocarbon degradation abilities (Oliveira et al., 2014). The only *Pseudomonadales* genus isolated, *Pseudomonas*, are often found to possess the *nah*-type gene which is involved in the breakdown of aromatic compounds (Singleton et al., 2009) and is associated with alkane degradation (Saul et al., 2005). Other root endophytic bacteria isolates have shown close association with hydrocarbon degradation, such as *Brevundimonas* (*Caulobacterales* order) which was revealed to increase PAH degradation potential and activity (Phillips et al., 2008). In addition, isolated orders such as *Rhodobacteriales* (Atlas et al., 2015), *Flavobacteriales* (Biswas et al., 2015), *Xanthomonadales* (Larik et al., 2015), and *Rhodospiriales* (Acosta-González et al., 2015) are linked to hydrocarbon degradation. Not only were the isolated endophytic bacteria related with hydrocarbon degradation but additional plant growth promoting abilities are associated with other orders such as *Actinomycetales*, *Rhizobiales*, *Bacilliales*, *Burkholderiales*, and *Enterobacteriales*. All of these taxa are associated with either the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole acetic acid (IAA) (Saif et al., 2014; Yaish et al., 2015), siderophore production (Jetiyanon, 2015; Prasad et al., 2015),

phosphate solubilisation (Jetiyanon, 2015; Saif et al., 2014), or nitrogen fixation (Okon et al., 2015).

Overall, endophytic communities identified through culture dependent techniques were considered to be less diverse than those analyzed from culture independent techniques (Table D.1). Although there were no significant differences in diversity indices for the isolates, similar to diversity indices calculated via high-throughput sequencing, the endophytic root bacteria of HT in the River Bank was found to be significantly more diverse that HT in the Processing Area. Low diversity in the Processing Area suggests that the root endophytic community was dominated by one or few species and the remainder are considered rare. This is apparent through the targeted 16S rRNA high-throughput sequencing of the endophytic communities, where the community of the HT root endosphere in the Processing Area was dominated by one order (*Acholeplasmatales*), and the HT root endosphere in the River Bank had close to equal dominance of three species (*Rhizobiales*, *Burkholderiales*, and *Actinomycetales*).

Compared to the sequencing of the extracted bacterial genomic DNA, which identified a total of 88 different orders, the culture dependent approach identified 13 orders. Each of the 13 isolates identified using culture dependent techniques were present in the 16S rRNA libraries and were within the top 20 most abundant orders based on both frequency and relative abundance. The ability to culture the dominant orders identified via high-throughput sequencing emphasizes their ability to become an effective inoculant. Microbial activity and abundance are shaped by inter-microbial competition (Thompson et al., 2005). The ability of an inoculated strain to persist in a natural environment is largely influence by its ability to compete with indigenous bacteria (Stefani et al., 2015). Thus if an inoculant strain is already dominant within an environment, they are more likely to persist and thrive. Since each of the isolated orders have known plant growth promoting abilities and are found to be dominant, there may be potential to identify inoculants that will promote plant establishment and survival in contaminated soils.

3.7 Summary

This study showed that the diverse plant species that have naturally recolonized Bitumount, support a wide variety of root endophytic bacterial communities. Culture dependent techniques demonstrated that a portion of the root-associated bacteria are culturable in a

laboratory system and that their abundance differed between plant species and sampling locations. Our findings determined that the abundance and community structure of root-associated bacteria was influenced by the interaction of plant species with sampling location, rather than a dominant effect of one or the other. Culture dependent techniques were able to accurately identify some of the more abundant orders characterized through high throughput sequencing. Members of the *Actinomycetales*, *Rhizobiales*, *Pseudomonadales*, *Burkholderiales*, and *Sphingomonadales* orders were the most commonly identified via both techniques. Their abundance emphasizes their potential ability to adjust to hydrocarbon contaminated soils that had varied levels of soil fertility. Since the natural vegetation at Bitumount harboured distinct microbial communities, further research is required to understand if these communities are aiding in plant establishment and growth in hydrocarbon contaminated soils. Studying the plant growth promoting abilities of these bacterial communities, especially hydrocarbon degradation, will allow us to gain an understanding of the functional role of these plant-associated bacteria.

4.0 HYDROCARBON DEGRADING GENES PRESENT WITHIN BACTERIAL ROOT ENDOPHYTES

4.1 Preface

In the previous chapter, it was demonstrated that the plants growing at Bitumount were able to support a wide variety of bacteria within the root endosphere despite high levels of soil hydrocarbon contamination. Results found that both plant species and sampling location significantly influenced the root endophytic bacterial community structure, as well as the abundance of rhizosphere and root endosphere bacteria. Overall, it was concluded that there was no single factor influencing the community composition of the root endophytic bacteria. In addition, many of the root endophytic bacteria identified at Bitumount have been linked with plant growth promoting abilities in other studies. Previous studies have found that the ability of plants to establish in hydrocarbon contaminated soils depends largely on the presence of hydrocarbon-degrading bacteria. However, the factors affecting hydrocarbon degrading bacteria within the root endosphere of naturally recolonized vegetation are not well resolved. To address this, two hydrocarbon degrading genes (CYP153 and *alkB*) were quantified within the root endosphere of the natural vegetation growing at Bitumount to determine if the abundance of the functional genes were related to plant species or soil properties.

4.2 Abstract

The Bitumount Provincial Historic site is the location of two of the world's first oil extracting and refining operations. Bitumount is thought to be contaminated through both natural and anthropogenic causes. Despite this, plants have successfully recolonized the site even though hydrocarbon levels range from 330-24700 mg kg^{-1} of soil. Due to this apparent resilience of the plant community, Bitumount represents a unique opportunity to study the hydrocarbon degrading genes of the root endophytic bacteria. This study was conducted to survey two hydrocarbon degrading genes (CYP153 and *alkB*) associated with the root endophytic community. Plant samples including smooth brome (*Bromus inermis*, SB), horsetail sp. (*Equisetum* sp., HT), slender wheatgrass (*Agropyron trachycaulum*, SW), Kentucky bluegrass (*Poa pratensis*, KB), an unspecified member of the pea family (*Fabaceae*, PF), and wild strawberry (*Fragaria virginiana*, WS) were collected in June 2014. Quantitative PCR was used to assess the gene copy abundance of 16S rRNA, CYP153, and *alkB* in root endophytic communities. Plant species and sampling location interacted to significantly affect ($p<0.05$) the abundance of 16S rRNA, CYP153, and *alkB* genes. Gene abundance was consistently lowest in the PF root endosphere and highest within SW in the samples collected from the Entrance and Processing Area. There was no apparent shift towards increased hydrocarbon degrading gene abundance in sampling locations that had higher soil hydrocarbon concentrations. Positive correlations between certain soil nutrients and *alkB* and 16S rRNA, indicate the beneficial effect of soil fertility on endophytic bacteria. In contrast, the three gene targets assessed were negatively correlated with soil hydrocarbon content. Results from this study suggest that grass species may be the most suitable for reclamation of hydrocarbon contaminated sites as they had increased hydrocarbon degrading genes within the root bacterial communities.

4.3 Introduction

Hydrocarbons represent one of fastest growing pollutants worldwide and are considered one of the most prevalent environmental contaminants in Canada (Canadian Council of Ministers of the Environment, 2014). Although hydrocarbon contamination can occur through natural causes, anthropogenic activities represent a major source of hydrocarbon contamination to soil. A large portion of hydrocarbon contamination occurs as the result of the increased use of petroleum products as an energy source. It is anticipated that from 2012 to 2040, the global demand for energy will rise 37% as countries continue to grow and the standard of living improves (Canadian Association of Petroleum Producers, 2015b). The Athabasca oil sands, located in Alberta, Canada, is one of the largest oil reserves in the world, thought to have over 170 billion barrels of recoverable oil (Lynam et al., 2015). Oil production in the Canadian oil sands is expected to increase from 2.2 million barrels a day in 2014, to 4.0 million barrels in 2030 (Canadian Association of Petroleum Producers, 2015b) increasing the potential for contamination. Hydrocarbons can enter the environment during extraction, transportation, shipping and through urban runoff and pollutant discharges (Lozada et al., 2014). Hydrocarbons are persistent and toxic (Alrumman et al., 2015); their accumulation in animal and plant tissue can cause death or mutations (Das and Chandran, 2011). Because of the risks involved with hydrocarbon contamination, industry is legally obligated to reclaim all land that is used for oil production.

There are several physical and chemical technologies used to treat petroleum-contaminated soils including combustion, photolysis, and adding dispersant (Xu et al., 2013). Not only can these approaches be expensive, but they have the potential to leave compounds that are more environmentally toxic than the initial contaminant (Shahi et al., 2016). On the other hand, biological approaches, such as bioremediation, can be used as an alternative for addressing soil hydrocarbon contamination. Bioremediation is the process of using microorganisms and plants to remove organic or inorganic pollutants from the environment. It is characterized as an effective, economic, and versatile method to treat hydrocarbon contaminated soils (Reddy et al., 2011). However, since petroleum hydrocarbons are comprised of a variety of toxic compounds including normal alkanes (n-alkanes), and cyclic alkanes (c-alkanes) (Fukuhara et al., 2013),

plant growth can be inhibited. Therefore, the success of bioremediation relies heavily on the plant-associated microorganisms, particularly the endophytic bacteria (Kukla et al., 2014).

Endophytes, defined as microorganisms that colonize the interior of the plant without causing any harm, are of great interest due to their close relationship with plants. Not only will endophytes not cause any deleterious effect to the host plants, but they also have the ability to alleviate plant stress. Plant growth promotion can occur through several mechanisms such as biofertilization and phytostimulation (Mercado-Blanco, 2015). However, the success of bioremediation relies heavily on the metabolic potential of the microorganisms to transform or detoxify pollutants (Megharaj et al., 2011). Bacteria are able to reduce the toxicity of the contaminant to plants by degrading and converting the pollutant into easily metabolizable substrates. The ability of bacteria to mineralize the alkanes is largely attributed to enzymes such as the alkane hydroxylases, AlkB and cytochrome P450, which are encoded by the *alkB* and CYP153 genes, respectively (Fatima et al., 2015).

The most widely studied alkane hydroxylases are the AlkB enzymes (Koch et al., 2009) that carry out a terminal hydroxylation of n-alkanes (Singh et al., 2012). *alkB* is responsible for the aerobic transformation of mid-length *n*-alkanes (C_5 to C_{16}) and in some cases degrade longer *n*-alkanes chains (Pérez-de-Mora et al., 2011). The cytochrome P450 is another alkane hydroxylase for the degradation of midlength *n*-alkanes (C_5 to C_{10}) (Rojo, 2010). The cytochrome P450 is versatile, able to introduce oxygen into a wide range of substrates and catalyze chemical reactions (Denaro et al., 2010). Not only can the same bacteria have genes that encode both CYP153 and *alkB* but the two functional genes can cooperatively control hydrocarbon oxidation (Ivanova et al., 2014). It is reported that more than 60 genera of aerobic bacteria and 5 genera of anaerobic bacteria are able to degrade n-alkanes (Nie et al., 2014).

Studying hydrocarbon degrading genes can be a helpful indicator to determine the bioremediation potential of a contaminated site by functionally characterising the bacterial community (Yang et al., 2014). Using culture dependent techniques to enumerate hydrocarbon degrading bacteria can be time consuming and may only represent a small portion of the community. However molecular methods have led to significant advancements in *in situ* bioremediation (Brow et al., 2013) and methods such as quantitative real-time polymerase chain

reaction (QPCR), are an effective alternative for estimating the degradative potential of the bacterial consortium within a contaminated environment (Cébron et al., 2008). During QPCR, specific primers target a functional gene and detect its abundance in the population of interest (Griffin, 2014). Therefore, QPCR assesses the potential microbial ability to degrade hydrocarbons, providing an indicator of remediation capacity in hydrocarbon contaminated sites.

The Bitumount Provincial Historic site, located within the Athabasca oil sands, is the location of two of the world's first oil extracting and refining operations. One of Bitumount's defining characteristics is that it has evidence of hydrocarbon contamination through both natural and anthropogenic process. The Athabasca oil sands deposits naturally lie near the surface of the soil, allowing for ease of exploitation of the resource. However, the increased hydrocarbon can lead to plant growth being inhibited. In addition, no reclamation efforts have occurred since Bitumount's closure, allowing for the persistence of hydrocarbons to remain, resulting from the decades of oil exploration. Despite this, vegetation has recolonized the site through means of natural revegetation.

In Chapter 3 of this thesis, the root endophytic bacteria of the natural vegetation at Bitumount were surveyed using culture dependent and independent methods. Results demonstrated that the root endosphere of the different plant species sampled were able to support diverse and abundant microbial communities, with members of the *Actinomycetales*, *Rhizobiales*, *Pseudomonadales*, *Burkholderiales*, and *Sphingomonadales* being the most commonly identified orders. Since the natural vegetation support diverse communities, despite high hydrocarbon concentration, there is a need to characterize their functional potential to determine if they are alleviating stress allowing for plant growth promotion. Therefore, this study was designed to determine if the presence of hydrocarbon degrading bacteria were associated with the natural vegetation growing at the Bitumount Provincial Historic site. The specific aim of this study was to quantify the presence of hydrocarbon degrading genes within the root endophytic bacteria throughout the site.

4.4 Materials and Methods

4.4.1 Experimental design

This study involves the quantification of hydrocarbon degrading genes present within the endophytic root community associated with the natural vegetation growing at the Bitumount Provincial Historic Site, Alberta. As previously mentioned in section 3.2 of this thesis, 12 sampling points were identified and grouped into sampling locations in June 2014. In total there were six different sampling locations, designated as River Bank, Pathway, Processing Area, Quarry, Quarry Border, and Entrance. At each sampling location, representative plant species were collected for further study, therefore not every plant species was sampled at each location and not every location has more than one plant species. They included smooth brome (*Bromus inermis*, SB), horsetail sp. (*Equisetum spp.*, HT), slender wheatgrass (*Agropyron trachycaulum*, SW), Kentucky bluegrass (*Poa pratensis*, KB), an unspecified member of the pea family (*Fabaceae*, PF), and wild strawberry (*Fragaria virginiana*, WS).

Triplicates of each plant sample were collected by excavating the roots. Plant samples were placed into plastic bags, and were stored in coolers for transport to the University of Saskatchewan. The aerial portion of each plant was removed and then plant roots were separated from the bulk soil and stored at -80^{o}C for further analysis.

4.4.2 Sample preparation for QPCR

Roots were surface sterilized using a modified protocol from Siciliano and Germida (1999). Briefly, excess soil was removed by washing roots in water. The roots were then placed in a 300 mL Erlenmeyer flask containing 100 mL NaClO (1.05% v/v) in phosphate-buffered saline (PBS) (1.2 Na_2HPO_4; 0.18 NaH_2PO_4; 8.5 NaCl (g L^{-1})). The Erlenmeyer flasks, containing the roots, were placed on a rotary shaker for 15 min at 150 rpm. The roots were then rinsed 10 times using 100mL of sterile tap water. To check surface sterility, 0.1 mL of each final wash was plated on 1/10 strength trypticase soy agar (1/10 TSB; trypticase soy broth (3g L^{-1}); solidified with agar (15 g L^{-1}), with cycloheximide (Phillips et al., 2010). Successful sterility of the roots was confirmed by visual checks of the plates after 72 h.

Total genomic DNA was extracted from 0.1 g of surface sterilized roots using the standard MoBio protocol from the PowerPlant® Pro DNA Isolation kit. Concentration of individual DNA extracts were then quantified using a Qubit Fluorometer (Qubit 2.0 Fluorometer, Life Technologies).

4.4.3 QPCR standard preparation

Template DNA for standards of CYP153 and *alkB* were synthesized using Biomatik services (Cambridge, Canada) with the flanking restriction sites for subcloning into the pUC18 plasmid. For construction of the 16S rRNA gene standard, DNA was extracted from a *Pseudomonas stutzeri* culture (Fierer et al., 2005) obtained from the culture collection of the Soil Microbiology Laboratory at the Department of Soil Science, University of Saskatchewan. Using the genomic DNA extracted from the pure culture, the eubacteria16S rRNA gene was PCR amplified and amplicon size was verified on 1.0% agarose gel. The bands of interest were excised under UV light and were gel purified using the standard operating procedure outlined by the Qiagen QIAquick gel extraction kit (Qiagen Inc., Toronto, Ontario). The target insert was then quantified using the Qubit Fluorometer (Qubit 2.0 Fluorometer, Life Technologies). After the target insert was prepared, it was ligated into the plasmid vector using Invitrogen TOPO TA Cloning Kit® (Thermo Fisher Scientific Inc., Waltham, MA) with One Shot ® MAX efficiency ™ DH5α –T1R Escherichia coli. Transformations were spread on liquid broth (LB) media with 50 µg ml^{-1} of ampicillin and X-gal for 16 h at 37.0 ^{o}C. Successful ligation, confirmed as white colonies, were then grown in 3 ml LB broth (16 h at 37.0 ^{o}C, 150rpm) with 50 µg ml^{-1} of ampicillin. To isolate the E. coli host genomic DNA, 2 ml of colonies with good turbid growth were selected for plasmid miniprep using the Qiagen QIAprep Spin Miniprep kit (Qiagen Inc., Toronto, Ontario).

Plasmids with inserts, including synthesized CYP153 and *alkB*, respectively, were then linearized using HindIII restriction enzyme (Thermo Fisher Scientific Inc., Waltham, MA). After linearization, plasmids were loaded onto an agarose gel to separate the linearized plasmid from the reaction components and uncut plasmid. Bands of interest were then excised and gel purified using Qiagen QIAquick gel extraction kit (Qiagen Inc., Toronto, Ontario). The linearized plasmid and insert were quantified using Qubit Fluorometer (Qubit 2.0 Fluorometer, Life

Technologies), and were then used in a dilution series to form a standard curve. Efficiency (E) of the QPCR and correlation coefficient (R^2) were calculated based on the acquired slopes of the standard curves achieved using a serial 10-fold dilution of standards. The following equation was used to achieve efficiency: $E\ (\%) = (10^{(-1/\text{slope})} - 1) \times 100$. For each QPCR run, a five point standard curve using technical triplicates was generated along with triplicate negative controls.

4.4.4 QPCR assays

A QPCR assay was conducted for each target gene (16S rRNA, CYP153, *alkB)* using the Applied Biosystems® One Plus Real-Time PCR System and the Platinum SYBR green QPCR Supermix (Thermo Fisher Scientific Inc., Waltham, MA). The primers and QPCR conditions used for each gene can be found in Table 4.1. For the universal 16S rRNA gene, serial plasmid dilutions from 10^7 to 10^2 gene copies μL^{-1} were used as standards. The PCR protocol for the universal 16S rRNA target consisted of 10 µl of Platinum SYBR mix, 0.75 µl of each primer, 0.4 µl of Rox (1:10) (Thermo Fisher Scientific Inc., Waltham, MA), 0.625 µl of 1:5 bovine serum albumine (BSA) (10 µg μl^{-1}) (Thermo Fisher Scientific Inc., Waltham, MA), 2 µl of template DNA (3 µg μl^{-1}), and 5.475 µl of ultra-pure water. Standard curves for 16S rRNA (r^2>0.99) were linear with a minimum efficiency of 86.3%.

Serial plasmid dilutions, for the CYP153 gene, from 10^6 to 10^1 gene copies μL^{-1} were used as standards. The CYP153 PCR assay contained of 10 µl of Platinum SYBR mix, 1.25 µl of the forward and reverse primer, 0.4 µl of Rox (1:10), 2 µl of 1:5 BSA (10 µg μl^{-1}), 2 µl of 3 µg μl^{-1} of template DNA, and 3.1 µl of ultra-pure water. The minimum efficiency for CYP153 analyzed was of 92.1% and all standard curves were linear (r^2>0.99). For the *alkB* gene, standards were formed from serial plasmid dilutions ranging from 10^6 to 10^1 gene copies μL^{-1}. The PCR assay for *alkB* comprised of 10 µl of Platinum SYBR mix, 0.4 µl of $MgCl_2$ (50mM), 0.4 µl of each primer, 0.4 µl of 1:10 Rox (1:10), 0.4 µl of 1:5 BSA(10 µg μl^{-1}), and 4 µl of both template DNA (3 µg μl^{-1}) and ultra-pure water. Standard curves were linear (r^2>0.99) for *alkB* with a minimum efficiency of 83.73%.

QPCR assays were run in three technical replicates for each root endophytic bacterial community sample. The threshold cycle (Ct) values and the gene copy numbers received were used to create standard curves. Data was expressed as gene copy number per gram of fresh root weight.

Table 4.1 Primers and conditions for quantitative real-time polymerase chain reaction (QPCR) of hydrocarbon degrading genes present within the endosphere of the natural vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada.

Target Gene Primer	Primer sequence (5'-3')	QPCR conditions	Reference
16S rDNA (Eubacteria)			
Eub338F	ACTCCTACGGGAGGCAGCAG	2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 1min, 30s of annealing at 53°C, 72°C for 1min, with data collection for 1 min at 80 °C.	(Fierer et al., 2005)
Eub518R	ATTACCGCGGCTGCTGG		
CYP153 (alkane hydroxylase)			
P450fw1	GTSGGCGGCAACGACACSAC	4 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30s, and elongation at 72°C for 45s. Data collection for 1 min at 80 °C.	(Arslan et al., 2014)
P450rv3	GCASCGGTGGATGCCGAAGCCRAA		
alkB (alkane hydroxylase)			
alkB-1f	AAYACIGCICAYGARCTIGGICAYAA	10 min at 95°C, followed by a touchdown PCR of 5 cycles of 95 °C for 45s, 1 min 62 °C and a stepwise reduction to 57 °C and 45s at 72 °C . Followed by 40 cycle of 95 °C for 45s, 57 °C for 1 min and 45s at 72 °C.	(Wallisch et al., 2014)
alkB-1r	GCRTGRTGRTCIGARTGICGYTG		

4.4.5 Statistical methods

Statistical analyses were performed using the SAS program (version 9.3) with a SAS Macro add in (The SAS system for Windows, SAS institute, 2003). The Shapiro-Wilk statistic was used to test the normality of the data. Analysis of variance (ANOVA) was used to determine the significant difference of values between the treatments and their interactions. A mixed model approach was used with sampling location defined as the random effect and plant species defined as the fixed effect. A Tukey's test for post hoc analysis at 5% significance level was used to analyse the differences between treatment means. Pearson correlation was used to determine possible relationships between gene abundance of 16S rRNA, CYP153, *alkB*, and soil properties (Table 3.2 and 3.3).

4.5 Results

Figure 4.1 illustrates the abundance of the universal 16S rRNA gene in root-associated DNA extractions from plants throughout Bitumount. Plant species and sampling location interacted significantly ($p<0.05$) to affect abundance of bacteria associated with plant roots at the site. Bacterial 16S rRNA gene abundance was lowest in the root endophytic community of the PF in the Quarry (3.08 log gene copies g^{-1} of plant root) and highest in the root endosphere of SW in the Entrance and Processing Area with abundances of 4.64 and 4.67 log gene copies g^{-1} of plant root, respectively. However, only the root endophytic community of PF in the Quarry and WS in the Quarry border harbored significantly ($p<0.05$) lower 16S rRNA gene copies than the other root endophytic communities.

Quantification of the two functional genes, CYP153 and *alkB*, was performed to assess the presence of degradative enzymes encoded within the genes. Similar to bacterial 16S rRNA gene abundances, the CYP153 gene copy number was lowest in the PF root endosphere of the Quarry and highest within the root endophytic community associated with SW of the entrance, with abundances of 3.11 log gene copies g^{-1} of plant root and 4.67 log gene copies g^{-1}, respectively (Fig. 4.2). Bacterial CYP153 gene copies were found to be significantly ($p<0.05$) influenced by the interaction of plant species with sampling location. However, only the root endophytic community of PF in the Quarry supported significantly lower abundances of the

CYP153 genes than the other endophytic communities, with exception of the endosphere of SB in the Entrance and Processing Area, and WS in the Quarry Border.

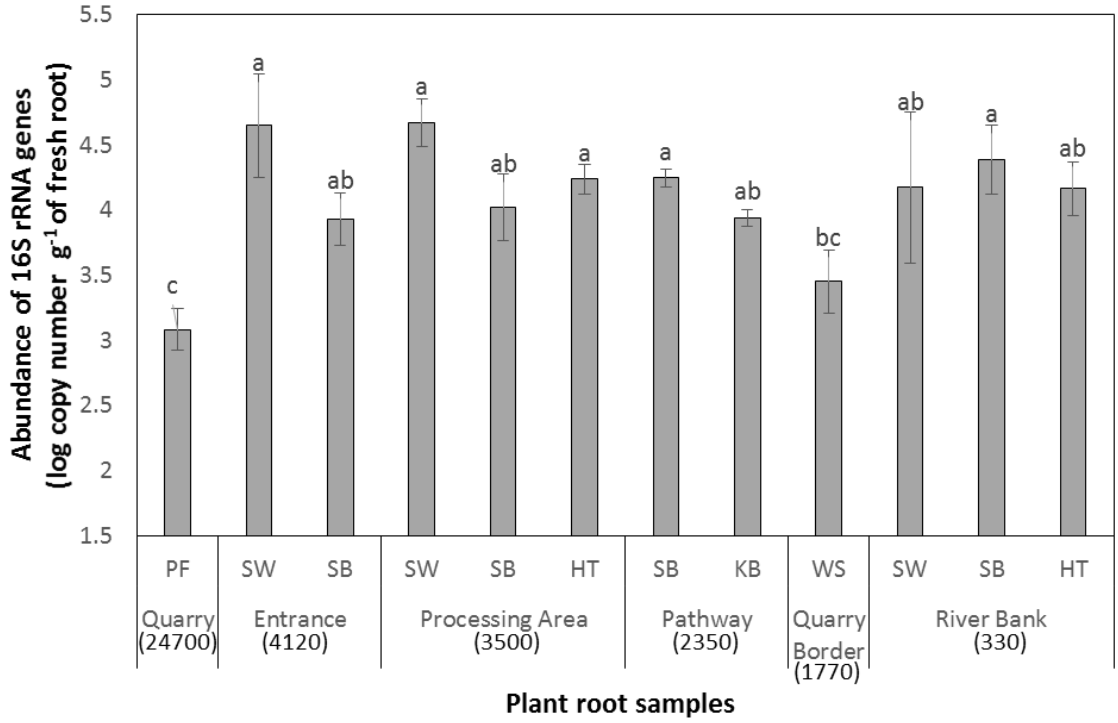

Figure 4.1 Abundance of 16S rRNA within the root endosphere of natural vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada. Error bars represent standard deviation. Different letters indicate significant difference ($p \leq 0.05$) using Tukey's Post-hoc. Values in parentheses on the x-axis represent total hydrocarbon content (mg kg^{-1}) for each sampling location. SW, slender wheatgrass (n=9); SB, smooth brome (n=12); HT, horsetail (n=6); KB, Kentucky bluegrass (n=3); PF, pea family (n=3); WS, wild strawberry (n=3).

The bacterial *alkB* gene abundance results are illustrated in Fig. 4.3. Gene abundance of *alkB* was consistently lower than the two other targeted genes and was significantly ($p<0.05$) influenced by the interaction of plant species and sampling location. Similarly to CYP153 gene abundance, the root endosphere of PF in the Quarry supported the lowest *alkB* gene abundance (2.69 log gene copies g^{-1} of plant root), which was found to be significantly lower than the other plants sampled with the exception of SB in the Entrance and WS in the Quarry Border.

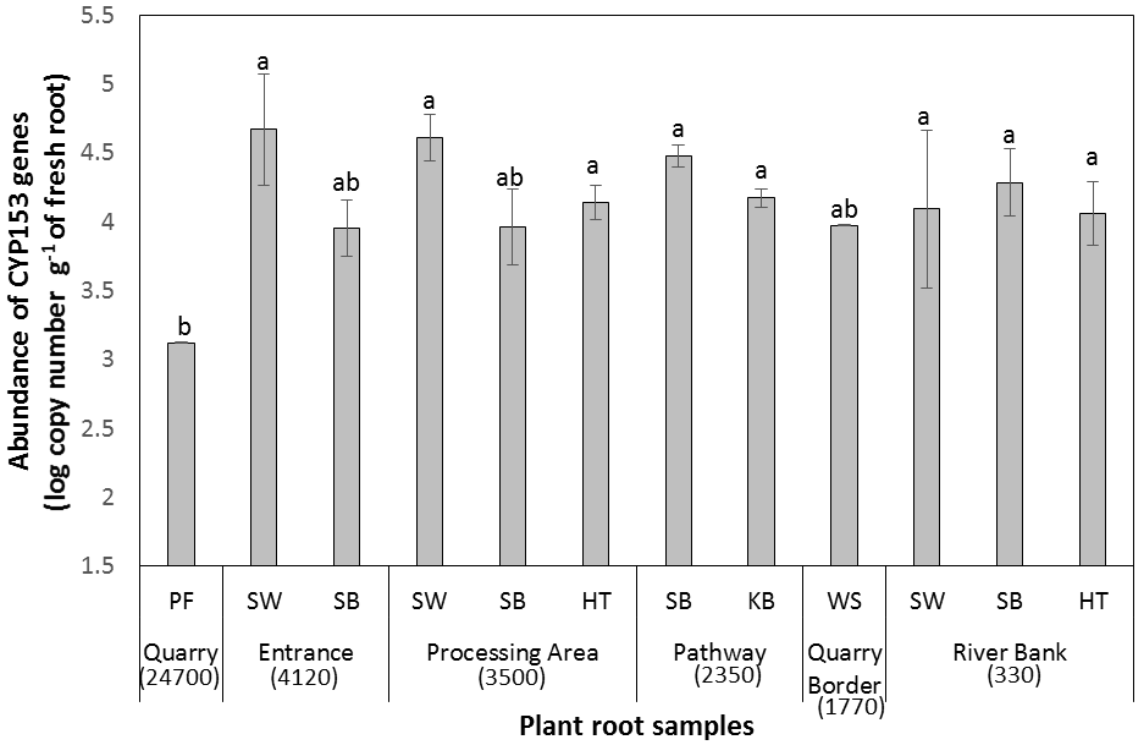

Figure 4.2 Log gene copy number of CYP153 within root endosphere of natural vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada. Error bars represent standard deviation. Different letters indicate significant difference ($p \leq 0.05$) using Tukey's Post-hoc. Values in parentheses on the x-axis represent total hydrocarbon content (mg kg^{-1}) for each sampling location. SW, slender wheatgrass (n=9); SB, smooth brome (n=12); HT, horsetail (n=6); KB, Kentucky bluegrass (n=3); PF, pea family (n=3); WS, wild strawberry (n=3).

Based on the gene copy abundances per fresh root weight, the ratio of the two functional genes to 16S rRNA were calculated to estimate if the proportion of functional genes within bacteria from the root endosphere differed throughout Bitumount (Fig. 4.4). The lowest ratio was associated with CYP153 gene abundance in the root endosphere was 0.97 within HT (River Bank and Processing Area) and SB (River Bank) and increased to 1.2 (WS in the Quarry Border). The lowest ratio associated with *alkB* was 0.87 in the root endosphere of PF (Quarry) and increased to a ratio of 0.97 within the SB root endosphere in the Pathway. There was no significant difference *($p>0.05$*) occurring between plant species and location for either CYP153 or *alkB* genes. This suggests that the proportion of hydrocarbon degrading genes found within a bacterial community was not dependent on plant species sampled or sampling location.

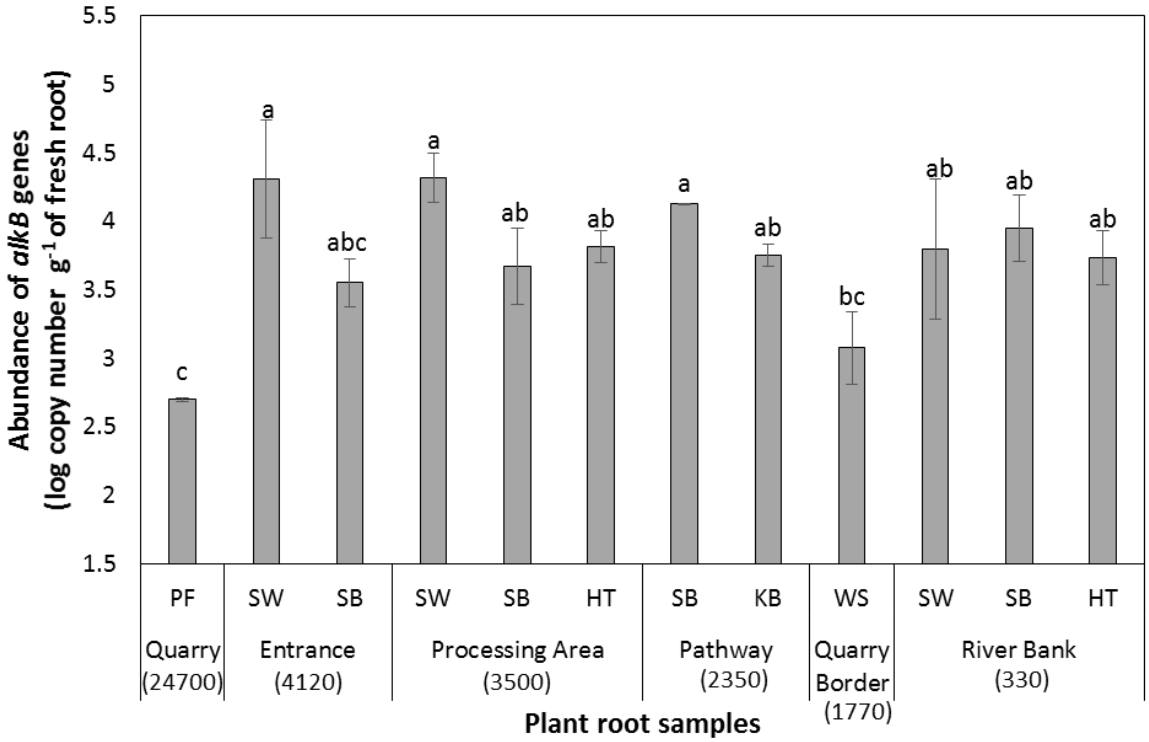

Figure 4.3 Log gene copy number of *alkB* within root endosphere of natural vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada. Error bars represent standard deviation. Different letters indicate significant difference ($p \leq 0.05$) using Tukey's Post-hoc. SW, slender wheatgrass (n=9); SB, smooth brome (n=12); HT, horsetail (n=6); KB, Kentucky bluegrass (n=3); PF, pea family (n=3); WS, wild strawberry (n=3).

Pearson correlation demonstrated significant relationships between the soil properties and the gene copy abundance for each gene quantified (Table 4.2). Hydrocarbon content demonstrated a strongly negative correlation with *alkB* ($p<0.01$), 16S rRNA, and CYP153 abundance ($p<0.001$) suggesting that hydrocarbon levels negatively influenced both total number of root endophytic bacteria, and bacteria harboring hydrocarbon degrading genes. Positive correlations between pH with *alkB* gene copies ($p<0.01$) and with 16S rRNA and CYP153 ($p<0.001$) were also found. In contrast, a significant positive correlation existed between gene copy numbers and available nutrients. Specifically, phosphate had a significant positive correlation ($p<0.05$) with 16S rRNA and *alkB* abundance. A positive correlation ($p<0.05$) also existed between the 16S rRNA gene abundance and potassium levels. To determine the effect of the high hydrocarbon content and low pH levels in the Quarry, a Pearson correlation statistic was

calculated for all three genes against pH and hydrocarbon content, without the inclusion of the Quarry (Table G.1); however, there was no significant correlation.

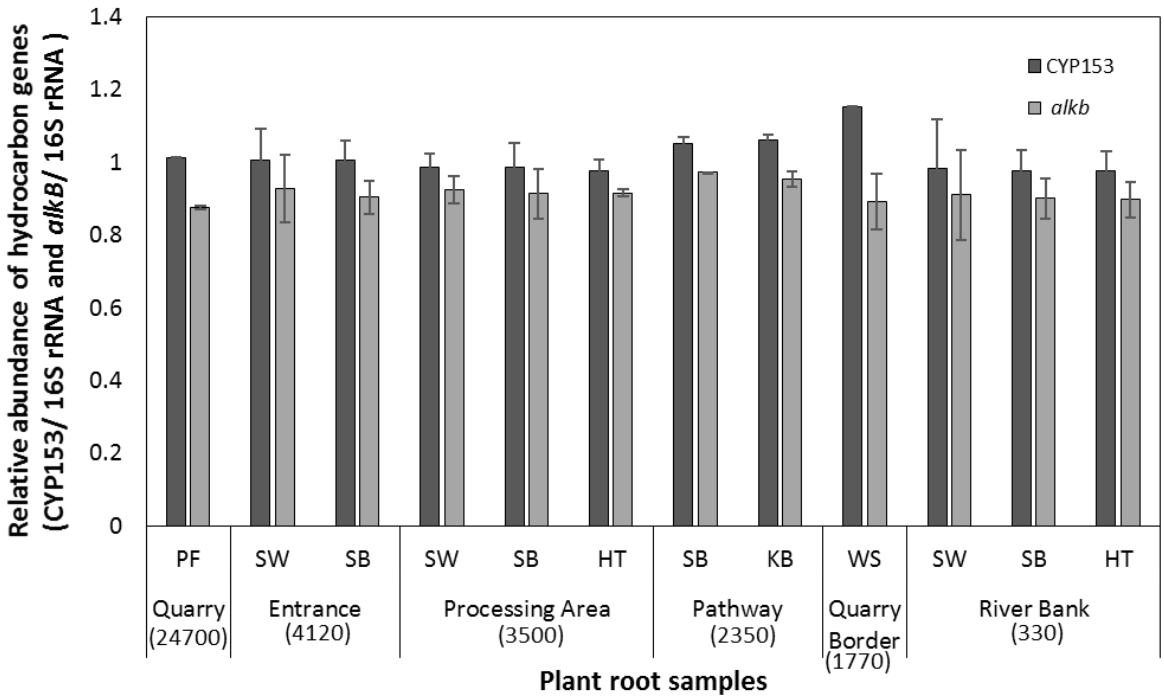

Figure 4.4 Relative abundance of hydrocarbon degrading genes (CYP153/ 16S rRNA and *alkB*/ 16S rRNA) in root endosphere of the natural vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada. Error bars represent standard deviation. Values in parentheses on the x-axis represent total hydrocarbon content (mg kg^{-1}) for each sampling location. SW, slender wheatgrass (n=9); SB, smooth brome (n=12); HT, horsetail (n=6); KB, Kentucky bluegrass (n=3); PF, pea family (n=3); WS, wild strawberry (n=3).

Table 4.2 Pearson correlation coefficients between soil properties and gene copy numbers of 16S rRNA, CYP153, and *alkB* of the root bacterial endophytes associated with the natural vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada.

Genes	pH	Nitrate	Sulfate	Phosphate	Potassium	Hydrocarbons
16S rRNA	0.53***	NS	NS	0.41*	0.40*	-0.58***
CYP153	0.56***	NS	NS	NS	NS	-0.57***
alkB	0.50**	NS	NS	0.36*	NS	-0.53**

*,**,***, Significant at $p \leq 0.05, 0.01, 0.001$

4.6 Discussion

Hydrocarbon contaminated soils are known to inhibit plant growth, thus influencing the ability to reclaim disturbed land. One of the main inhibiting factors to plant survival is the toxicity of low molecular weight compounds and the hydrophobic properties caused by hydrocarbons that limit plant water and nutrient absorption (Tara et al., 2014). The ability of plants to persist in hydrocarbon contaminated soils depends dominantly on the capacity of the root associated bacteria to reduce the contaminant through the presence hydrocarbon degrading genes.

In this study, the abundance of two hydrocarbon degrading bacterial genes (CYP153 and *alkB*) and the 16S rRNA gene were quantified from the root endosphere of the natural vegetation growing at Bitumount. There was an interaction effect between plant species and sampling location on the abundance of 16S rRNA, CYP153, and *alkB* genes. Although no comparable studies have been conducted on sites similar to Bitumount, the effect of plant species and soil environment on gene copy abundance are documented. Distinct microbial communities are known to be selected depending on different plant species. In a study conducted by Yousaf et al. (2011), the colonization of a bacterium with the CYP153 gene was partially dependent on the plant species, where the rhizosphere and endosphere of Italian ryegrass (*Lolium ultiflorum)* harbored the highest gene abundance compared to birdsfoot trefoil (*Lotus corniculatus*) and alfalfa (*Medicago sativa*). Additionally, Siciliano et al. (2001) found that plant species, along with contaminant type and level, influenced the abundance of hydrocarbon degrading bacteria, including those harbouring the *alkB* gene .

Plant species-specific factors that influence bacterial abundance and colonization both pre- and post-colonization include root surface structure, root architecture, root exudates, apoplastic fluid nutrient composition, and intercellular space (Hallmann and Berg, 2006). Similar to our results, Yousaf et al. (2010a) found that a higher abundance of alkane-degrading bacteria were associated with the grass species than with a flowering plant from the pea family. Although it is species-dependent, grasses are well suited for remediation due to their extensive root systems, vigorous growth following establishment, and tolerance to acidic soils, dry conditions, and cold temperatures (Cook and Hesterberg, 2013). Grasses have extensive fibrous

roots that provide an increased surface area for the colonization of bacteria and nutrient uptake (Fatima et al., 2015), thus increasing the potential for harbouring hydrocarbon degraders. Their roots also physically disrupt the soil leading to an introduction of oxygen, which is required to catalyze the oxidation of alkanes by alkane hydroxylases (Tsuboi et al., 2015). In addition, grass roots and rhizomes both release alkanes into the rhizosphere (Marseille et al., 1999) and absorb alkanes from the soil (Khan et al., 2013), thus favoring the colonization of hydrocarbon degrading bacteria.

Conversely, previous studies also demonstrate the effect that soil type and soil nutrient status have on the hydrocarbon degrading gene abundance in contaminated soil. The colonization capacity of the bacterium carrying CYP153 (*Pantoea* sp. BTRH79) was found to be dependent largely on soil type, where sandy soils harboured lower abundance compared to loamy soils (Afzal et al., 2011). Low gene abundance associated with sandy soils, was partially attributed to increased nutrient competition between plant roots and microbe caused by the low organic matter in sandy soil (Afzal et al., 2011). Similarly, the effect of nutrient levels were seen in a second study where not only the abundance and expression of CYP153, but the colonization and metabolic activity of inoculated bacteria (*Pantoea* sp. BTRH79) in the rhizosphere of annual ryegrass was also enhanced (Arslan et al., 2014). Furthermore, contaminant type and levels have a large effect on the structure of the root bacterial communities, as plants growing on petroleum contaminated soils reportedly recruit hydrocarbon degrading bacteria (Kukla et al., 2014). For example, Phillips et al. (2009), found Altai wild rye selectively recruited and maintained hexadecane degrading root endophytes during stressed conditioned caused by an increase in total petroleum hydrocarbons.

Root endophytic bacteria can experience a shift in community structure after exposure to stress caused by a contaminant (Kandalepas et al., 2015). Previous studies demonstrate a shift towards the dominance of hydrocarbon degraders in soils that are more critically contaminated with hydrocarbon than in less polluted soils (Siciliano et al., 2001; Saul et al., 2005; Vázquez et al., 2009). Conversely, no shift towards increased hydrocarbon degrading genes was detected in the current study, despite varying levels of hydrocarbon content. This suggests that hydrocarbon content was not directly influencing the abundance of hydrocarbon degrading genes, but rather by simply decreasing the size of the total bacterial community. The evenness in the distribution

of the functional genes associated with the root endosphere of vegetation growing at Bitumount highlights that the potential for hydrocarbon degradation is present within many soil bacteria (Kloos et al., 2006). This may result from the fact that some hydrocarbon degraders are known to be facultative, therefore they do not solely rely of the on the presence of hydrocarbons for survival, but can use other substrates as carbon sources (Pérez-de-Mora et al., 2011).

When comparing the abundance of the CYP153 genes to the 16S rRNA gene present within the root endosphere, it is apparent that in certain cases abundance of the CYP153 gene is higher or nearly equal to the 16S rRNA gene. Similar results have been documented in studies that focused of the bacterial gene abundance of hydrocarbon degraders. For example, Richardson et al. (2015) observed that the level of hydrocarbon genes in soil bacteria were higher than that of the total bacterial population (calculated by *rpoB*) in both contaminated and uncontaminated soils at a remediation site of an aged diesel spill in east Antarctica. Additionally, Yergeau et al. (2012) observed high abundances of hydrocarbon degrading genes within the soil at a bioremediation site in the Canadian Artic. They noted that the abundance of genes encoding the hydrocarbon degrading enzymes were above genome equivalents, suggesting that a large portion of the bacteria possessed the hydrocarbon degrading genes, or that the genes were present in multiple copies within individual bacteria (Yergeau et al., 2012). Many alkane degraders are known to possess more than one alkane hydroxylase with overlapping substrate ranges (van Beilen and Funhoff, 2007). *Pseudomonas putida* and *Pseudomonas fluorescens* have demonstrated to functionally express eight CYP153 genes along with a CYP153 family ferredoxin and ferredoxin reductase protein, allowing the host to use a wide range of substrates as carbon and energy sources (Funhoff et al., 2006). *Sphingomonas* sp. HXN-200 have also been known to contain five CYP153 genes, whereas *R. Erythropolis* isolates may possesses up to three CYP153s (Denaro et al., 2010). These results emphasize the importance of being cautious when interpreting results from QPCR as direct indicators of the cell number as there may be multiple ribosomal operons in the bacterial genome (Daghio et al., 2015). Although the results give a general overview into the functional diversity of the root endophytic community of the vegetation growing at Bitumount, the gene copy numbers are not indicative of the abundance of hydrocarbon degrading bacteria.

A strong negative correlation was observed between hydrocarbon content and all three genes present within the root endosphere. This is in disagreement with several studies, as it is often observed that hydrocarbon content can increase microbial abundance, including hydrocarbon degrading genes (Kostka et al., 2011; Wasmund et al., 2009). This was demonstrated by Whyte et al. (2002), who showed that *Pseudomonas putida* GPo1 *alkB* genotype was absent within pristine polar soils but were present within hydrocarbon-contaminated polar soils. This suggest that the hydrocarbon contamination enriched the *alkB* genotype (Whyte et al., 2002). Microbial communities can experience enhanced growth as hydrocarbons provide a major source of carbon, however detrimental effects on soil bacteria may occur as hydrocarbon content increases and becomes toxic. Bacteria tend to become inhibited by toxic conditions when hydrocarbon content reaches levels of 25 000 mg kg^{-1} of soil (Yergeau et al., 2009). Correlation performed following removal of the Quarry location samples (hydrocarbon content of 24 700 mg kg^{-1}) demonstrated that there was no significant effect of hydrocarbon content on gene copy number for any of the three genes. This is in accordance with a study conducted by Perez-de-Mora et al. (2011), who hypothesized that the abundance would increase with long term contamination compared to control soil, but found that the abundance of hydrocarbon degrading genes was not significantly influenced by the different sampling locations and contaminant levels. These results indicate that hydrocarbons may have no effect or may increase bacterial abundance at certain levels; however, the toxic effect caused by too high contaminant concentrations may impede the bacterial community and therefore functional genes.

Soil pH is an important determinant of bacterial community structure which can affect the form, concentration, and availability of substrates, therefore influencing cell growth and activity (Nicol et al., 2008). A positive correlation between pH levels and gene copy abundance of all three genes was observed. However, with the exception of the Quarry (pH=7), pH levels throughout the site were quite similar (pH 8.2-8.5) despite varying hydrocarbon levels. When a correlation was performed with the removal of the extreme data point, there was no longer a significant relationship, suggesting that gene copy numbers abundance are not sensitive to changes in pH.

Nutrient availability has often been reported to be the limiting factor in not only plant growth but also microbial proliferation in oil contaminated soils. The hydrophobic nature of

hydrocarbons decreases the availability of nutrients to plants and bacteria, and can influence the abundance and activity of plant-associated bacteria. The influence of nutrient levels on gene abundance is observed in this study, as 16S rRNA gene copies were positively correlated with both phosphate and potassium and *alkB* abundance was positively correlated with phosphate. In addition to the previously mentioned influence of nutrients on CYP153 abundance, nutrient enrichments have been found to increase total bacteria number in hydrocarbon-contaminated Antarctic soils (Powell et al., 2006), as well as *alkB* gene copies in an oil contaminated soil originating from an industrial zone in Slovenia (Wallisch et al., 2014). The influence of nutrients on hydrocarbon degradation was demonstrated by Siciliano et al. (2003), who studied the influence of planted and non-planted soils on the mineralization of hexadecane and phenanthrene in microcosms. Under no additional fertilization, no differences in mineralization occurred between treatments; however, when fertilizer was added, the planted treatments mineralized significantly more hexadecane and phenanthrene than the unplanted control (Siciliano et al., 2003). This suggests that the hydrocarbon degrading populations were highest when associated with plants, however they were inhibited by low nutrient levels (Siciliano et al., 2003). The results seen in this study, along with others, demonstrate the importance of nutrients and their role in hydrocarbon contaminated soils, as they serve as a method to promote the growth and activity of hydrocarbon degrading bacteria and the plants they interact with.

4.7 Summary

In this study, the presence of hydrocarbon degrading genes associated with the root endophytic bacteria of the natural vegetation growing at Bitumount was demonstrated. Gene copy abundance not only varied throughout the site, but there were multiple factors influencing gene abundance. The 16S rRNA, CYP153, and *alkB* gene abundance were influenced by the interaction of plant species and sampling location. Overall, the positive effect of improved soil properties on microbial communities were revealed. The influence of nutrient levels on gene abundance reflected the need for increased fertility within contaminated soils to enhance microbial proliferation. In contrast, an inhibitory effect of hydrocarbon content (≈ 25 000 mg kg^{-1}) on microbial communities on bacterial abundance within the Quarry was demonstrated. Contrary to other research, the proportion of functional genes to 16S rRNA did not differ significantly throughout Bitumount. This suggests that despite increased hydrocarbon content at

different sampling locations, there was no selective recruitment of hydrocarbon degrading bacteria within the endosphere of the natural vegetation where hydrocarbon concentration of the soil increased. These results further indicate that CYP153 and *alkB* would likely be present within the root endophytic community of these plant species grown in non-contaminated soils. The results of this study suggest that grass species may be more suitable for bioremediation due to their higher hydrocarbon degrading gene abundance within the endosphere. This increased colonization of potential degraders in SB and SW may be attributed to their fibrous roots which allow for increased bacterial colonization as well as their ability to release alkanes into the rhizosphere. These findings provide insight into the ability of root associated bacteria to degrade hydrocarbon thus enhancing potential for natural revegetation to occur in the Bitumount Provincial Historic site.

5.0 SUMMARY AND CONCLUSIONS

The Bitumount Provincial Historic site is thought to be impacted by hydrocarbons through both natural and anthropogenic causes. The oil sands deposits associated with Bitumount are close to the soil surface which makes the deposits more easily mined; however, the proximity to the surface also increases the hydrocarbon content of the soil, making it more difficult for plant growth. In terms of anthropogenic influences, no reclamation efforts have occurred at Bitumount since its closure in 1958. This has allowed hydrocarbons resulting from the several decades of oil mining on the site to persist. Despite these two sources of contamination, plants have been able to recolonize the site through means of natural revegetation. Prior to this work, very little was known about Bitumount and how plants were able to recolonize the site regardless of the hostile conditions.

The effect of hydrocarbon content on plant growth is studied in many diverse environments. Hydrocarbon levels can cause toxicity in plants, and can limit the ability of plants to gain available water and nutrients due to hydrophobic conditions. However, root-associated microorganism are known to alleviate this stress through plant growth promoting abilities, such as hydrocarbon degradation. To date, the plant-microbe partnerships occurring within naturally revegetated hydrocarbon soils is not well understood. Therefore, Bitumount offered a unique opportunity to study this relationship. In addition, gaining a better understanding of root-associated bacteria occurring in Bitumount may lead to enhanced reclamation efforts of disturbed land used for oil exploration and mining.

The first objective of this study was to use both culture dependent and culture independent techniques to conduct a survey of the endophytic root bacteria associated with the natural vegetation growing at the Bitumount Provincial Historic site. Plant species and sampling location interacted to significantly affect the abundance of both rhizosphere and root endosphere populations. Rhizosphere bacterial abundance displayed a significant negative relationship with hydrocarbon levels and a positive relationship with soil nutrients, indicating that improved soil

conditions (i.e. high fertility and low contaminants) enhanced rhizosphere bacterial abundance. However, no significant relationship was exhibited for root endophytic bacterial abundance and soil properties. Plant species and sampling location significantly affected the root endophytic community structure, indicating that there was not one dominant factor influencing bacterial root endophytes. Many of the dominant orders identified using high-throughput sequencing were isolated using culture dependent techniques. Overall, members of the *Actinomycetales*, *Rhizobiales*, *Pseudomonadales*, *Burkholderiales*, and *Sphingomonadales* were the most commonly identified orders indicating that they are well adjusted to hydrocarbon contaminated soils with low soil fertility. In addition, many of these orders contain bacteria that are known to help promote plant growth through several mechanisms such as nutrient acquisition, phytohormone production, and contaminant degradation.

Since the vegetation at Bitumount was found to support diverse endophytic root bacterial communities, the characterization of their functional potential was analyzed to determine if the endophytic community was alleviating plant stress, thus enhancing revegetation. The second objective of this study was to quantify hydrocarbon degrading genes (CYP153 and *alkB*) and universal 16S rRNA gene present within the root bacterial endophytic communities associated with the natural vegetation at Bitumount. Abundance of 16S rRNA, CYP153, and *alkB* genes within the root endosphere was influenced by the interaction of plant species and sampling location. Hydrocarbon content levels were not directly promoting a shift towards greater abundance of hydrocarbon degrading bacteria within plant roots as indicated by not significant differences in functional gene abundance to 16S rRNA. Similar to the rhizosphere bacterial abundance, a positive relationship between gene abundance and soil nutrient levels reflected the effect soil fertility had on microbial proliferation. Generally, hydrocarbon degrading genes were highest within grass species (SB and SW), emphasizing their known role of being good plants for reclamation and bioremediation purposes.

The general conclusion of this thesis is that the natural vegetation growing at Bitumount supported diverse root endophytic bacterial communities. Root endophytic bacterial community was significantly influenced by both sampling location and plant species. Culture dependent techniques were able to accurately identify dominant bacteria associated with the root endosphere, as seen when compared to high-throughput sequencing analysis. In addition, gene

copy abundance of hydrocarbon degrading genes were dependent on plant species, as well as sampling location. Although hydrocarbon content did not promote a shift towards more abundant hydrocarbon degrading populations, the negative effect of hydrocarbon levels on microbial proliferation were demonstrated.

5.1 Future Research Directions

Further analysis of the root-associated endophytic bacteria of Bitumount should focus on the screening of the root endophytic bacteria isolates for plant growth promoting capabilities, such as hydrocarbon degradation and nutrient acquisitions. Screening of the isolates would provide a more in depth analysis of the potential of plant growth promotion. In addition, isolates positive for plant growth promoting abilities should be tested within a greenhouse study as inoculants. Various studies have analyzed the influence of inoculating a bacterium harboring hydrocarbon degrading genes on plant growth (Afzal et al., 2012; Tara et al., 2014) and plant systems are found be more effective in remediation when microbial inoculants are in use compared to noninoculated plants (Siciliano et al., 2001). Inoculation studies will enable us to determine if targeted bacteria are able to increase survival rate of specified plant species.

Testing other soil parameters (e.g. nitrogen content, soil texture, organic matter content) at Bitumount would be valuable in determining the influence on the population abundance, diversity, and hydrocarbon degrading genes of the root endophytic bacterial within a naturally revegetated site. Additional analysis of other plant growth promoting mechanisms could lead to a better understanding of the plant-microbe interactions occurring on site. Lastly, additional studies should focus on natural revegetation within other sites with contaminated soils. This will allow a broader understanding of the mechanisms and influences involved in plant recolonization.

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APPENDIX A

Photographs of the Bitumount Provincial Historic site, Alberta Canada.

Fig. A.1 The quarry at the Bitumount Provincial Historic site Alberta, Canada (October 2013).

Fig. A.2 The power plant within the processing area at the Bitumount Provincial Historic site, Alberta, Canada (June 2014).

Fig. A.3 General view of the oil separation plant at the Bitumount Provincial Historic site Alberta, Canada (June 2014).

APPENDIX B

Alpha diversity indices of root endophytic communities associated with the vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada (rarefied to the median).

Table B.1 Alpha diversity indices of root bacterial endophytic communities (rarefied to the median) associated with the vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada.

Location	Hydrocarbon content (mg kg^{-1})	Plant†	Shannon Index (H')§	Inverse Simpson Index (1/D)§	Chao 1 Index
Quarry					
	24700	PF	3.55ab	16.01ab	1411
Entrance					
	4120	SW	3.56ab	19.13ab	1055
		SB	3.23ab	12.83ab	1366
Processing Area					
	3500	SW	3.42ab	16.48ab	1350
		SB	3.19ab	14.92ab	1893
		HT	2.01b	4.87b	1315
Pathway					
	2350	SB	3.28ab	15.87ab	1316
		KB	2.57ab	10.53ab	2169
Quarry Border					
	1770	WS	2.78ab	10.54ab	2673
River Bank					
	330	SW	3.03ab	10.58ab	3331
		SB	2.67ab	6.86b	1922
		HT	3.85a	28.96a	2162

†SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.
‡Number of sequences and alpha diversity are presented as means of each replicated sample.
§Different letters in same column indicated significant difference ($p \leq 0.05$) using Tukey's Post-hoc.

APPENDIX C

Rarefaction curves relating the observed number of sequences to the observed number of operational taxonomic units (OTUs) at a distance of 3%.

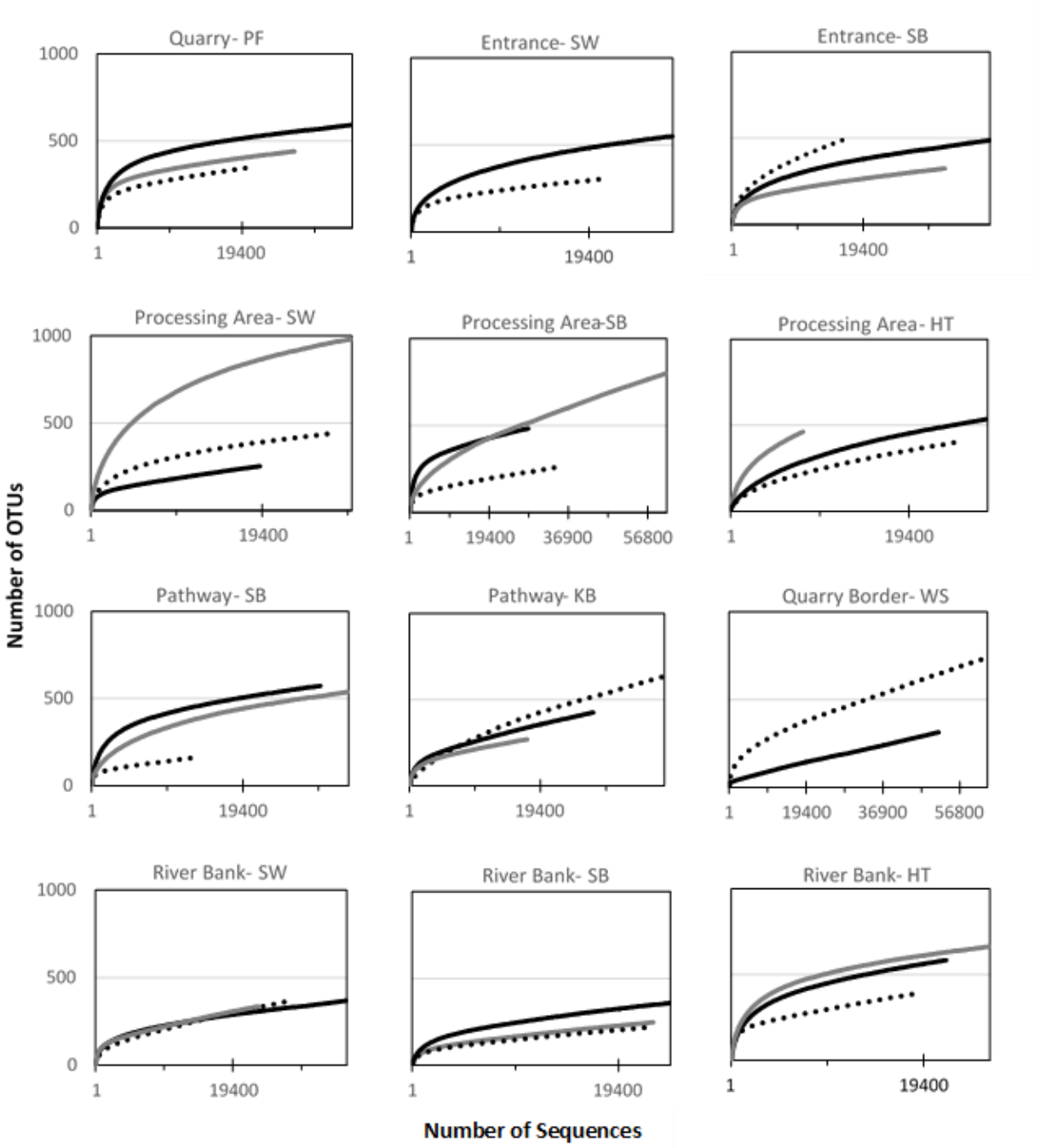

Fig. C.1 Rarefaction curves relating the observed number of sequences from the root endosphere of vegetation at the Bitumount Provincial Historic site, Alberta, Canada to the observed number of operational taxonomic units (OTUs) at a distance of 3%. Each curve represents the replicate sequenced for each sample (n=3). Locations ordered from high to low total hydrocarbon levels (24700-330 mg kg^{-1}). Note: Quarry Border- WS and Entrance- SW have n=2. SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.

APPENDIX D

Diversity index of culturable bacteria.

Table D.1 Alpha diversity of isolated root endophytic bacteria from the natural vegetation at the Bitumount Provincial Historic site, Alberta, Canada.

Location	Hydrocarbon content (mg kg^{-1})	Plant†	Shannon Index (H')‡	Inverse Simpson Index (1/D)‡
Quarry				
	24700	PF	2.06	9
Entrance				
	4120	SW	2.21	11.88
		SB	2.16	12.21
Processing Area				
	3500	SW	1.94	7.77
		SB	2.55	28.5
		HT	1.94	5.14
Pathway				
	2350	SB	2.25	13.57
		KB	1.82	5.43
Quarry Border				
	1770	WS	2.16	11.4
River Bank				
	330	SW	1.97	7.31
		SB	2.45	21.38
		HT	2.55	15.55

†SW, Slender Wheatgrass; SB, Smooth Brome; HT, Horsetail; KB, Kentucky Bluegrass; PF, Pea Family; WS, Wild Strawberry.
‡Based on genera level.

APPENDIX E

Identification of culture endophytic bacteria from the natural vegetation growing at the Bitumount Provincial Historic site. Identification are based on Sanger Sequencing.

Table E.1 Sanger Sequencing description of endophytes cultured from the natural vegetation growing at the Bitumount Provincial Historic site, Alberta, Canada.

Entry Identification	Closest Relative	Percent similarity	Accession number
Quarry- Pea Family			
L7-2	Pseudomonas tolaasii strain ATCC 33618	99%	gi|636558425|NR_114481.1
L7-5	Novosphingobium sp. PP1Y strain PP1Y	96%	gi|444303825|NR_074247.1
L7-6	Leucobacter denitrificans strain M1T8B10	98%	gi|566085010|NR_108568.1
L7-8	Brevundimonas mediterranea strain V4.BO.10	97%	gi|310975244|NR_037108.1
L7-10	Rhizobium herbae strain CCBAU 83011	96%	gi|645320290|NR_117530.1
L7-12	Rhizobium herbae strain CCBAU 83011	98%	gi|645320290|NR_117530.1
L7-14	Rhizobium herbae strain CCBAU 83011	93%	gi|645320290|NR_117530.1
L7-16	Pseudoxanthomonas spadix BD-a59 strain BD-a59	99%	gi|444439641|NR_074956.1
L7-17	Brevundimonas mediterranea strain V4.BO.10	98%	gi|310975244|NR_037108.1
L7-21	Uncultured bacterium clone LNH_9_9_11_Water.46398	88%	gi|674127276|KM128344.1
L7-22	Uncultured bacterium clone B176_261	90%	gi|683851123|KM500888.1
L7-23	Luteibacter rhizovicinus strain LJ96	96%	gi|343201471|NR_042197.1
L7-24	Flavobacterium subsaxonicum strain WB4.1-42	96%	gi|636559028|NR_115085.1
L7-29	Rhizobium galegae strain NBRC 14965	99%	gi|631252515|NR_113713.1
L7-31	Pseudomonas frederiksbergensis strain DSM 13022	99%	gi|645319817|NR_117177.1
L7-34	Pseudomonas tolaasii strain ATCC 33618	99%	gi|636558425|NR_114481.1

L7-36	Asticcacaulis benevestitus strain Z-0023	99%	gi|343201707|NR_042433.1
L7-37	Xanthomonas fuscans subsp. fuscans	99%	gi|659364994|NR_121770.1
L7-38	Pseudomonas tolaasii strain ATCC 33618	98%	gi|636558425|NR_114481.1
L7-42	Pseudomonas frederiksbergensis strain DSM 13022	99%	gi|645319817|NR_117177.1
Entrance- Slender Wheatgrass			
L11-5	Leifsonia psychrotolerans strain LI1	98%	gi|566085031|NR_108604.1
L11-7	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	96%	gi|485099118|NR_102514.1
L11-8	Sphingomonas roseiflava strain MK341	99%	gi|645320532|NR_117716.1
L11-10	Streptomyces anulatus strain NRRL B-2000	97%	gi|343202984|NR_043489.1
L11-16	Bacillus aryabhattai strain B8W22	98%	gi|645321488|NR_118442.1
L11-18	Leifsonia psychrotolerans strain LI1	97%	gi|566085031|NR_108604.1
L11-22	Yersinia ruckeri strain ATCC 29473	97%	gi|343201122|NR_041833.1
L11-23	Yersinia ruckeri strain ATCC 29473	95%	gi|343201122|NR_041833.1
L11-28	Leucobacter denitrificans strain M1T8B10	97%	gi|566085010|NR_108568.1
L11-30	Yersinia ruckeri strain ATCC 29473	96%	gi|343201122|NR_041833.1
L11-31	Agaricicola taiwanensis strain CC-SBABM117	91%	gi|672238945|NR_125534.1
L11-32	Yersinia ruckeri strain ATCC 29473	96%	gi|343201122|NR_041833.1
L11-34	Leifsonia psychrotolerans strain LI1	98%	gi|566085031|NR_108604.1
L11-43	Leucobacter denitrificans strain M1T8B10	98%	gi|566085010|NR_108568.1
L11-45	Bacillus halosaccharovorans strain E33	99%	gi|566085271|NR_109116.1
L11-46	Stenotrophomonas pavanii strain LMG 25348	96%	gi|645320911|NR_118008.1
L11-48	Arthrobacter subterraneus strain CH7	99%	gi|343203024|NR_043546.1

L11-56	Staphylococcus epidermidis RP62A strain RP62A	98%	gi|444439680|NR_074995.1
L11-60	Bacillus halosaccharovorans strain E33	99%	gi|566085271|NR_109116.1
L11-62	Bacillus aryabhattai strain B8W22	97%	gi|645321488|NR_118442.1
Entrance- Smooth Brome			
L12-5	Rhizobium herbae strain CCBAU 83011	99%	gi|645320290|NR_117530.1
L12-7	Flavobacterium oncorhynchi strain 631-08	97%	gi|645319654|NR_117031.1
L12-8	Pseudomonas mediterranea strain CFBP 5447	97%	gi|265678522|NR_028826.1
L12-10	Flavobacterium pectinovorum strain NBRC 15945	97%	gi|559795131|NR_104717.1
L12-16	Pseudomonas kuykendallii strain H2	99%	gi|645321108|NR_118155.1
L12-18	Leifsonia lichenia strain 2Sb	97%	gi|631251447|NR_112644.1
L12-22	Flavobacterium oncorhynchi strain 631-08	96%	gi|645319654|NR_117031.1
L12-23	Arthrobacter subterraneus strain CH7	99%	gi|343203024|NR_043546.1
L12-28	Pedobacter alluvionis strain NWER-II11	97%	gi|343205897|NR_044382.1
L12-30	Flavobacterium oncorhynchi strain 631-08	96%	gi|645319654|NR_117031.1
L12-31	Brevundimonas abyssalis strain TAR-001	98%	gi|631253110|NR_114308.1
L12-32	Lentzea jiangxiensis strain FXJ1.034	98%	gi|566084908|NR_108456.1
L12-34	Sphingomonas hankookensis strain ODN7	98%	gi|636560510|NR_116570.1
L12-43	Microbacterium invictum strain DC-200	96%	gi|343202422|NR_042708.1
L12-45	Microbacterium saccharophilum strain K-1	96%	gi|631253144|NR_114342.1
L12-46	Pedobacter alluvionis strain NWER-II11	97%	gi|343205897|NR_044382.1
L12-48	Pedobacter alluvionis strain NWER-II11	97%	gi|343205897|NR_044382.1
L12-56	Microbacterium invictum strain DC-200	98%	gi|343202422|NR_042708.1

L12-60	Unclassified		
L12-62	Leifsonia psychrotolerans strain LI1	97%	gi|566085031|NR_108604.1
Processing Area- Slender Wheatgrass			
L9-2	Rhizobium herbae strain CCBAU 83011	99%	gi|645320290|NR_117530.1
L9-10	Leifsonia psychrotolerans strain LI1	97%	gi|566085031|NR_108604.1
L9-20	Pedobacter alluvionis strain NWER-II11	97%	gi|343205897|NR_044382.1
L9-27	Leifsonia psychrotolerans strain LI1	98%	gi|566085031|NR_108604.1
L9-30	Leifsonia lichenia strain 2Sb	96%	gi|631251447|NR_112644.1
L9-31	Leifsonia lichenia strain 2Sb	97%	gi|631251447|NR_112644.1
L9-37	Unclassified		
L9-41	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	99%	gi|485099118|NR_102514.1
L9-42	Leucobacter denitrificans strain M1T8B10	97%	gi|566085010|NR_108568.1
L9-43	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	99%	gi|485099118|NR_102514.1
L9-45	Rhodococcus cerastrii strain C5	98%	gi|645319726|NR_117103.1
L9-46	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	98%	gi|485099118|NR_102514.1
L9-47	Microbacterium lacus strain A5E-52	97%	gi|343200876|NR_041563.1
L9-48	Demequina sediminicola strain HR08-43	99%	gi|631252369|NR_113567.1
L9-49	Microbacterium lacus strain A5E-52	96%	gi|343200876|NR_041563.1
L9-51	Microbacterium mangrovi strain MUSC 115	96%	gi|699005414|NR_126283.1
L9-52	Sphingomonas roseiflava strain MK341	99%	gi|645320532|NR_117716.1
L9-55	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	99%	gi|485099118|NR_102514.1
L9-57	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	99%	gi|485099118|NR_102514.1

L9-63	Microbacterium lacus strain A5E-52	96%	gi|343200876|NR_041563.1
Processing Area- Smooth Brome			
L8-2	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	99%	gi|485099118|NR_102514.1
L8-5	Stenotrophomonas humi strain R-32729	98%	gi|343202282|NR_042568.1
L8-6	Pedobacter aquatilis strain AR107	97%	gi|343201720|NR_042446.1
L8-8	Serratia liquefaciens strain ATCC 27592	95%	gi|659364569|NR_121703.1
L8-10	Ochrobactrum anthropi strain ATCC 49188	96%	gi|444303821|NR_074243.1
L8-12	Leifsonia psychrotolerans strain LI1	99%	gi|566085031|NR_108604.1
L8-14	Xanthomonas fuscans subsp. fuscans	99%	gi|659364994|NR_121770.1
L8-16	Xanthomonas fuscans subsp. fuscans	97%	gi|659364994|NR_121770.1
L8-17	Xanthobacter autotrophicus strain NBRC 102463	95%	gi|631252906|NR_114104.1
L8-23	Luteibacter anthropi strain CCUG 25036	97%	gi|636560851|NR_116911.1
L8-21	Unclassified		
L8-22	Xanthomonas fuscans subsp. fuscans	98%	gi|659364994|NR_121770.1
L8-24	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	98%	gi|485099118|NR_102514.1
L8-29	Rhizobium herbae strain CCBAU 83011	98%	gi|645320290|NR_117530.1
L8-31	Dyadobacter psychrophilus strain BZ26	99%	gi|645319865|NR_117212.1
L8-34	Pedobacter alluvionis strain NWER-II11	97%	gi|343205897|NR_044382.1
L8-36	Flavobacterium pectinovorum strain NBRC 15945	97%	gi|559795131|NR_104717.1
L8-37	Rathayibacter caricis strain VKM Ac-1799	98%	gi|265678454|NR_028756.1
L8-38	Rhizobium herbae strain CCBAU 83011	97%	gi|645320290|NR_117530.1
L8-42	Microbacterium murale strain 01-Gi-001	96%	gi|645320385|NR_117603.1

Processing Area- Horsetail

L10-1	Rhodococcus globerulus strain DSM 43954	96%	gi|645321716|NR_118617.1
L10-2	Pseudomonas fluorescens Pf0-1 strain Pf0-1	97%	gi|507148028|NR_102835.1
L10-8	Staphylococcus epidermidis RP62A strain RP62A	95%	gi|444439680|NR_074995.1
L10-9	Sanguibacter keddieii strain DSM 10542	99%	gi|444439401|NR_074716.1
L10-17	Actinokineospora diospyrosa strain NRRL B-24047	98%	gi|586661232|NR_024962.2
L10-20	Chryseobacterium lactis strain KC1864	97%	gi|699005387|NR_126256.1
L10-23	Agromyces indicus strain NIO-1018	97%	gi|566085177|NR_108908.1
L10-25	Pseudomonas mediterranea strain CFBP 5447	97%	gi|265678522|NR_028826.1
L10-26	Serratia liquefaciens strain ATCC 27592	95%	gi|659365258|NR_122057.1
L10-29	Pseudomonas mediterranea strain CFBP 5447	97%	gi|265678522|NR_028826.1
L10-31	Microbacterium testaceum StLB037 strain StLB037	97%	gi|444304216|NR_074641.1
L10-33	Pseudomonas mediterranea strain CFBP 5447	98%	gi|265678522|NR_028826.1
L10-39	Pseudomonas mediterranea strain CFBP 5447	98%	gi|265678522|NR_028826.1
L10-43	Pseudomonas mediterranea strain CFBP 5447	98%	gi|265678522|NR_028826.1
L10-44	Pseudomonas mediterranea strain CFBP 5447	97%	gi|265678522|NR_028826.1
L10-47	Sanguibacter keddieii strain DSM 10542	99%	gi|444439401|NR_074716.1
L10-48	Bacillus halosaccharovorans strain E33	99%	gi|566085271|NR_109116.1
L10-50	Arthrobacter subterraneus strain CH7	98%	gi|343203024|NR_043546.1
L10-57	Pseudomonas mediterranea strain CFBP 5447	97%	gi|265678522|NR_028826.1
L10-58	Pseudomonas mediterranea strain CFBP 5447	97%	gi|265678522|NR_028826.2

Pathway- Smooth Brome

L5-2	Pseudomonas tolaasii strain ATCC 33618	99%	gi|636558425|NR_114481.1
L5-13	Variovorax defluvii strain 2C1-b	86%	gi|566085263|NR_109102.1
L5-15	Microbacterium invictum strain DC-200	98%	gi|343202422|NR_042708.1
L5-16	Niabella hirudinis strain E96	95%	gi|566085646|NR_109743.1
L5-17	Brevundimonas mediterranea strain V4.BO.10	97%	gi|310975244|NR_037108.1
L5-22	Novosphingobium sp. PP1Y strain PP1Y	93%	gi|444303825|NR_074247.1
L5-24	Flavobacterium subsaxonicum strain WB4.1-42	95%	gi|636559028|NR_115085.1
L5-25	Devosia riboflavina strain NBRC 13584	96%	gi|631252420|NR_113618.1
L5-26	Pseudoxanthomonas japonensis strain NBRC 101033	98%	gi|631252774|NR_113972.1
L5-27	Rhizobium galegae strain NBRC 14965	96%	gi|631252515|NR_113713.1
L5-28	Brevundimonas mediterranea strain V4.BO.10	99%	gi|310975244|NR_037108.1
L5-29	Rhizobium galegae strain NBRC 14965	97%	gi|631252515|NR_113713.1
L5-31	Brevundimonas subvibrioides strain ATCC 15264	98%	gi|470466710|NR_074136.1
L5-37	Rhizobium galegae strain NBRC 14965	96%	gi|631252515|NR_113713.1
L5-38	Novosphingobium sp. PP1Y strain PP1Y	97%	gi|444303825|NR_074247.1
L5-39	Rhizobium galegae strain NBRC 14965	97%	gi|631252515|NR_113713.1
L5-41	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	96%	gi|485099118|NR_102514.1
L5-42	Pseudomonas poae RE*1-1-14 strain RE*1-1-14	98%	gi|485099118|NR_102514.1
L5-44	Xanthomonas fuscans subsp. fuscans	99%	gi|659364994|NR_121770.1
L5-45	Microbacterium invictum strain DC-200	98%	gi|343202422|NR_042708.1
Pathway- Kentucky Bluegrass			
L4-1	Rhizobium leguminosarum bv. viciae 3841 strain 3841	96%	gi|526641922|NR_103919.1

L4-5	Microbacterium lacus strain A5E-52	96%	gi|343200876|NR_041563.1
L4-15	Paenibacillus sacheonensis strain SY01	99%	gi|645320168|NR_117438.
L4-17	Leucobacter denitrificans strain M1T8B10	98%	gi|566085010|NR_108568.1
L4-22	Rhizobium leguminosarum bv. viciae 3841 strain 3841	97%	gi|526641922|NR_103919.1
L4-25	Leucobacter denitrificans strain M1T8B10	97%	gi|566085010|NR_108568.1
L4-28	Leucobacter denitrificans strain M1T8B10	95%	gi|566085010|NR_108568.1
L4-29	Rhizobium herbae strain CCBAU 83011	97%	gi|645320290|NR_117530.1
L4-32	Mucilaginibacter rigui strain NBRC 101115	95%	gi|631252778|NR_113976.1
L4-34	Rhizobium skierniewicense strain CH11	98%	gi|645321643|NR_118559.1
L4-36	Rhizobium leguminosarum bv. viciae 3841 strain 3841	99%	gi|526641922|NR_103919.1
L4-37	Burkholderia glathei strain N15	99%	gi|449372120|NR_037065.2
L4-38	Pseudomonas frederiksbergensis strain DSM 13022	98%	gi|645319817|NR_117177.1
L4-40	Leifsonia psychrotolerans strain LI1	96%	gi|566085031|NR_108604.1
L4-41	Leucobacter denitrificans strain M1T8B10	96%	gi|566085010|NR_108568.1
L4-42	Pseudomonas frederiksbergensis strain DSM 13022	98%	gi|645319817|NR_117177.1
L4-43	Pedobacter alluvionis strain NWER-II11	98%	gi|343205897|NR_044382.1
L4-48	Rhizobium leguminosarum bv. viciae 3841 strain 3841	99%	gi|526641922|NR_103919.1
L4-52	Rhizobium leguminosarum bv. viciae 3841 strain 3841	96%	gi|526641922|NR_103919.1
L4-61	Rhizobium leguminosarum bv. viciae 3841 strain 3841	97%	gi|526641922|NR_103919.1
Quarry Border- Wild Strawberry			
L6-2	Rhizobium herbae strain CCBAU 83011	99%	gi|645320290|NR_117530.1
L6-7	Sphingomonas cynarae strain SPC-1	99%	gi|566085281|NR_109167.1

L6-8	Unclassified		
L6-9	Rhizobium galegae strain NBRC 14965	97%	gi|631252515|NR_113713.1
L6-17	Pseudoxanthomonas spadix BD-a59 strain BD-a59	96%	gi|444439641|NR_074956.1
L6-21	Novosphingobium sp. PP1Y strain PP1Y	99%	gi|444303825|NR_074247.1
L6-22	Rhizobium skierniewicense strain CH11	98%	gi|645321643|NR_118559.1
L6-24	Luteibacter rhizovicinus strain LJ96	98%	gi|343201471|NR_042197.1
L6-28	Pseudoxanthomonas japonensis strain NBRC 101033	98%	gi|631252774|NR_113972.1
L6-29	Pseudomonas tolaasii strain ATCC 33618	99%	gi|636558425|NR_114481.1
L6-30	Asticcacaulis benevestitus strain Z-0023	99%	gi|343201707|NR_042433.1
L6-35	Sinorhizobium fredii NGR234 strain NGR234	96%	gi|507148112|NR_102919.1
L6-36	Rhizobium skierniewicense strain CH11	97%	gi|645321643|NR_118559.1
L6-37	Luteibacter rhizovicinus strain LJ96	98%	gi|343201471|NR_042197.1
L6-40	Novosphingobium sp. PP1Y strain PP1Y	98%	gi|444303825|NR_074247.1
L6-44	Sphingomonas astaxanthinifaciens strain NBRC 102146	99%	gi|631252839|NR_114037.1
L6-45	Rhizobium galegae strain NBRC 14965	99%	gi|631252515|NR_113713.1
L6-46	Brevundimonas faecalis strain CS20.3	99%	gi|645319831|NR_117187.1
L6-48	Xanthomonas fuscans subsp. fuscans	99%	gi|659364994|NR_121770.1
L6-49	Brevundimonas mediterranea strain V4.BO.10	99%	gi|310975244|NR_037108.1
River Bank- Slender Wheatgrass			
L1-4	Lysinibacillus contaminans strain FSt3A	97%	gi|566085642|NR_109740.1
L1-6	Variovorax paradoxus S110 strain S110	99%	gi|444304229|NR_074654.1
L1-8	Rhizobium galegae strain NBRC 14965	97%	gi|631252515|NR_113713.1

L1-9	Streptomyces tacrolimicus strain ATCC 55098	99%	gi|694275221|NR_116991.2
L1-10	Phyllobacterium trifolii strain PETP02	98%	gi|343202755|NR_043193.1
L1-11	Streptomyces acidiscabies strain ATCC 49003	96%	gi|636560474|NR_116534.1
L1-25	Rhizobium galegae strain NBRC 14965	96%	gi|631252515|NR_113713.1
L1-28	Phyllobacterium trifolii strain PETP02	98%	gi|343202755|NR_043193.1
L1-29	Phyllobacterium trifolii strain PETP02	90%	gi|343202755|NR_043193.1
L1-31	Bacillus aryabhattai strain B8W22 16S	99%	gi|645321488|NR_118442.1
L1-32	Variovorax paradoxus S110 strain S110	99%	gi|444304229|NR_074654.1
L1-35	Pseudomonas brassicacearum subsp. brassicacearum NFM421 strain NFM421	98%	gi|444439519|NR_074834.1
L1-40	Paracraurococcus ruber strain NS89	96%	gi|310974972|NR_036836.1
L1-44	Phaeobacter gallaeciensis strain 155	89%	gi|636559276|NR_115333.1
L1-45	Phyllobacterium trifolii strain PETP02	96%	gi|343202755|NR_043193.1
L1-56	Pseudomonas brassicacearum subsp. brassicacearum NFM421 strain NFM421	97%	gi|444439519|NR_074834.1
L1-58	Bacillus toyonensis strain BCT-7112	98%	gi|659364953|NR_121761.1
L1-59	Phyllobacterium trifolii strain PETP02	99%	gi|343202755|NR_043193.1
L1-64	Phyllobacterium trifolii strain PETP02	97%	gi|343202755|NR_043193.1
L1-66	Phyllobacterium trifolii strain PETP02	98%	gi|343202755|NR_043193.1
River Bank- Smooth Brome			
L2-1-1	Promicromonospora umidemergens strain 09-Be-007	97%	gi|645319547|NR_116951.1
L2-1-10	Pseudomonas brassicacearum subsp. brassicacearum NFM421 strain NFM421	97%	gi|444439519|NR_074834.1

L2-1-11	Leucobacter denitrificans strain M1T8B10	97%	gi|566085010|NR_108568.1
L2-1-12	Sphingomonas cynarae strain SPC-1	98%	gi|566085281|NR_109167.1
L2-1-13	Rathayibacter caricis strain VKM Ac-1799	97%	gi|265678454|NR_028756.1
L2-1-17	Nubsella zeaxanthinifaciens strain NBRC 102579	94%	gi|631252948|NR_114146.1
L2-1-19	Flavobacterium pectinovorum strain NBRC 15945	96%	gi|559795131|NR_104717.1
L2-1-23	Leucobacter denitrificans strain M1T8B10	94%	gi|566085010|NR_108568.1
L2-1-27	Microbacterium saccharophilum strain K-1	97%	gi|631253144|NR_114342.1
L2-1-28	Pseudomonas frederiksbergensis strain DSM 13022	98%	gi|645319817|NR_117177.1
L2-1-31	Unclassified		
L2-1-33	Sphingobium aromaticiconvertens strain RW16	98%	gi|343201753|NR_042479.1
L2-1-35	Leifsonia psychrotolerans strain LI1	97%	gi|566085031|NR_108604.1
L2-1-45	Sphingobium aromaticiconvertens strain RW16	99%	gi|343201753|NR_042479.1
L2-1-46	Sphingomonas aestuarii strain K4	99%	gi|343205865|NR_044341.1
L2-1-49	Rhizobium lemnae strain L6-16	94%	gi|699005305|NR_126174.1
L2-1-54	Pseudomonas lini strain DLE411J	97%	gi|602270614|NR_029042.2
L2-1-55	Micrococcus luteus strain BGDa135 M20	83%	gi|294860848|GQ289369.1
L2-1-61	Brevundimonas abyssalis strain TAR-001	80%	gi|631253110|NR_114308.1
L2-1-63	Leucobacter denitrificans strain M1T8B10	98%	gi|566085010|NR_108568.1
River Bank- Horsetail			
L2-2-1	Agromyces subbeticus strain Z33	97%	gi|343202723|NR_043149.1
L2-2-2	Microbacterium invictum strain DC-200	98%	gi|343202422|NR_042708.1
L2-2-3	Sphingomonas roseiflava strain MK341	96%	gi|645320532|NR_117716.1

L2-2-9	Leucobacter denitrificans strain M1T8B10	98%	gi|566085010|NR_108568.1
L2-2-16	Microbacterium invictum strain DC-200	97%	gi|343202422|NR_042708.1
L2-2-18	Pseudomonas kuykendallii strain H2	98%	gi|645321108|NR_118155.1
L2-2-22	Streptomyces tacrolimicus strain ATCC 55098	97%	gi|694275221|NR_116991.2
L2-2-27	Arthrobacter phenanthrenivorans strain Sphe3	98%	gi|444439455|NR_074770.1
L2-2-31	Unclassified		
L2-2-39	Cellulomonas pakistanensis strain NCCP-11	97%	gi|672238864|NR_125452.1
L2-2-40	Streptomyces michiganensis strain CSSP181	96%	gi|636559303|NR_115360.1
L2-2-42	Microbacterium invictum strain DC-200	96%	gi|343202422|NR_042708.1
L2-2-44	Rathayibacter caricis strain VKM Ac-1799	96%	gi|265678454|NR_028756.1
L2-2-48	Methylobacterium populi strain BJ001	99%	gi|636558842|NR_114899.1
L2-2-49	Leucobacter denitrificans strain M1T8B10	97%	gi|566085010|NR_108568.1
L2-2-50	Sphingomonas cynarae strain SPC-1	98%	gi|566085281|NR_109167.1
L2-2-51	Leucobacter denitrificans strain M1T8B10	97%	gi|566085010|NR_108568.1
L2-2-53	Leucobacter denitrificans strain M1T8B10	94%	gi|566085010|NR_108568.1
L2-2-56	Flavobacterium pectinovorum strain NBRC 15945	97%	gi|559795131|NR_104717.1
L2-2-59	Rhizobium leguminosarum bv. viciae 3841 strain 3841	99%	gi|526641922|NR_103919.1

APPENDIX F

Gene sequence for CYP153 (339 bp) and *alkB* (548 bp) for synthesis of standard from Biomatik services (Cambridge, Canada).

CYP153 (339 bp)

```
  1 gtgggcggca acgacaccac tcgaaactcg atgagtggcg gtgtgcttgc gctcaaccgg
 61 ttccccgacc agttcgagaa gctgaaggcg aaccccgacc tgatccccaa catggtctcg
121 gagatcatcc ggtggcagac cccgctggcc tacatgcgcc ggatcgccaa gaccgacacc
181 atgctgaagg gccagttcat ccgcaagggc gacaaagtcg tgatgtggta cgcctcgggc
241 aaccgcgacg aaagcgtgtt cgaacggccc gatgagttga tcatcgatcg gagcaacgcc
301 cgccaccaca tctccttcgg cttcggcatc caccgctgc
```

alkB (548 bp)

```
1 Aacacggcgc acgaactagg gcacaagaag accgccatcg aacgctggct ggccaagctg
61 gccctggcgc cgaccggcta tggccatttc tgtatcgagc acaaccgcgg gcatcaccgg
121 gacgtggcca cgccggagga ttccgcctca tcacgtatgg gcgagagcta ctatcgcttc
181 atcaagcgcg agatccccgg ggccttccgc cgcgcctggg ccatcgaggg cgatcgcatg
241 gcccgcaagg gactgagccg ctggtcgttg cagaacgata tcgtccacac ggccctggta
301 accgtcgtgc tgtggggcgg gctgattttc tggctgggtc tcgcggtgct gcccttcctg
361 ctgctgcagg cgctgatcgc ctattcgctg ctttcctccg cgaactacgt agagcattac
421 ggactgttgc gccagagatt ggccagcggt cgctacgaac gccccgagcc gcgccactcc
481 tggaacagca atcatgtgct gtcgaacatc ctcctctatc agctccagcg ccactcggac
541 caccacgc
```

APPENDIX G

Pearson correlation (p values) between gene abundance and pH and Hydrocarbon content of sampling locations (excluding the Quarry) through the Bitumount Provincial Historic site, Alberta, Canada.

Table G.1 Pearson correlation (p values) between gene abundance and pH and hydrocarbon content of sampling locations (excluding the Quarry) through the Bitumount Provincial Historic site, Alberta, Canada.

Genes	pH	Hydrocarbon content
16S rRNA	p=0.13	p=0.43
CYP153	p=0.18	p=0.29
alkB	p=0.66	p=0.20