Culture-Dependent and -Independent Microbial Analyses of Petroleum Hydrocarbon Contaminated Arctic Soil in a Mesocosm System

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Abstract

Microbial-based strategies were investigated for eventual bioremediation of petroleum hydrocarbon-contaminated, acidic soils from Resolution Island (RI), Nunavut. A biotreatability assessment phase one study determined that supplementation of soil with commercial fertilizer and lime enhanced hydrocarbon mineralization. Phase two applied these conditions to large scale mesocosm trials, containing ~150 kg soil, incubated in a temperature cycle that represented the ambient summer conditions on RI (10 d of 1°C - 10°C for 60 d). Culture-dependent and –independent analyses of RI soil microbial communities showed the mesocosm treatment enhanced hexadecane mineralization, increased the enumerations of total microbes and viable, cold-adapted hydrocarbon-degrading microorganisms. DGGE analyses indicated emergence of a hydrocarbon-degrading community and 16S rRNA gene clone libraries showed bacterial population shift in mesocosm soils. Potentially novel isolated strains included those able to grow on hydrocarbons alone while under acidic or sub-zero conditions. This microbiological study addressed RI site conditions and presents a potential bioremediation.

Résumé

Des techniques s'appuyant sur la microbiologie ont été utilisée pour évaluer la biorestauration future de sols acides, contaminés par des hydocarbures pétroliers, à Resolution Island (RI), Nunavut. Premièrement, une étude de biotraitabilité a permis de determiner que l'amendement du sol avec des fertilisants de type commercial et de la chaux améliore la dégradation des hydrocarbures. La phase deux a consisté en l'application de ces conditions à des essais de mesocosmes à grande échelle incubés à des températures représentant les conditions estivales de RI, i.e. cycle de 10 jrs (1°C-10°C) pendant 60 jrs. Des analyses de microbiologie classique et de biologie moléculaire des communatés microbiennes du sol de RI ont démontré que l'amendement des mésocosmes a permis une augmentaion de la minéralisation de l'hexadécane et un accroîssement du dénombrement de total de microorganismes ainsi que des microorganismes viables, adaptés au froid et dégradant les hydrocarbures. Des analyses par DGGE ont démontré l'apparition d'un communauté microbienne dégradant les hydrocarbures et une librairie de clones d'ARNr 16S a souligné un réarrangement des populations microbiennes présentes dans les sols de mesocosmes. Des nouvelles souches ont été isolées, incluant certaines pouvant croître sur une source unique d'hydrocarbures sous des conditions acides ou sous-zéro. Cet étude microbiologique a été faite sous des conditions respectant celles présente à RI et présente des procédés pouvant être utilisées pour la bioremediation du site.

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Chapter One. Introduction

1.1. Resolution Island, hydrocarbon contaminated site

Resolution Island (RI; 61°30'N 65°00'W) is located off the southern tip of Baffin Island, in the Canadian territory of Nunavut, and is part of the Canadian Arctic Archipelago (Fig. 1.1). The Arctic zone surrounds the North Pole and can be defined geographically, as north of the Arctic Circle latitude parallel at 66° 33'N, or by the climate, which would be the zone with a mean isotherm of 10°C in the month of July (Fig. 1.2). The isotherm of 10°C closely corresponds to the tree line or the zone of transition from where fully-crowned forests can grow unimpeded by weather conditions to where weather inhibits tree growth. Russia, Iceland, Norway, Sweden, Denmark, the United States and Canada all have regions within the Arctic Circle. The sub-Arctic, which lies immediately south of the Arctic, is generally defined as the region between the 50°N and 70°N lines of latitude, where mean monthly temperatures are above 10°C for one to three months any given year (Fig. 1.1; AMAP 1998). Geologically, the Arctic Archipelago has been subjected to repeated glaciation and deglaciation events, the most recent being ~25,000 years ago (Aiken et al. 2003). The periglaciation of the Precambrian bedrock of the Canadian Shield, which makes up RI, formed a soil profile consisting of course sands, gravels and bare rock. The soils of these regions tend to be nutrient deficient and acidic due to the relatively young or immature age of the soils, and the texture and physical composition of the soil particles (Aiken et al. 2003). RI sits ~30 miles long by ~20 miles wide with the majority of the soil on the island at the edge of the water and is vegetated mainly by lichens, moss and algae (Aiken et al. 2003). Historical recordkeeping of RI weather has shown typical sub-Arctic weather conditions, remaining below 0°C for the majority of the year from about September to May, while during the summer months of June to August, a

cyclical temperature range from ~0°C to ~10°C occur in a 24 h period (Environment Canada *http://www.weatheroffice.ec.gc.ca/*).

Human presence on RI can be traced back to the bipeds that crossed the Bering straight, their decedents the Inuit, and the European explorers who followed. Since the mid-twentieth century, anthropogenic activity on RI focused on the construction and operation of a military radar base; part of the Pinetree Line in the Distant Early Warning system with the United States Air Force (USAF), in collaboration with the Canadian government, in charge of and responsible for the construction and maintenance of the RI base. Construction of the base started in 1951 and lasted three years, and concluded with more than 20 buildings, an airstrip, radar arrays and eight dump sites. It was built on the highest point on the island, a bluff on the Western part of the island overlooking the Atlantic Ocean. The USAF decommissioned military activities on RI in 1961, but an Air Communication and Control Squadron remained operational on RI until 1973 (Pinetree line *http://www.pinetreeline.org/site8.html*). In 1974, the site was turned completely over to the Canadian government and is currently administered by Indian and Northern Affairs Canada (INAC) (INAC: http://www.ainc-inac.gc.ca/nu/nuv/zxca_e.html). Between 1987 and 1990, site investigations on RI uncovered the environmental footprint left by the military occupants; soils were found to be contaminated with polychlorinated biphenyls (PCB), asbestos, heavy metals and petroleum hydrocarbons (INAC http://www.ainc-inac.gc.ca/nu/nuv/zxca_e.html; Chang et al. 2007).

1.1.1. Hydrocarbon chemistry and spill/ soil dynamics

Hydrocarbons, the major constituent of petroleum, include saturated alkanes and cycloalkanes, and unsaturated alkenes, alkynes and aromatic hydrocarbons. The usual composition of light crude oil is 78% saturates, 18% aromatics, 4% resins (pyridines, quinolines) and <2% asphaltenes (phenols, porphyrins) (Olah and Molnár 2003). Refinement is based on the

distillation of hydrocarbon fractions, boiling off smaller, lighter molecules until the desired carbon content is reached with upgrades or blends of fractions combined for higher quality or specialty mixtures. The hydrocarbon fractions in order of decreasing volatility are F1 (C_6 - C_{10}); F2 (C_{10} – C_{16}); F3 (C_{16} – C_{34}) and F4 (C_{34} – C_{50}) with C_x referring to the number of carbon molecules in the alkane backbone.

The spill profile, or spatial area directly contacted with discharged hydrocarbons, depends on the time of year, amount/ extent, type of petroleum product and soil particle size of the spill site. Hydrocarbons flow down through the soil and larger soil particles generally allow greater migration. The state of the ground on which the spill is discharged affects the vertical and horizontal spill profile, with diminished oil distribution concurrent with lower temperatures (Chuvilin et al. 2001). In frozen ground, like seasonal or permanent ice-layers, hydrocarbon movement is restricted mostly to small cracks and fissures or unfrozen water films (Chuvilin et al. 2001). Surface ice will halt the penetration of hydrocarbons into the soil, but can cause wider horizontal spread of the contamination, so spills during the winter, when the ground active layer is frozen, tend to have different profiles than summer spills, where oil can seep vertically down to the permafrost layer. The hydraulic conductivity of the ice-layer, permafrost or active layer is the quantitative measure of how water flows though these layers, and in frozen sections, liquid movement is confined to microscopic layers of liquid water surrounding minerals or other soil particles (McCauley et al. 2002). These tiny coatings allow passage of non-aqueous liquids deep into the otherwise impenetrable frozen ground levels (McCauley et al. 2002). The type of petroleum product discharged will also influence the terrestrial migration; in general, the more viscous the product, the slower it will migrate. Smaller more volatile fractions will move quickly, or evaporate, while larger fractions will take more time to flow through soil. The overall viscosity of the spilled petroleum product is also dependent on the ambient temperature, with colder temperatures

effectively increasing the viscosity and slowing the rate of hydrocarbon movement (Olah and Molnár 2003).

As the hydrocarbons move through the soil, some of the organic carbon will be removed from the system or become unavailable to microbes by abiotic processes. In the soil, hydrocarbon degrading microorganisms must be able to come in physical contact with the hydrocarbon molecules, so that the molecule can be catabolized. This contact depends on the concentration of hydrocarbons in soil and the physical interplay between the hydrocarbons and the soil environment. Some hydrocarbons can be adsorbed to humic substances (Leahy and Colwell 1990), like polycyclic aromatic hydrocarbons (PAHs) which adsorb more readily to humic substances as the pH of the system becomes more acidic and/ or the temperature is decreased (Lesage et al. 2001; Ping et al. 2006). The sorption characteristics of hydrocarbons to soil can further depend on the soil matrix; in a marsh environment with multiple soil types, a greater reduction of hydrocarbons was observed in sandy soils than in mineral soils (Lin et al. 1999). Generally, hydrocarbons in soil become less available to microorganisms with increasing concentration of soil organic matter and soil clay proportion (Löser et al. 1999). The lipophilic nature of hydrocarbons can also prevent interaction and possible removal by microbes. Ghoshal et al. (1996) examined the bioavailability of naphthalene in coal tar, a non-aqueous phase liquid (NAPL), which can cause chronic contamination of the surrounding environment due to the slow PAH dissolution rate. The rate of naphthalene mineralization by microorganisms was influenced by the naphthalene mass transfer, and reduction of a large fraction of naphthalene in coal tar was possible if made bioavailable (Ghoshal et al. 1996).

Abiotic uptake or loss of hydrocarbons in a system is finite, reaching a saturation point that, unless conditions change, will prevent further removal (Lesage et al. 2001; Ping et al. 2006). This is apparent in aged

spills where the residual hydrocarbons are usually the larger more recalcitrant molecules and can remain in the soil for long periods of time without any apparent loss of concentration from either abiotic or biotic processes (Trindade et al. 2005).Possibly to a greater extent than aged spills, polar region hydrocarbon contamination can remain unchanged for very long periods of time. Volitization of hydrocarbons can remove hydrocarbons, mostly in the F1 fraction, from a system, but the cold temperatures that characterize polar regions will greatly reduce this volitization. Polar regions also reduce the exposure of ultraviolet (UV) radiation, which can break down hydrocarbon molecules (Weissenfels et al. 1992) due to the long periods of total darkness in winter and the cover created by snow and ice that can come year-round. Even after oil contamination is removed, the effect of an oil spill in the Arctic or sub-Arctic has a great affect on the environment, prolonging the time for recovery as Arctic flora and fauna tend to recuperate and grow at relatively slower rates than their counterparts at lower latitudes (Jorgenson 1995). This recovery time would be even longer if not for the presence and diversity of soil microorganisms able to degrade hydrocarbons.

1.2. Bioremediation

Bioremediation can be defined as any process that uses organisms to remove contaminants from an environment in an attempt to return the environment to pre-contaminated conditions. Bioremediation is an inclusive term that can include bacteria, archaea, fungi and other eukaryotes. Here, bioremediation refers to mainly bacterial activity, unless otherwise noted. Bioremediation as a technology and environmental clean-up strategy has been developing for some 40 years, covering a diverse range of contaminants and environments. Petroleum hydrocarbon contamination has affected a wide range of environments, and approaches for the bioremediation these sites have included the three major processes; monitored natural attenuation; bioaugmentation and;

biostimulation (Head et al. 2003). Environmental conditions determine which bioremediation approach, or combination, is most appropriate.

1.2.1. Bioremediation of hydrocarbon contaminated environments

Monitored natural attenuation is considered the simplest bioremediation approach and comprises checking the intrinsic degradation of contaminants in an environment. Takahata et al. (2006) concluded that monitored natural attenuation could be a possible remediation strategy for a BTX-contaminated aquifer. In another monitored natural attenuation study, Bradley et al. (1995) found higher rates of toluene mineralization in an hydrocarbon-contaminated aquifer in Adak, AK, USA at 5°C than in a Hanahan, SC, USA aquifer at 20°C. However, monitored natural attenuation is not generally considered a feasible option for terrestrial ecosystems, which do not have a constant nutrient flow. Contaminated sites that have remained unchanged for long periods of time represent situations not suited for monitored natural attenuation because there is no evidence that once monitoring of the site starts, the contamination level would decrease without anthropogenic intervention, as was found to be the case with hydrocarbon contamination in Antarctic soils (Aislabie et al. 2004).

Bioaugmentation is the addition to a system of biologically active organisms known to degrade the target contamination. The increase number of contaminatnt-degraders in the system would then remove the pollution faster. Presently, there is some debate as to the success of a bioaugmentation approach for bioremediation. Whyte et al. (1999, 1998) increased the rate of hexadecane mineralization by inoculating hydrocarbon contaminated Arctic soil with a consortium of hydrocarbon degrading organisms and a single hydrocarbon degrading *Rhodococcus* sp. strain. However, the authors concluded that the indigenous microbial community consisted of hydrocarbonclastic microbes well adapted to the environmental conditions and the microbial levels were plentiful enough

for bioremediation of the soil without additional supplementation with hydrocarbon degrading microorganisms (Whyte et al. 1999). Another bioaugmentation study of hydrocarbon contaminated soil used an excessive inoculum size of 10^9 CFU g^{-1} soil of indigenous and non-indigenous microorganisms and found the lag time of ^{14}C-dodecane mineralization in microcosm mineralization assays was reduced (Mohn et al. 2000). Bioaugmentation treatment was found to neither increase the rate of hydrocarbon degradation nor the extent of total petroleum hydrocarbon (TPH) removal in small scale biopiles at an Arctic soil site contaminated with weathered diesel fuel (Thomassin-Lacroix et al. 2002). Similar conclusions were drawn concerning the bioremediation projects for the crude oil contaminated shorelines affected by the Exxon Valdez accident (Atlas et al. 1995; Braddock et al. 1995). Stallwood et al. (2005) concluded in their study of oil contaminated Antarctic soil that bioaugmentation with an inoculum of indigenous microorganisms may speed-up the rate of TPH degradation if applied soon after the initial spill. Head et al. (2003) stated that bioaugmentation is only an effective treatment in a contaminated environment when no microorganisms able to degrade the contamination are naturally present in the environment. These examples demonstrate the need for investigation into a contaminated environment before proceeding with a bioremediation strategy.

Biostimulation addresses the deficiencies of the environment, providing the “ideal” conditions for microbial growth, activity and thus biodegradation. Many of the beforehand mentioned studies have also included successful biostimulation approaches (Atlas et al. 1995; Whyte et al. 1999; Mohn et al. 2000; Thomassin-Lacroix et al. 2002; Stallwood et al. 2005). Common biostimulation practices include supplementation with necessary or additional nutrients, water or air. More site-specific treatments may include chelating agents to detoxify metals or surfactants to increase hydrocarbon bioavailability. The application of biostimulants to

a contaminated environment can be an important a factor that should account for the environmental conditions and other biodegradation limitations of the system. *Ex situ* strategies that dig up, and remove contaminated soils may not be feasible for widely-spread contaminated areas or for contaminated areas that are extremely remote, leaving various *in situ* strategies more common for polar bioremediation projects (Aislabie et al. 2006). Biopiles, like the *ex situ* strategies, depend on excavation of the contaminated soils, which can then be covered to increase soil temperature and have air and nutrients piped through them (Aislabie et al. 2006). Bioventing (pumping of air) and biosparging (pumping of air and nutrients) are both similar to biopiles, but instead of the contaminated soil being excavated, the desired pipes are put directly into the earth and, therefore, the microorganisms are biostimulated below ground (Aislabie et al. 2006). Landfarming is an above ground approach where nutrients, water, etc. are spread onto the soil surface and mixed into the contaminated soil (Aislabie et al. 2006). Additional soil tilling will promote increased aeration and distribution of microorganisms, nutrients and contaminants.

1.2.2. Hydrocarbon biodegradation at cold temperatures

The constant cold temperature of the Arctic plays an important role in the ability of microorganisms to degrade hydrocarbons *in vivo*. Soil moisture content affects the bioavailability of hydrocarbons as well, due to the hydrophobic nature of hydrocarbons and the obligatory use of water by microorganisms. Soil moisture content also affects the growth characteristics of soil microorganisms and various studies have determined different “optimal” soil moisture guidelines (Sommers et al. 1981, King et al. 1992, EPA 1995, Dibble et al. 1979). However, a soil with high moisture content will have reduced air space in the soil matrix, increasing the proportion of anaerobic micro-environments in the soil, thus slowing overall hydrocarbon biodegradation. Linn and Doran (1984) found that once soil moisture content was raised above 60%, the oxygen

available became the limiting factor for microbial growth. Børresen et al. (2006) investigated different soil moisture levels in an Arctic soil and found reduced hexadecane mineralization with 20% soil moisture content, the highest level tested, which they conclude to be because of the oxygen limited system. Wetting and drying cycles also affect aerobic/ anaerobic zones in the soil matrix, and White et al. (1998) found wet/ dry cycles help to remove phenanthrene from test soils.

Wet/ dry cycles play an important role in soil dynamics by changing the flow and distribution of nutrients used by microorganisms. Soil nutrient availability can hinder or help microbial growth and metabolism. The *in situ* soil carbon:nitrogen:phosphorus (C:N:P) ratio may not be optimal for microbial growth in a system, and various C:N:P soil optimization studies have yielded a range of values with favorable results. Alexander (1999) suggested a C:N:P ratio of 100:3:0.6, while the U.S. Environmental Protection Agency (EPA) found that a C:N:P range of 100:10:1 to 100:1:0.5 was successful (EPA 1995). At highly contaminated sites, the high concentration of nitrogen required to achieve these C:N:P ratios can cause adverse effects on microbial growth. Addition of inorganic salts, which will dissolve into the aqueous phase of the soil matrix, will subsequently increase the salinity (Walworth et al. 2001). Different microorganisms have different optimal osmotic conditions, and a sudden osmotic change could inhibit microbial activity (Harris 1981), not to mention the possible deleterious effects a sudden boost in soil nutrient level would have on organisms adapted to a low nutrient system (Margesin 2000). Kästner et al. (1998) found a decrease in PAH biodegradation when soil salinity increased while Rhykerd et al. (1995) showed that increased soil salinity reduced hydrocarbon mineralization activity as the hydrocarbon-degrading bacterial population levels remained constant. Multiple studies tested a range of nitrogen salt concentrations in hydrocarbon contaminated soil and found greater total hydrocarbon mineralization from experiments that did not use the highest nitrogen salt

concentrations since these levels can be toxic or inhibitory for microbes (Børresen et al. 2006; Walworth et al. 2001; Mohn et al. 2000; Braddock et al. 1997). Direct nitrogen-to-soil values from 100 mg N kg^{-1} soil to 250 mg N kg^{-1} soil have been used with success in selected cases (Huesemann 1995; Whyte et al. 1999).

Osmotic pressure can additionally be increased in the aqueous phase of the soil matrix in sub-zero (<0°C) temperatures. This occurs by salt exclusion from frozen water into the surrounding aqueous film, which remains unfrozen due to the freezing-point depression effect of the higher salinity levels (Torrance et al. 2006). Accordingly, freeze-thaw cycles will impact the soil microbial community, changing both nutrient availability and salinity in the liquid section within the soil. Eriksson et al. (2001) found that freeze-thaw cycles may have been responsible for increased hydrocarbon biodegradation in microcosm experiments using diesel fuel contaminated Arctic soil. The authors surmise that the repeated freeze-thaw cycles could make nutrients more bioavailable. In another investigation into hydrocarbon biodegradation under freeze-thaw cycles, Børresen et al. (2007) monitored mineralization of radiolabeled hexadecane and phenanthrene in Arctic soils. They found that hexacane mineralization activity increased in the freeze-thaw cycle, when compared to the constant temperature assays. Conversely, phenanthrene mineralization activity was reduced in the freeze-thaw cycles (Børresen et al. 2007). They speculated that differences in the sensitivity of the specific hydrocarbon degrading populations to cold temperature effected mineralization ability.

Another environmental factor that may be important for hydrocarbon biodegradation is soil pH (Aislabie et al. 2006; Margesin et al. 2001; Leahy et al. 1990), with a neutral pH = 6-8 described as optimal (van Agteren et al. 1998; Norris et al. 1993). Hamamura et al. (2006) found comparable amounts of *n*-alkanes of C_{12} to C_{24} mineralization in seven disparate soils

with ranges in pH from 5.4 - 8.8. Hydrocarbon biodegradation has been observed under much more acidic conditions by Stapleton et al. (1998), who found aromatic hydrocarbon mineralization in soil downstream of a coal pile with pH = 2.0 and Hamamura et al. (2005) isolated an organism with an alkane degradation gene homologue (*alkB*) from natural hydrocarbon seeps of Rainbow Springs, Yellowstone National Park soils with pH values of 2.8 – 3.8. Uyttebroek et al. (2007) successfully used PAH contaminated soils at pH = 2 as inoculums to enrich cultures growing on phenanthrene and pyrene at pH = 3, 5 and 7. These examples demonstrate the hydrocarbon-degrading ability of microorganisms even under acidic conditions. Investigations into hydrocarbon biodegradation in alkaline conditions have been less extensively published than those in acidic conditions. However, bacteria optimally degraded monocrotophos (MCP), an organophosphorus insecticide, in wastewater at pH = 8.0 (Bhadbhade et al. 2002) and Maltseva et al. (1996) isolated an haloalkaliphilic bacterium able to degrade 2,4-dichlorophenoxyacetic acid at pH 8.4 – 9.4.

1.2.3. Hydrocarbon-degrading soil microorganisms

Representatives from all three major domains of life have demonstrated the ability to oxidize and break down hydrocarbon molecules for growth (van Hamme et al. 2003). The terminal oxidation pathway in Bacteria sequentially oxidizes of one end of the alkane chain by a monooxygenase (van Beilen et al. 1994). The resultant primary alcohol can then be further oxidized by dehydrogenases to aldehydes, then carboxylic acids, which can subsequently enter the *beta*-oxidation pathway (van Beilen et al. 1994). The most well characterized genes that encode the catabolic alkane enzymes are from the OCT plasmid (van Beilen et al. 1994; van Beilen et al. 2001) A wide range of bacterial and fungal species have been shown to have homologues to catabolic genes related to those found on the OCT plasmid (van Beilen et al. 2003). *Rhodococcus* sp. strains Q15 demonstrated multiple alkane hydroxylase systems (Whyte et al. 2002)

that enabled it to metabolize a range of alkane chain lengths (Whyte et al. 1998). The biterminal oxidation pathway is similar to the terminal but both ends of the chain are oxidized, and the sub-terminal oxidation pathway does not start at an end of the alkane chain, but in the middle and results in a secondary alcohol (van Beilen et al. 2003). Multiple aromatic degradation pathways have been described, though much attention has been given to the biodegradation of naphthalene in microorganisms, most notably from *Pseudomonas.* The first oxidation step of in this pathway is by the action of dioxygenases and dehydrogenases, which convert the aromatic hydrocarbon a *cis*-dihydrodiol and then a catechol (Cerniglia 1992). Different enzymes can then cleave the ring, yielding *cis, cis*-muconic acid or 2-hydroxymuconic semialdehyde, depending on the specific structure of the PAH molecule (Cerniglia 1992). The naphthalene biodegradation pathway model has shed light on the metabolism of other related molecules, like phenanthrene (Kiyohara et al. 1994) though some other pathways have been noted. Instead of dioxygenases, some PAH degradation pathways involve monooxygenases in the first stage, and other molecules, like benzoate, are converted to protocatechuate instead of catechol as the central intermediate (Fritsche W. and Hofrichter M. 2000). As with alkanes, a wide variety of organisms are able to use PAH as a sole carbon source (Cerniglia 1992; Widada et al. 2002). Foght et al. (1990) tested hexadecane and phenanthrene degradation of 138 isolates and found that both were readily biodegraded, but not a single isolate could degrade both compounds. A later investigation by Whyte et al. (1997) isolated a cold-adapted *Pseudomonas* sp. with the ability to degrade both PAH and alkanes. *Alkanindiges illinoisensis*, first isolated and described by Bogan et al. (2003) displayed the ability to degrade a variety of straight chain and branched alkanes, along with floruene. Anaerobic hydrocarbon biodegradation has also been observed and involves nitrate, ferric iron or sulphate as electron acceptors, syntrophic or anoxygenic photosynthetic growth, or unique mechanisms to replace the

action of molecular oxygen in aerobic hydrocarbon biodegradation (Widdel et al. 2001). Some examples of anaerobic hydrocarbon biodegradion include *Azoarcus* sp. strain HxN1, which was shown to degrade short chain alkanes by denitrification (Ehrenreich et al. 2000). *Geobacter metallireducens* reduced ferric iron to metabolize toluene (Lovley et al. 1989), while *Desulfobacula toluolica* reduced sulfate (Rabus et al. 1993) and the phototrophic *Blastochloris sulfoviridis* strain ToP1 used light to also degrade toluene (Zengler et al. 1999). The processes of anaerobic hydrocarbon biodegradation share the common trait of being relatively slow when compared to aerobic processes (Widdel et al. 2001). The capacity of microorganisms to degrade hydrocarbons depends on other factors besides the absence or presence of oxygen. The structure of the hydrocarbon molecule will greatly impact the degradation ability by an organism. As mentioned, some organisms may not be genetically enabled to degrade a certain class of hydrocarbon, for example alkanes or will only be able to degrade specific members of an hydrocarbon class, for instance only the F2 alkanes. Generally, longer chain alkanes and larger aromatics are more difficult for biodegradation (Leahy and Colwell 1990; Huesemann 1995). Microcosm mineralization assays at 5°C of alkanes with various chain lengths showed *Rhodococcus* Q15 to more readily degrade shorter chain length alkanes (Whyte et al. 1998).

1.2.4. Cold adaptations and hydrocarbon impacts

The microbial cold temperature adaptations include various physiological changes. One example is desaturation of membrane lipids to increase membrane fluidity, as exemplified by *Bacillus subtilis.* The *des* system in *B. subtilis* is involved in the formation of unsaturated fatty acids, and is induced by cold shock (Aguilar et al. 1999). Both the *des* transcript and the bulk mRNA were stable longer at cooler test temperatures (Aguilar et al. 1999). Many other difficulties face cold environment living microorganisms, from the formation of intracellular ice to reduced catalytic efficiency and stabilization of nucleic acids (Cavicchioli et al. 2002).

Microorganisms have evolved cold-active or anti-freeze proteins to counteract such problems (reviewed by Cavicchioli et al. 2002). In terms of hydrocarbon-degrading cold adapted microorganisms, as previously described, a variety of organisms have displayed the ability to degrade hydrocarbons at low or sub-zero temperatures. Whyte et al. (1999) described the physiological adaptations of a *Rhodococcus* sp. strain Q15 grown at low temperatures on hydrocarbons. Q15 demonstrated an increased cell surface hydrophobicity with production of biosurfactant(s) when grown on hydrocarbons compared to growth on glucose-acetate. This biosurfactant would increase the bioavailibity of naturally hydrophobic oil constituents. Q15 was also able to adhere to solid and liquid hydrocarbon phases via the change in cell surface hydrophobicity and a specialized extracellular polymeric substance which formed during growth on hydrocarbons caused cells to form clusters (Whyte et al. 1999).

Hydrocarbon contamination in cold environments, where cold-adapted microorganisms are present, will impact the microbial community as a whole. Juck et al. (2000) investigated the bacterial communities from two soils from northern Canada and the Canadian high Arctic, contaminated with hydrocarbons. Hydrocarbon-impacted soils and, pristine soils from both sites showed similar plate counts of viable organisms cultured at 5°C that ranged from ~10^6 – 10^8 colony forming units (CFU) g^{-1} soil (Juck et al. 2000). Conflicting results were observed concerning bacterial diversity of the sites when the hydrocarbon-impacted and pristine soils were compared. The northern Canada soil showed hydrocarbon contamination decreased soil bacterial diversity compared to pristine while the opposite was found for the high Arcitic soil (Juck et al. 2000), underscoring the specificity of individual sites. Labbé et al. (2007) examined the phylogenetic difference between hydrocarbon contaminated and pristine soil from Alpine soils in Tyrol, Austria. DGGE analyses revealed similar proportions of *Actinobacteria,* ranging from 18-20%, and *Proteobacteria,* from 73-76%, in the hydrocarbon-contaminated and pristine soils (Labbé

et al. 2007). Among the *Proteobacteria*, the *alpha*-class was nearly double (46%) in the pristine soil compared to the contaminated soil (24%) and the *beta*- and *gamma*- classes were only detected in the hydrocarbon-contaminated soil (Labbé et al. 2007). The authors further note that pristine soil had greater quantity of potential novel phylotypes.

1.3. Soil microorganisms

1.3.1. Isolation and characterization

Though soil has proved to be an extraordinarily difficult and complex environment for isolation and classification of microorganisms (Gewin 2006), members from the three major domains of life (*Bacteria*, *Archaea* and *Eukarya*) can be isolated. To date, various microbiological analytical tools and techniques have indicated that the most abundant and diverse soil microorganisms belong to the *Bacteria* (Killham 1994). Two main metrics for soil bacterial analyses involve classic isolation and culturing of pure strains for further physiological and biochemical characterization, and relatively modern culture-independent molecular classification by direct sequencing of the small subunit ribosomal RNA gene (16S rRNA gene). Both have distinct advantages coupled with significant drawbacks and biases.

Classic microbiology can be traced to the first microscopic observations of bacteria in the latter half of the 1600's, by Antony van Leeuwenhoek and 200 years later to the beginnings of bacteriology and microbial taxonomic classification by Ferdinand Cohn. The subsequent work of Louis Pasteur and Robert Koch famously disproved the theory of spontaneous generation, created the burgeoning field of medical microbiology and focused on isolation and characterization of bacteria in pure cultures. But it was at the end of the 19th and early part of the 20th century that Martinus Beijerinck and Sergei Winogradsky revealed the unimaginable breadth of microbial life in the environment, with the discovery of viruses, the development of enrichment cultures and the amazing significance

microbial metabolic functions have on global geochemical processes. Isolation of bacteria from the environment, at this point, principally involved the plating of environmental samples on solid, nutrient rich media. Recent technological advances involving microscopic manipulations for individual cell isolation and novel culturing approaches that mimic the natural environment have improved the power to isolate pure bacterial cultures but still remain extremely limited in this respect (Kaeberlein et al. 2002; Zengler et al. 2002; Ferrari et al. 2005).

Soil, though more difficult to work with than the marine environment, is thought to contain the greatest biodiversity of any environment on Earth (Roesch et al. 2007). Different investigation strategies into the biodiversity in soil exist that involve of environmental sampling and extraction of target molecules and include analysis of key biogenic molecules like membrane lipid and/ or respiratory quinone profiles. The most commonly used method for microbial classification is by sequencing the 16S rRNA gene (Pace 1997; DeLong and Pace 2001). The 16S rRNA gene provides a highly conserved marker, with a slow and constant mutational rate that can be used to measure taxonomic distances between species based on differences in the DNA sequence (Woese and Fox 1977; Woese 1987). Many molecular phylogenetic environmental studies using 16S gene analyses have uncovered numerous, potentially new microbial species, genera and even domains lurking, with no cultured, laboratory strain representative for comparison (e.g. Barns et al. 1994; Pace 1997; Hugenholtz et al. 1998; Dojka et al. 2000; Hugenholtz et al. 2001; Sogin et al. 2006). Speculation of the order of magnitude concerning the total number of bacterial species is debated by microbiologists (Hong et al. 2006), making it impossible to precisely quantify the significance of the cultured laboratory stains, which may only represent ~1% of the total number of species on the planet (Amann et al. 1995). Torsvik et al. (1990) used culture-independent methods to explore the number of bacterial species in a gram of soil by using DNA:DNA hybridization from bacterial

genomes extracted from cells and calculated the reassociation of these genomes. This study concluded that ~10,000 different bacterial species were present in a gram of boreal forest. Gans et al. (2005) followed the experimental procedure of Torsvik et al. (1990), but used computational improvements to calculate the number of bacterial species, and estimated that the actual quantity was nearly three orders of magnitude larger. Roesch et al. (2007) constructed one of the largest 16S rRNA gene clone libraries to date, with 25,000 gene fragment sequences from each of four test soils. The authors used high-throughput pyrosequencing (Margulies et al. 2005) and estimated a more conservative figure of ~52,000 bacterial speices per gram soil. Clearly, a comprehensive community profile of any environmental system based on the organisms cultured alone would be incomplete and inaccurately represent reality. On the other hand, there are cases of organisms cultured from an environment that were not detected by molecular analysis of that same environment (Donachie et al. 2007) underscoring the bias inherent in the molecular techniques used.

1.3.2. Molecular Analyses

16S rRNA gene analysis introduces biases and limits the practicality of basing community profiles solely on DNA isolation, amplification and sequencing. The process can be divided into three major stages, each of which can introduce bias; DNA extraction; polymerase chain reaction (PCR); DNA sequencing and bioinformatic analyses. Various chemical and mechanical techniques exist that are designed to extract DNA from within cells and the surrounding physical matrix, and purify this separated DNA (Sambrook and Russell 2001). The efficiency for DNA extraction depends on the methods used, the physical matrix, and the cell type (Whyte and Greer 2005). Although extraction methods are designed to deal with distinct matrices and cell types, for instance Gram-positive cells are generally more resistant than Gram-negative cells to lysis, no method is considered infallible (Krsek and Wellington 1999; Martin-Laurent et al. 2001).

PCR, the powerful technique developed by Kary Mullis in 1983 that exponentially amplifies specific segments of DNA, is based on a repeating cycle of different temperatures, the physical reaction of DNA to these temperatures and the biochemical activity of, most commonly, an enzyme isolated from *Thermus aquaticus, taq* DNA polymerase. Although widely used and authoritative, each step of the PCR cycle can introduce bias and due to the exponential nature of the PCR, small imprecisions can become prevalent in the PCR products. The first step is the melting, or denaturing, of double stranded template DNA, the kinetics of which is determined by the DNA sequence, and more specifically the percent guanine and cytosine (%GC). With a higher %GC, less efficient melting occurs, possibly necessitating higher melting temperatures for different lengths of time for different samples. Once single stranded, primers can anneal to target locations allowing the necessary starting point for DNA extension by *taq* polyermase. Primer design can be tailored for the level of specificity desired and is based on known DNA sequences and the variable and conserved regions of those DNA sequences. Of course, the more "universal" the primer pair, the more broad the amplification, which can result in unwanted PCR artifacts, while conversely, more specific primers may eliminate some artifacts but lose some desired targets. Since the primers are based on known sequences, along with the previously mentioned biases, it cannot be assumed that complete coverage of an environment can be achieved using a singular molecular inquiry. Additionally, *taq* polyermase has a known error rate (Saiki et al. 1988) that will affect the subsequent PCR product sequence. Also, once the DNA that makes up the rRNA genes is single stranded, it can form the secondary structures that rRNA is known for, so *taq* must overcome these obstacles. Once a reliable PCR product has been obtained, the DNA sequence can be determined by automated DNA sequencing machines, of which only very well funded laboratories can maintain in-house, leaving the majority of researchers to send their DNA products to outside

sequencing centers. With reliable sequences, online databases (National Center for Biotechnology Information, NCBI; Ribosomal Database Project, RDP) are used to help classify the unknown sequence, though strict regulation of the online databases is ambiguous. Subsequent phylogenetic analyses (e.g. dendograms) have no standard protocols making generation and interpretation somewhat uncertain. PCR can also be used to target and amplify any section of DNA, including catabolic genes or genetic regulatory regions (van Beilen et al. 2001) and more recently in conjunction with other molecular techniques, whole genomes of organisms or the metagenome of an entire ecosystem (Handelsman 2004).

Discrimination of the different multiple 16S rRNA gene PCR products from the sampled environment is the next step in molecular microbial ecology. Two widely used and respected techniques for the discrimination of any PCR products are denaturing gradient gel electrophoresis (DGGE) and the clone library, used singularly or in combination by many of the above mentioned studies. DGGE uses the difference in DNA sequence, more specifically the unique denaturing kinetics of a DNA fragment that is determined by %GC in that DNA fragment, and the negative charge of DNA to separate distinct fragments (Muyzer et al. 1993). PCR products are separated on a polyacrylamide gel with the denaturants urea and formamide increasing in concentration along a gradient in the gel. The PCR products are pulled through the gel by electric current and as the DNA fragments encounter increasing concentrations of the denaturants, the molecules separate and migration through the gel is retarded. The DNA fragment does not fully separate because special DGGE primers are used during the PCR amplification stage that have a GC-clamp, which is a DNA sequence of ~40 bp added to the end of the PCR product, consisting entirely of GC, thus preventing complete denaturation. Because the unique sequence of each PCR product determines the position in the gel where migration stops, the DNA bands that form tend to represent different organisms. The individual bands can subsequently be cut, re-

amplified by PCR and sequenced. Muyzer et al. (1993) displayed the sensitivity of DGGE finding it possible to resolve representatives at only 1% of the total population. Applications for these molecular fingerprints or DNA community profiles have included comparisons between two different environmental samples or to mark the community change in one sample over time, among others (reviewed in Muyzer et al. 1998).

Clone libraries based on 16S rRNA genes are another method for molecular community investigation, starting again with extraction of DNA from the organisms in an environmental sample and PCR-amplification of the 16S rRNA gene. The PCR product is then ligated into a suitable vector followed by transformation into a suitable host, most commonly *Escherichia coli*. Host bacteria are grown and plated on selective media with the subsequent colonies individually prepared for vector DNA isolation and PCR-amplification of the ligated section of the vector. The resultant PCR products can then be sequenced, and the community representative clone library can be scrutinized with various statistical tools. Unlike DGGE, which gives a qualitative approximation of the population proportion of an individual microorganism, clone libraries can quantify the proportions of each distinct group represented in the community. As mentioned, both DGGE and clone libraries have been used extensively in many studies, some of which have employed both (e.g. Perreault et al. 2007), and each having common and unique applications and shortcomings (Spiegelman et al. 2005).

1.4. The present study

This collaborative project was supported by INAC and Qikiqtaaluk Environmental (QE) (Montreal, Canada) and involved the Department of Civil Engineering and Applied Mechanics (CEAM) and the Department of Natural Resource Sciences (NRS) at McGill University. The analyses of PhD candidate Wonjae Chang in the laboratory of Dr. Subhasis Ghoshal of CAEM were used in conjunction with the results presented here to

achieve the future on-site bioremediation of petroleum contaminated soils from a former military radar station located on RI. The overall project focused on increasing the rate of hydrocarbon mineralization by landfarming through biostimulation of the indigenous microbial populations of RI soil under conditions similar to that found naturally on RI. The microbiological aspects of this research, presented here, included investigation of the soil bacterial community via culture-dependent and -independent methods and characterization and monitoring the response of the community to the various treatments. This microbiology investigation was divided into two phases; biotreatability assessment and mesocosm trials. Because the long-term goals of this project are the eventual bioremediation of the actual RI site, mesocosm scale investigations are essential to obtain cost and design data (Schmidt and Scow 2001). The scale-up procedure is also important, as early identification of potential problems will translate into smooth field-scale implementation (Battaglia and Morgan 1994).

The first phase of biotreatability assessment was a relatively minor component of the overall project, but essential to identify possible treatment strategies for the main component of the project, large scale mesocosm trials. The biotreatability assessment used small scale microcosm mineralization assays of ~20 g soil and representative ^{14}C-labelled hydrocarbons (hexadecane, phenanthrene and naphthalene) to monitor soil microbial mineralization activity at a constant temperature of 5°C. Different soil treatments were tested to amend the naturally nutrient-deficient and acidic RI soil. Due to the small scale, many microcosm assays could be tested to identify the optimal soil treatment. Culture-dependent analyses were also conducted to enumerate the heterotrophic and hydrocarbon-degrading microbial populations on solid media plates. Culture-independent analysis included total soil community DNA extracted and examined for the presence of bacterial catabolic genes involved in hydrocarbon degradation. Additionally, the soil bacterial community was

investigated by PCR-DGGE analysis. The information gleaned from these initial investigations was then scaled up and applied in phase two, the mesocosm trials. This scale up process also served a troubleshooting role, as it identified unique problems for the actual scale up procedure under controlled laboratory conditions.

The major component of this project was the mesocosm trial because it offered a closer representation of the actual on-site conditions found at RI, thus increasing the likelihood that the data obtained would accurately reflect the future on-site bioremediation project. The mesocosm tanks, which were designed, constructed and maintained by W. Chang at CEAM, contained ~150 kg RI soil and the mesocosm trials were conducted under conditions that closely mimicked the RI summer, when the indigenous microbial population is most metabolically active and when the highest rate of hydrocarbon degradation would be expected to take place. The mesocom tanks were housed in an unique indoor facility for cold-temperature remediation research at CEAM, where temperatures can be programmed to follow a constant cyclical pattern. Following the pattern of a typical RI summer, the trials lasted 60 days and the temperature was set to cycle from 1°C to 10°C.

The microbial analyses during the mesocosm trial used soil sampled at four time points (days 0, 20, 40 and 60) from three layers(top, middle and bottom). Microbial hydrocarbon mineralization activity, population level and community were measured. Microbial hydrocarbon mineralization activity was monitored by spiking the mesocosm soil with ^{14}C-hexadecane. These activity studies were designed to give a snap-shot measurement of the microbial mineralization activity of the mesocosm at that specific time from that specific layer. Microbial population levels were quantified by direct epifluorescent microscopy, and by plate culturing of cultivatable, viable, aerobic, hydrocarbon-degrading organisms. DGGE was used to track the changes in bacterial population due to the mesocosm treatment,

and clone libraries were constructed to determine the overall alteration of the bacterial community from the beginning of the mesocosm experiment to the end.

Lastly, cultured isolates were purified and identified by 16S rRNA gene sequencing to further understand the cultivatable proportion of the community. Growth of isolated strains at different temperatures and on different media was also investigated, for possible future bioaugmentation studies. Potentially novel isolates could also improve the general knowledge concerning biodiversity in soil microbiology from an unique site.

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Figure 1.1 Resolution Island.

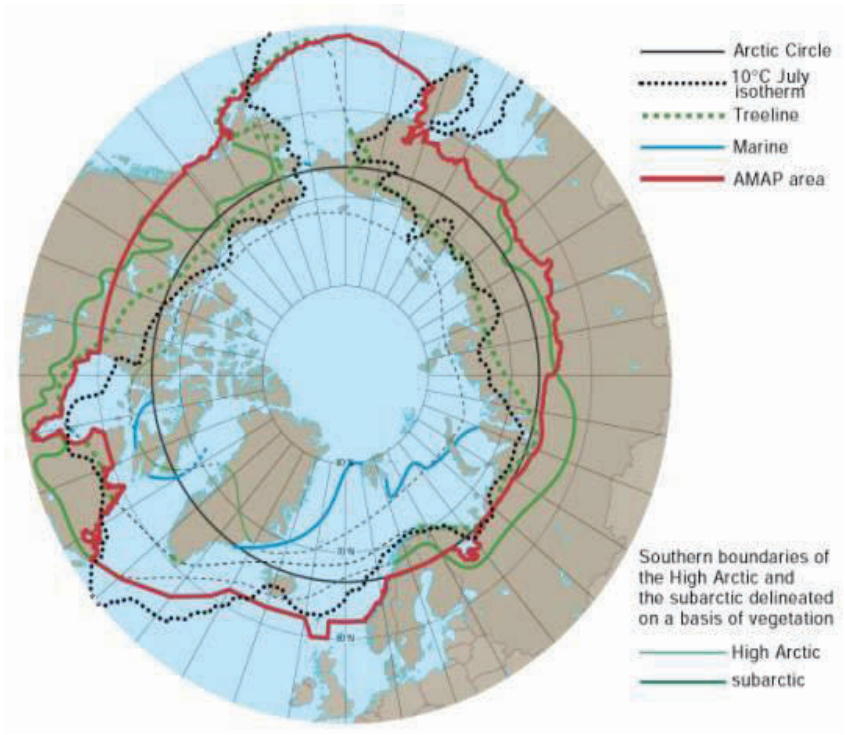

Figure 1.2. The Arctic (adapted from AMAP 1998).

Chapter Two. Materials and Methods

2.1. General laboratory practices

All microbiological analyses conducted in NRS were carried out following rigorous aseptic techniques and procedures. Unless otherwise noted, all equipment was sterilized by autoclaving at 121°C for at least 15 min at 15 pounds per square inch (103 kPa), or provided sterile by the manufacturer. All solutions and media were autoclaved or sterilized by passage through sterile MCE 0.22 µm pore size, 25 mm syringe filters (Fisher Scientific, Waltham, MA), or guaranteed sterile by the manufacturer. Metal or glass spatulas, tweezers, spreading sticks, inoculating loops or other manipulation tools were sterilized in 70 – 90% ethanol with subsequent flaming, or flaming to a red hot state. All manipulation tools were cooled before use. All microbiological analyses were conducted in a Thermo Forma class II A2 biological safety cabinet under laminar flow (Thermo Forma, Marietta, OH) or under a flame to maintain a sterile environment. Latex or non-latex gloves were worn in order to reduce the risk of contamination by microorganisms or enzymes. All chemical solutions, reagents or other ingredients were of reagent-grade quality and purity as provided by the manufacturer and prepared with sterile deionized water (ddH_20). All solutions or equipment used for DNA analyses were subjected to a minimum of 20 min of UV light in either a Thermo Forma class II A2 biological safety cabinet under laminar flow (Thermo Forma, Marietta, OH) or a Fisherbrand UV sterilization cabinet (Fisher Scientific, Waltham, MA).

2.2. Soil samples

RI soil for this project was provided by INAC and QE, and received by CEAM, who were responsible for transportation and maintenance of the samples until, delivered for microbiological analyses to NRS. Soil provided by INAC and QE included samples that were labeled as both pristine (uncontaminated with hydrocarbons) and hydrocarbon contaminated soil.

The first sample bags of pristine and contaminated soils came in summer of 2005 and designated PS and HC, respectively. The bulk soils used for all mesocosm assays were received by CEAM in October of 2005 and designated PSB (for pristine soils-bulk) and HCB (for hydrocarbon contaminated soils-bulk). All samples for microbiological analyses received from CEAM were in sterile Whirl-pak sampling bags (Nasco, Fort Atkinson, WI) having been frozen at -20°C. Samples were transported to NRS in coolers with ice to maintain low temperatures, and were stored at -20°C and handled aseptically in NRS. Soil physical – chemical analyses and hydrocarbon constituent analysis were conducted by Maxxam Analytique Inc. (Montreal, Canada) and CEAM. Soil lettuce seed germination (Greene et al. 1989), earthworm lethality (EPA 1989) and Microtox (Environment Canada 1992) tests were conducted at the Biotechnology Research Institute (BRI) (Montreal, Canada).

2.3. Phase one: Biotreatability assessment

2.3.1. ^{14}C-Hydrocarbon mineralization microcosm assays

Microcosm assays were set up in 100 mL serum bottles (Fisher Scientific, Waltham, MA) capped with either gray butyl (Wheaton, Millville, NJ) or Septa-Teflon (Supelco, Bellefonte, PA) stoppers. Microcosms were aseptically monitored by wiping the stopper with 70% ethanol before sampling. To maintain the *in situ* temperature of each microcosm, all sampling was conducted on ice in a fume hood and the time each microcosm was removed from an incubator was minimized. Each microcosm contained ~20 g (wet weight) of soil and 1 borosilicate glass 12 x 75 mm disposable culture tube (Fisher Scientific, Waltham, MA) that held 1 mL of CO_2 trap solution. All trap solutions consisted of 1 M KOH plus ethylene glycol (Fisher Scientific, Waltham, MA) at the following concentrations: 5°C incubation, 10%; -5°C and -10°C incubation, 20%; -15°C incubation, 30%. Radiolabeled substrates, 9-^{14}C-phenanthrene (Sigma-Aldrich), 1-^{14}C-naphthalene (Sigma-Aldrich) or 1-^{14}C-hexadecane,

(Amersham Piscataway, NJ) were added to a final disintegrations per minute (dpm) counts of ~80,000 - 100,000, plus 100 ppm cold (not radiolabelled) hexadecane or 10 ppm cold phenanthrene or naphthalene. Hexadecane solutions were prepared in hexanes (Fisher Scientific, Waltham, MA), while naphthalene and phenanthrene solutions were prepared in methanol and ethanol (Fisher Scientific, Waltham, MA), respectively. Sterile controls were autoclaved with soil at 121°C for at least 30 min at 15 pounds per square inch (103 kPa) on two consecutive days prior to the beginning of the experiment. Fertilizer amendments used either mono-ammonium phosphate fertilizer (MAP; Yunnan Newswift Company Ltd.) which is guaranteed by the suppler to contain ≥49% available phosphate and ≥9% total nitrogen (N) or Plantex 20:20:20 All Purpose Fertilizer (20:20:20; Plant Products Company Ltd.) which is guaranteed by the manufacturer to contain 20% total N, 20% available phosphate and 20% soluble potash. Fertilizers were added to have a final N concentration of 250 $\mu g\ g^{-1}$. When added alone, potassium phosphate was added to a final concentration of 50 $\mu g\ g^{-1}$. When added alone, ammonium nitrate was added to a final concentration of 87.5 $\mu g\ g^{-1}$. $CaCO_3$ was added to a final concentration of 10 mg g^{-1}, which was found to give a soil pH of 6.5 – 7 (Chang et al. 2007). All amendments and radiolabeled substrate solutions were added to the soil, vigorously vortexed to ensure even distribution within the microcosm bottle. Microcosm assays incubated at 5°C were monitored for eight weeks and were sampled once a week, while those incubated at sub-zero temperatures were sampled every 2 – 4 weeks. For sampling, the CO_2 trap solution was drawn out of the glass test tube and washed with an additional 1 mL of fresh trap solution, and another 1 mL of trap solution added. The 2 mL of sampled trap solution was added to 20 mL glass scintillation vials (Fisher Scientific, Waltham, MA) with 18 mL of ScintiVerse scintillation fluid (BD, Franklin Lakes, NJ) and counts were read on a Beckman-Coulter LS 6500 Multi-Purpose Scintillation Counter

and the supplied software (Beckman Coulter, Inc., Fullerton, CA) with a count time of 5 min for each vial. Radioactive counts from the extracted collection plus scintillation liquid solution represented the proportion of the radioactive substrate mineralized to $^{14}CO_2$ and retained in the collection solution as $K_2{}^{14}CO_3$ (no precipitates observed). Ethylene glycol, a quenching agent, added to the collection solution prevented the collection solution from freezing and had only minor quenching effects (Steven et al. submitted for publication). All microcosm assays had three replicates for each individual assay.

2.3.2. Microscopy

Light microscopy of samples was undertaken using a Nikon Eclipse E600 microscope (Nikon, Canada), with wet and dry mounts for eukaryotic cell morphology determination (Sambrook and Russell 2001). Epifluorescent microscopy also used the Nikon Eclipse E600 microscope equipped with a Nikon super high pressure mercury lamp, for total soil microbial enumerations. Samples for epifluorescent microscopy were prepared as follows: 1 g of soil was placed into a sterile test tube with 2.5 g of sterile 3 mm glass beads (Fisher Scientific, Waltham, MA) and 9 mL of 0.1% $Na_4P_2O_7 \cdot 10\ H_2O$ and vortexed for 2 min. Appropriate dilution series were prepared using 0.1% $Na_4P_2O_7 \cdot 10\ H_2O$ and 900 μL from the appropriate dilution was added to 100 μL 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein hydrochloride solution(DTAF; Sigma-Aldrich). DTAF solution was made fresh and kept in the dark for each use. The solution consisted of 5 mg DTAF mL^{-1} dissolved in 0.05 M Na_2PO_4. The soil dilution and DTAF solution mixture was allowed to stain, in the dark, for at least 30 min, then filtered through a Poretics polycarbonate black 0.22 micron 25 mm filter (Osmonics Inc., Westborough, MA). The filter was then air dryed, in the dark, after which a drop of non-drying immersion oil for fluorescence microscopy type FF (Cargille Laboratories Inc., Cedar Grove, NJ) was added with a cover slip placed on top and another drop of the same

immersion oil on top of the cover slip for oil immersion viewing. DTAF covalently binds to the primary hydroxyl groups of carbohydrates and N-termini of proteins with an excitation range in blue light of 450–490 nm and gives a yellow-green emission in the range of 515–565 nm (Schumann et al. 1998). Enumeration calculations were generally conducted as described Kepner and Pratt (1994) by counting ten random fields from the filter with the mean number from the ten fields reported, and accounts for the dilution series and field size, resulting in a "cells g^{-1} soil" value.

2.3.3. DNA extraction and purification

Total community DNA from RI soil was extracted from soil slurries (section 2.3) using an UltraClean Soil DNA Kit (Mo Bio, Solana Beach, CA), following the manufacturers protocol. Individual isolate DNA was either extracted using a DNeasy Tissue Kit (Qiagen, Mississauga, ON) following the manufacturers protocol for Gram-positive bacterial DNA extraction or via the boiling lysis technique that calls for colonies scraped from a plate and suspended in 500 μL of ddH_2O; boiled for 10 min; cooled at -80°C for 15 min; boiled for 10 min; cooled on ice and centrifuged for 2 min at 13, 600 x *g*. Cell debris was discarded and the supernatant stored. Boiling lysis was also used for DNA extraction of clones. All soil DNA extracts were stored at -20°C.

Soil DNA extracts from soil was purified by polyvinylpolypyrrolidone (PVPP) solution spin columns (Berthelet et al. 1996). Briefly, 300 g of insoluble PVPP was suspended in 4 L of 3 M HCl for 12 to 16 hours at room temperature. The suspension was filtered, and the captured PVPP was resuspended in 20 mM KH_2PO_4 (pH 7.4) and stirred for 1 to 2 hours. The filtering and resuspension process was repeated until the suspension reached pH 7.0. The PVPP suspension was stored at 4 °C until used. Approximately 450 μL of PVPP mixture was aliquoted into sterile microspin columns (Amersham Biosciences, Buckinghamshire, UK) and these columns with PVPP were placed in sterile microcentrifuge tubes.

Columns were centrifuged twice at 800 x *g* for 3 min at room temperature, in order to remove all extra PVPP mixture liquid, after which columns were placed in new, sterile microcentrifuge tubes and DNA extracts were applied to the centre of columns. Columns were spun at 800 x *g* for 3 min at room temperature and the eluted DNA extract was stored at -20°C.

2.3.4. PCR and agarose gel electrophoresis

PCR was performed in 0.2 mL thin walled PCR tubes, thin walled 12 tube strips, or 96-well PCR plates (Diamed Lab Supplies Inc., Mississauga, ON) using either a Touchgene Gradient thermocycler machine or TC-312 thermocycler machine (both from Techne Inc., Burlington, NJ). All PCR reagents were supplied by Invitrogen Canada, Burlington, ON, and stored at -20°C, unless otherwise noted. Standard PCR reactions contained 1x PCR buffer, 0.2 mM of each dNTP, 0.75 - 1.5 mM $MgCl_2$, 1 – 4 μL template DNA, 0.5 μM each primer (Table 2.2), 2 – 3 units of *Taq* polymerase, 10 mg mL^{-1} bovine serum albumin (BSA; Fisher Scientific, Waltham, MA), and the final volume was 25 – 50 μL in H_2O. All H_2O for molecular analyses was pretreated through a Millipore Simplicity 185 (Millipore Corp., Billerica, MA) and UV irradiated for 30 min. Except for the *Taq*-polymerase, primers, dNTPs and DNA, all PCR reagents were UV-treated prior to each reaction. PCR primer sequences, and the specific DNA target for those primers are listed in Table 2.2. All primers were purchased from MWG-Biotech (High Point, NC), and stored in 100 mM stocks at -20°C, unless otherwise noted. Negative PCR controls were prepared as above but with the template DNA replaced with H_2O pretreated through a Millipore Simplicity 185 (Millipore Corp., Billerica, MA) and UV irradiated for 30 min to ensure no extraneous DNA contamination of reagents. Positive controls used DNA known to amplify under the given PCR conditions, and were used to ensure that proper reaction conditions were achieved. The PCR protocol for universal bacterial 16S rRNA gene amplification was as follows: 3 min at 95°C; 30

cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension of 7 min at 72°C. Amplification of DNA for DGGE analysis consisted of 5 min at 96°C; 10 cycles of 1 min at 96°C, 45 s at 68°C in the first cycle touching down 0.9°C per cycle to 59°C in the last cycle, and 1 min 30 s at 72°C; 15 cycles of 96°C for 1 min, 59°C for 45 s and 72°C for 1 min 30 s; and a final extension of 5 min at 72°C. The products of multiple PCR were pooled in order to obtain the necessary 500 ng DNA for DGGE analysis. Pooled PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen, Mississauga, ON) following the protocol provided by the manufacturer. The PCR protocols for amplification of bacterial catabolic genes was as follows: alkane monooxygenase (*alkB*); 5 min at 96°C; 25 cycles of 1 min at 94°C, 1 min 10 s at 55°C, 1 min 10 s at 72°C; final extension of 10 min at 72°C: catechol-2,3-dioxygenase and naphthalene dioxygenase, (*xylE* and *ndoB*, respectively); 96°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C; 3 min at 72°C: phenanthrene dioxygenase (*phnAc*); 96°C for 5 min; 35 cycles of 30 s at 94°C, 30 s at 52°C, 1 min at 72°C; 10 min at 72°C. Amplification of eukaryotic 18S rRNA gene used the following PCR protocol; 96°C for 5 min; 40 cycles of 1 min at 94°C, 1 min at 55°C 1 min at 72°C; 10 min at 72°C. The PCR protocol for amplification of eukaryotic 18S rRNA gene for DGGE analysis was 96°C at 5 min; 19 cycles of 1 min at 94°C, 1 min at 64°C, 1 min at 55°C, 3 min at 72°C; 10 cycles of 1 min at 94°C, 1 min at 55°C, 3 min at 72°C; 5 min at 72°C. DNA in PCR products was quantified by gel electrophoresis using the Chemi Genius BioImaging System with the GeneTools software (Syngene, Frederick, MD) or by measuring absorbance of extracts at 260 nm (Sambrook and Russell 2001) on an Ultrospec 2100 Pro UV/visible spectrophotometer (Biochrome Corporation, Cambridge, UK).

Gel electrophoresis was performed with horizontal 0.8 - 1.0% agarose (Fisher Scientific, Waltham, MA) gels buffered with either TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) or SB buffer (5 mM $Na_2B_4O_4 \cdot 10$

H_2O, pH adjusted to 8.5 with H_3BO_3) with gels containing 0.5 μg mL^{-1} ethidium bromide. TAE gels were generally run at 85 volts for 45 min, while SB gels were run from 100 – 300 volts for 7 – 15 min. DNA samples were mixed with 5x loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in H_2O]. Molecular weight standards λ DNA *Hind*III and 100 bp ladder (Invitrogen Canada, Burlington, ON) were prepared by addition of 46 μL of 500 μg mL^{-1} molecular weight standard stock to 20 μL 1 M NaCl, 0.2 μL 0.5 M EDTA, 20 μL 1 M Tris-Cl pH 7.8 and sterile H_2O to a final volume of 900 μL and heated 10 min at 65°C then cooled on ice for 10 min and added to 100 μL 10x loading buffer. 5 μL of the molecular weight standards were loaded into gels. Gels were visualized on a Chemi Genius BioImaging System with the GeneSnap software (Syngene, Frederick, MD). DNA extracted from bands used the QIAquick Gel Extraction Kit (Qiagen Inc., Mississauga, ON) following the manufacturers protocol.

2.3.5. DGGE analyses

Primers 341F-GC and 758-R (Table 2.2) were used to amplify a ~400 bp region of the bacterial 16S rRNA gene, which included a ~40 bp GC clamp. PCR products were quantified, and pooled to have 500 ng of PCR product for each sample. DGGE was performed using a BioRad DCode Universal Mutation Detection Systen (BioRad Hercules, CA), following the instructions provided by the manufacturer. Samples were loaded onto an 8% (v/v) acrylamide gel with a denaturing gradient generated by urea and formamide, ranging from 35% - 65%, in TAE buffer with a final volume of 11.5 mL. A 6% (v/v) acrylamide gel with 0% denaturant was used as a spacer gel, final volume of 10 mL, where the samples were loaded, but was discarded after the completion of the run. Each DNA sample was loaded into the wells of the spacer gel, previously having been mixed with DGGE 2x loading dye (0.05 % bromophenol blue, 0.05 % xylene cyanol, and 70 % glycerol in deionized water). Electrophoresis was performed for

16 h at 60 V and 60°C. Gels were stained for at least 1 h in Vistra Green Nucleic Acid Stain (1:10000 dilution; GE Healthcare Bio-Sciences AB, Uppsala,Sweden). Gels were visualized on a Bio Rad Molecular Imager FX equipped with an External Laser Imager FX (Bio-Rad Laboratories Inc., Hercules, CA). The DGGE procedure, starting with the soil extracted community DNA, was repeated at least once to ensure that a similar banding pattern appeared in the DGGE gel. DNA was extracted from DGGE gels by elution of cut bands in 20 µL of UV-treated ddH_2O overnight, followed by re-amplification of the DGGE-band DNA by PCR. DGGE analyses were conducted on HC soil set up in parallel to microcosm mineralization assays, but excluding the radioactive substrate. After two months of incubation at 5°C, DNA was extracted and purified and otherwise prepared for DGGE analysis. For mesocosm soils, DNA prepared for DGGE analyses as above and stored at -20°C until the end of the mesocosm trials so samples from all mesocosm time points could be run on the same DGGE gel.

2.3.5. DNA sequencing and online databases

Primers 341-F and 758-R (Table 2.2) were used to amplify a ~400 bp region of the bacterial 16S rRNA gene from total soil community DNA. PCR products and primer 341F, were sent for sequencing to either the McGill University and Genome Quebec Innovation Centre (Montreal, QC), which uses a 3730XL DNA Analyzer system or the Plate-forme d'analyses biomoléculaires at the Université Laval (Québec, Canada), which employs two 16-capillary genetic analyzers: an ABI Prism 3130XL and an ABI Prism 3100XL. 16S rRNA gene sequences obtained were compared to the public, online databases of NCBI with BLAST (BLAST; Altschul *et al.,* 1990) and the Ribosomal Database Project II (RDP; Cole et al. 2007).

2.3.6. Microbial enumeration and isolation

Microorganisms were isolated and enumerated from soil samples on solid media using the spread plate technique (Sambrook and Russell 2001).

Screw-cap test tubes with 2.5 g of 3 mm glass beads (Fisher Scientific, Waltham, MA) were sterilized by autoclaving and ~5 g soil added to tubes. Dilution solution (0.1% $Na_4P2O_7 \cdot 10\ H_2O$) was added to 3-4x v/w of soil to create a soil slurry. The soil slurry was vortexed for ~2 min after which an appropriate dilution series was prepared in dilution solution. Aliquots were plated onto appropriate solid media plates. For solid media, 15% Bacto-Agar (Difco Laboratories, Detriot, MI) was added, while liquid versions were the same recipes minus the agar. Incubation of inoculated plates for viable plate enumerations was conducted at either 5°C or ambient room temperature (~24°C). Luria-Bertani (LB) broth contained per litre: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g. Minimal salts medium (MSM) contained: 1 M NaH_2PO_4; 1 M K_2HPO_4; 1 M $(NH_4)_2SO_4$; 0.5 M $MgSO_4 \bullet 7\ H_2O$; 1 mL L^{-1} of trace mineral solution. Trace mineral solution contained (mM): $Co(NO_2)_2 \bullet 6\ H_2O$ (1); $AlK(SO_4)_2 \bullet 12\ H_2O$ (1); $CuSO_4$ (1); $ZnSO_4 \bullet 7\ H_2O$ (10); $MnSO_4 \bullet H_2O$ (10); $FeSO_4 \bullet 7\ H_2O$ (10); $Na_2MoO_4 \cdot 2\ H_2O$ (2); $Ca(NO_3)_2 \bullet H_2O$ (10). MSM was amended with 200 μL of Arctic diesel (Shell Canada) or hexadecane (Acros Organics, New Jersey, USA) soaked onto a sterile 2 cm^2 piece of filter paper stuck to the inside lid of the petri plate. The pH for MSM was either ~7.2 or ~4.5. R2A (Difco, Detroit, MI), contained 0.5 g yeast extract, 0.5 g proteose peptone No. 3, 0.5 g casamino acids, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \bullet 7\ H_2O$, (pH 7.2 ± 0.2). Media pH was adjusted with HCl or NaOH and measured with an Accumet basic AB15 pH meter (Fisher Scientific, Waltham, MA). Colonies were incubated and counted for two months maximum. Colonies with different morphology and emergence time were selected and re-streaked at least 3 times before performing DNA isolation or making stocks prepared in either 10% R2A or LB supplemented with 20% v/v glycerol, and stored frozen at -80°C. All plate enumerations contained three replicates for each individual dilution.

Isolated strains were tested for growth at sub-zero temperatures, -5°C, -10°C, -15°C. R2A plates were supplemented with one of the following freezing point depressants: 1%, 2.5%, or 5% v/v ethylene glycol (Fisher Scientific, Waltham, MA); 7% or 10% w/v sucrose (Fisher Scientific, Waltham, MA). Sub-zero culturing on MSM - Arctic diesel plates was supplemented with either 1.35 M or 2.5 M NaCl (Fisher Scientific, Waltham, MA). Isolated organisms were identified by sequence analyses (section 2.3.5) of the extracted and PCR amplified (sections 2.3.3 and 2.3.4) 16S rRNA gene or 18S rRNA gene. Primers 27F and Euk20F (Table 2.2) were supplied to sequencing facilities (section 2.3.5), which usually yielded ~800 bp region DNA sequence.

Enrichment cultures were conducted in 125 – 250 mL liquid media in appropriately sized Erlenmeyer flasks (Fisher Scientific, Waltham, MA) on a rotating shaker at ~150 rpm. Cultures were either maintained at room temperature (~24°C) or at 4°C. The two basic procedures followed that of Whyte et al. (1999) or Bogan et al. (2003). The first consisted of 30 g soil in 100 mL of MSM supplemented with 500 ppm yeast extract (Fisher Scientific, Waltham, MA) initially, with subsequent addition of 100 ppm diesel after 1 week of incubation. The second used 4 g soil in 50 mL MSM (pH 7.2 or pH 4.5) supplemented with 800 µL of either hexadecane (Acros Organics, New Jersey, USA) or Arctic diesel (Shell, Canada), incubated for 3 – 7 days, then transferred to new MSM-hydrocarbon media.

2.4. Phase two: Mesocosm trials

2.4.1. Mesocosm set-up and soil sampling

Mesocosm construction, maintenance, control, operation and sampling were conducted by W. Chang in CEAM, and soil samples for microbiological analyses were transferred to NRS. The cold-temperature facility of CAEM, which housed the mesocosm trials, was set to run a temperature cycle that mimicked the *in situ* RI summer temperature and duration. Correspondingly, the temperature was set to oscillate between

1°C and 10°C on a ten day cycle (Fig. 2.1) and the mesocosm trials lasted 60 days. The general sampling procedure used by W. Chang was as follows (as in Chang et al. 2007): soil for mesocosm trials were stored at -4°C while preparation for mesocosm trials was conducted at -4°C – 4°C. Approximately 150 kg of soil for each mesocosm tank was sieved through sterilized sieves with a pore opening size of 4.75 mm. Mesocosm tanks (Fig. 2.2) were made of stainless steel and the dimensions of the tank were 1.0 m long, 0.65 m wide and 0.35 m deep. The soil depth inside a mesocosm tank was ~22 cm. Five separate mesocosm tanks were prepared, and designated P1, P2, P3, P4 and P5, the different amendments and tilling regimes for each are as in Table 2.1. Amendment applications were conducted on two consecutive days and thoroughly mixed the soil with the given amendment. All mixing/ tilling of mesocosm soils was conducted with sterilized hand shovels and a pitchfork. Soils sampled for microbial analyses were removed from the mesocosm tank prior to the tilling events. Mesocosm soils for microbial analyses was sampled with a sterilized auger drilled to three specific depth ranges (Fig. 2.3); the top layer (T) was from the surface to a depth of 5 cm; the middle layer (M) was between 5 cm and 15 cm of depth, and; the bottom layer (B) was below 15 cm depth to the base of the mesocosm tank. To maximize the soil sample representation of the mesocosm tank as a whole, emphasis was placed on evenness of the soil sample from each layer. Accordingly, composite samples from each sample layer were prepared from an amalgamation of the soil recovered from 5 - 7 auger-drilled soil sub-samplings. The auger drill locations were spaced relatively equidistant across the mesocosm tank (Fig. 2.4). An approximately equal quantity of soil was recovered from each auger-drilled soil sub-sample totaling ~150 g soil for each individual sampling. Microbial analyses were completed from soil samples taken after Day 0, Day 20, Day 40 and Day 60 of the mesocosm trials. Mesocosm soil samples were placed in sterile Whirl-Pak bags (Fisher Scientific, Waltham, MA) and stored at -20°C before being

transported on ice to NRS where, again, they were stored at -20°C. Microbial analyses on mesocosm soil samples were conducted no longer than a week after soil samples were received at NRS.

Comprehensive microbial analyses were only completed on the P1 and P2 mesocosms soil samples from all three layers at all four time points. Comprehensive analyses included ^{14}C-hexadecane mineralization microcosm assays (set-up as in section 2.3.1 but without additional nutrient supplementation), epifluorescent microscopy for total soil microbial enumerations (section 2.3.2), viable plate enumerations (section 2.3.6); total community DNA extraction (section 2.3.3) for PCR amplification of 16S rRNA genes (section 2.3.4) and DGGE analyses (section 2.3.5) and sequencing and database comparison (section 2.3.5). Microbial analysis of the P3, P4 and P5 mesocosm trials was limited to DGGE analyses (section 2.3.5) of composite samples made from the three sampling layers from each of the four time points and subsequent online comparisons (section 2.3.5). Mesocosm samples are named as follows: a sample from the middle layer taken at day 20 from P1 would be labeled P1 M20; in the cases of P3, P4 and P5. For composite samples, the layer indication is omitted; for example, a sample from day 20 from P3 was labelled P3 D20.

2.4.2. Clone library construction from P1 B0 and P1 B60 soils

Soil community DNA was extracted and prepared (section 2.3.3) for PCR amplification of the bacterial 16S rRNA gene (section 2.3.4) using primers 27F and 758R. The cloning procedure used the pGEM-T Easy Vector System I (Promega Corporation, Madison, WI) kit, following the protocol provided by the manufacturer (or as in Steven et al. 2007). PCR product volume to vector volume ratios used were 1:1, 2:1, and 3:1 for the ligation reaction. All control reactions followed the instructions provided by the manufacturer and the kit included all necessary components. To ensure successful ligation, a positive control reaction was completed that used a

control insert for ligation into the vector. To determine the proportion of the ligation reaction in which the digested vector re-circularized, a background control was run that excluded insert DNA. To ensure the vector was completely digested, a ligation reaction was prepared that excluded insert DNA and the T4 DNA ligase. Negative controls for each sample checked possible vector contamination and consisted of a ligation reaction with 1 µL of PCR product and no vector.

Subcloning efficiency DH5*alpha* competent cells (Invitrogen Canada, Burlington, ON), a commercial *E. coli* strain, were used for the transformation procedure. All steps during the transformation procedure were conducted on ice, unless otherwise noted, and the protocol was as indicated by the manufacturer, with slight modifications. Competent cells were thawed on ice and divided into 50 µL aliquots. From each ligation reaction, 2 µL of PCR product was gently added and mixed with competent cells, and incubated on ice for 25 min. Tubes with competent cells and ligation reaction products were placed in a 42°C water bath for 30 s and immediately returned to ice for 2 min, after which 950 µL of room temperature SOC media (ingredients per liter: 20 g tryptone, 5 g yeast extract, 10 mL of 1 M NaCl, 2.5 mL of 1 M KCl, 10 mL of 2 M dextrose, and 10 mL of 2 M Mg^{2+} (prepared as 203.3 g L^{-1} $MgCl_2 \bullet 6\ H_2O$ and 246.5 g L^{-1} $MgSO_4 \bullet 7\ H_2O$) (pH 7.0 ± 0.2) was added and tubes were incubated at 37°C on a rotating shaker at 150 rpm for 1.25 h. Aliquots of 100 µL from each transformation reaction were spread plated on LB + ampicillin (100 µg mL^{-1} final amplicillin concentration) plates spread with 100 µL of 40 mM isopropyl-*beta*-D-thiogalactopyranoside (IPTG) and 100 µL 2% w/v 5 bromo 4 chloro 3 indolyl-*beta*-D-galactopyranoside (X-Gal) prepared in N,N-dimethyl formamide 30 min prior to inoculation. Plates were incubated overnight at 37°C, after which recombinants were analyzed by blue/white screening. White colonies, which should contain vector with 16S rRNA gene insert, were picked randomly from plates with a sterile toothpick and inoculated into 50 µL of sterile water, in 96-well plates (Diamed Lab

Supplies Inc., Mississauga, ON), for boiling lysis (section 2.), and 160 µL of sterile LB + amplicillin broth, in 96-well plates (Diamed Lab Supplies Inc., Mississauga, ON) for storage of clones. After boiling lysis (Section 2.3.3), the DNA was PCR amplified using primers SP6 and T7 (Table 2.2). The PCR protocol for amplification of cloned inserts consisted of 5 min at 95°C; 30 cycles of 45 s at 94°C, 30 s at 57°C, and 1 min at 72°C; and a final extension of 5 min at 72°C. Amplification was verified by gel electrophoresis (section 2.3.4) and sent for sequencing and compared to online databases (section 2.3.5). Inoculated LB broth cultures were incubated overnight at 37 °C, sterile glycerol added to a final concentration of 20 % v/v, and stored at -80 °C.

2.4.3. Clone library analyses

Phylogenetic trees were constructed from the CLUSTALW alignments in the program MacVector 7.0 (Oxford Molecular Group Ltd., Oxford, UK). Neighbor joining (Saitou et al. 1987) best trees were constructed using the Jukes-Cantor (1969) correction and the reliability of the tree branch points was assessed by bootstrap analysis of 1000 replicates. Distance matrices of clone sequences were constructed from CLUSTALW alignments using the DNADIST function, with the Jukes-Cantor (1969) correction, in the PHYLIP program version 3.65 (Felsenstein 2005) for clone library analyses in the programs DOTUR (Schloss et al. 2005) and webLIBSHUFF version 0.96 (Henriksen 2004). The DOTUR program calculated richness, by the Chao1 richness estimator (Chao 1984) and the ACE richness estimator (Chao et al. 1993); diversity by the Shannon's diversity index (H′) (Shannon et al. 1949) and the reciprocal of the Simpson's diversity index (1/D) (Simpson 1949). webLIBSHUFF compared the statistically significant difference of the compositions of the two libraries. Library coverage was calculated according to the formula C = (1-n′/N) x 100, where n′ is the number of phylotypes appearing once in the library and N is the library size (Good 1953).

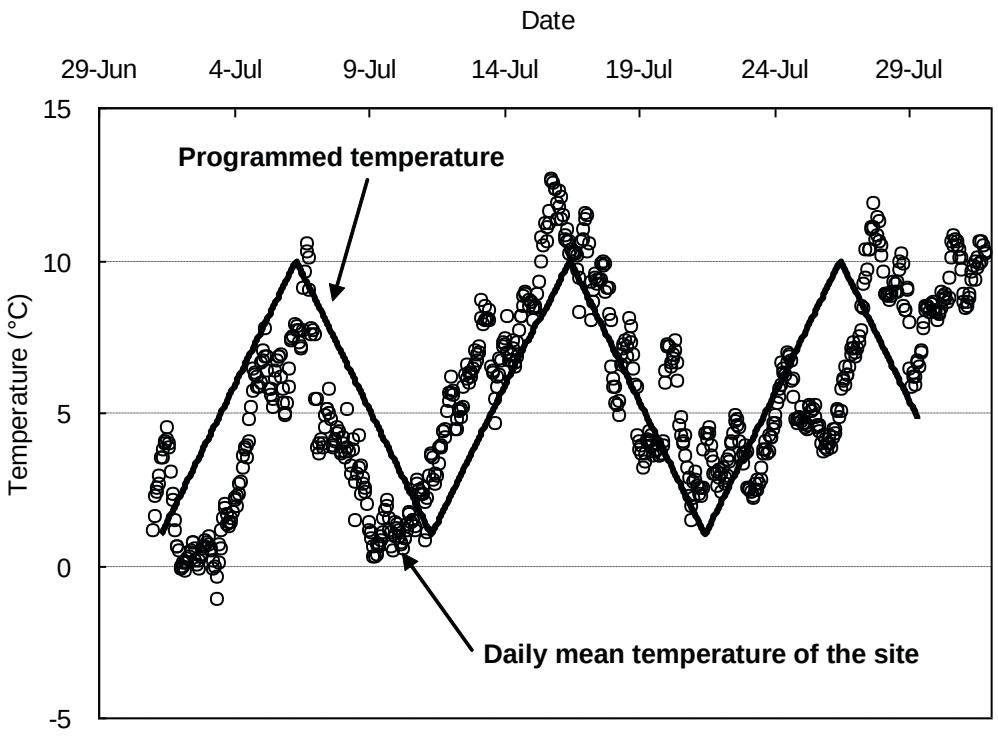

Figure 2.1. Variation in mean daily temperature at RI.

July temperatures for the past 3 years is shown in circles. Solid line shows the cold room temperature settings employed for the 60 day mesocosm trials (Chang et al. 2007).

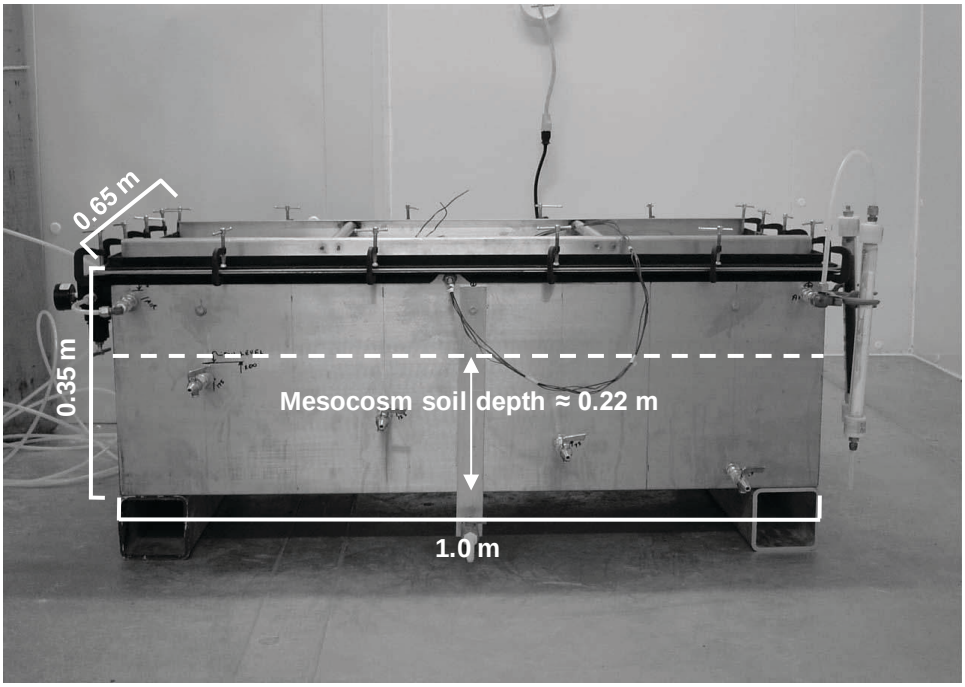

Figure 2.2. Mesocosm tank.

Tank dimensions are indicated along with the approximate soil depth inside the tank (dashed line) (Photo courtesy of W. Chang).

Table 2.1. Mesocosm treatments.

Mesocosm	Total Nitrogen (mg N kg^{-1})	$CaCO_3$ (mg kg^{-1})	Tilling Regime
P1	250	2000	1 per 10 days
P2	0	0	N / A
P3	100	2000	1 per 10 days
P4	250	0	1 per 10 days
P5	250	2000	2 per 7 days

Addition of 2000 mg $CaCO_3$ kg^{-1} RI soil was found to raise the soil pH from ~4.5 to 6.5 – 7.0 (see text).

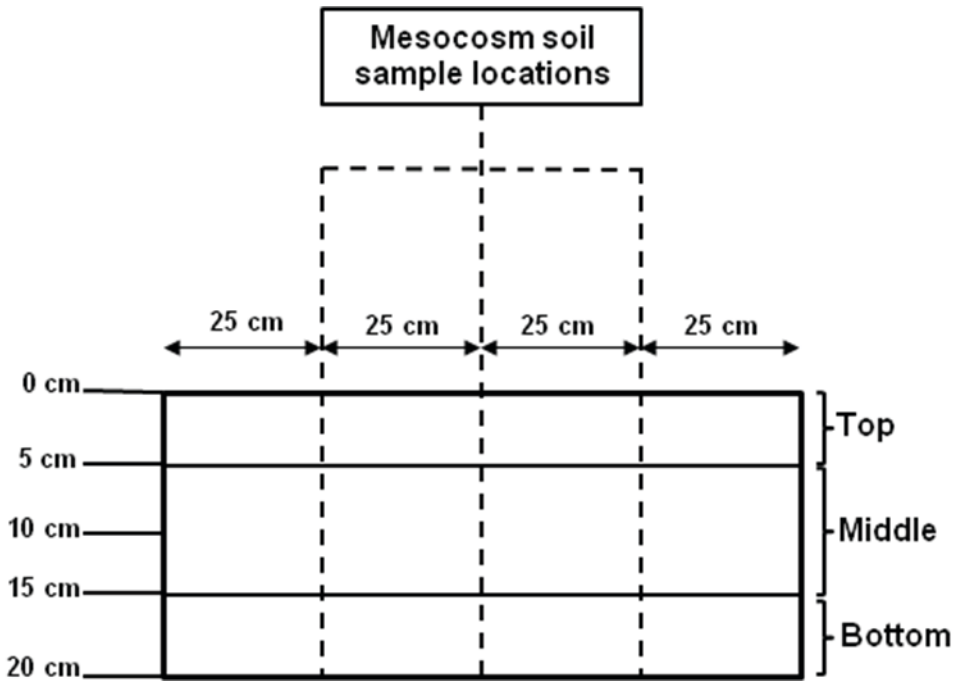

Figure 2.3. Side view schematic of mesocosm tank.

Approximate auger drill sampling locations indicated by dashed lines, taken from with the specific layer range, Top, Middle or Bottom. The Top layer includes the 2 – 3 cm of additional surface soil (Chang et al. 2007).

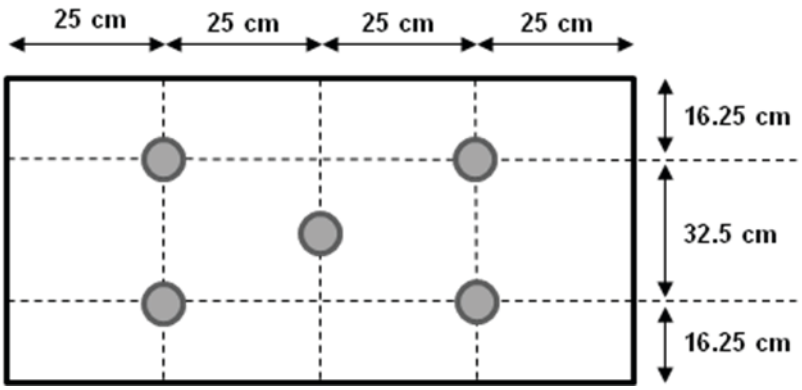

Figure 2.4. Top view schematic of mesocosm tank.

Approximate auger drill sampling locations indicated by circles, drilled to specific depth (Fig. 2.3). Microbial analyses used ~150 g of mesocosm soil, and each auger drill sample recovered 20 – 30 g soil. If more than the five drill locations were necessary to recovery the required soil quantity, additional holes, spaced as equidistant from one another as possible, were drilled (Chang et al. 2007).

Table 2.2. PCR primer sequences, gene targets, references.

Primer	Sequence 5′ to 3′	Target	Reference
341F-GC*	CCT ACG GGA GGC AGC AG	Bacterial 16S rRNA gene	Muyzer et al. (1996)
27F	AGA GTT TGA TCC TGG CTC AG	Bacterial 16S rRNA gene	de la Torre et al. (2003)
758R	CTA CCA GGG TAT CTA ATC C	Bacterial 16S rRNA gene	Woese (1987)
1492R	GGT TAC CTT GTT ACG ACT T	Bacterial 16S rRNA gene	de la Torre et al. (2003)
Euk20F	GTA GTC ATA TGC TTG TCT C	Eukaryotic 18S rRNA gene	Aguilera et al. (2006)
Euk516R-GC*	ACC AGA CTT GCC CTC C	Eukaryotic 18S rRNA gene	Aguilera et al. (2006)
Euk581F	GTG CCA GCA GCC GCG	Eukaryotic 18S rRNA gene	Bower et al. (2004)
Euk1134R	TTT AAG TTT CAG CCT TGC G	Eukaryotic 18S rRNA gene	Bower et al. (2004)
alkB-HIF	CIGIICACGAIITIGGICACAAGAAGG	Alkane monooxygenase	Chénier et al. (2003)
alk-H3R	IGCITGITGATCIIIGTCICGCTGIAG	Alkane monooxygenase	Chénier et al. (2003)
ndoBF	CAC TCA TGA TAG CCT GAT TCC TGC CCC CGG CG	Naphthalene dioxygenase	Whyte et al. (1996)
ndoBR	CCG TCC CAC AAC ACA CCC ATG CCG CTG CCG	Naphthalene dioxygenase	Whyte et al. (1996)
xylEF	GTG CAG CTG CGT GTA CTG GAC ATG AGC AAG	Catachol dioxygenase	Whyte et al. (1996)
xylER	GCC CAG CTG GTG GGT GGT CCA GGT CAC CGG	Catachol dioxygenase	Whyte et al. (1996)
phnAcF	CAA TTA CGG TGA TTT CGT GAC C	PAH dioxygenase	Laurie et al. (1999)
phnAcR	ACA AAA TTC TCT GAC GGC GC	PAH dioxygenase	Laurie et al. (1999)
SP6	CAT TTA GGT GAC ACT ATA G	Cloning vector	Promega**
T7	TAA TAC GAC TCA CTA TAG GG	Cloning vector	Promega**

-GC refers to GC-clamp, with sequence GCG GGC GGG GCG GGG GCA CGC GGG GCG CGG CGG GCG attached on 5′ end only for primers denoted.

Chapter Three. Results

3.1. Soil physical – chemical and catabolic gene analyses

The hydrocarbon contaminated and pristine (uncontaminated) RI soils were found to be mainly sand based, as defined by USDA classification. The particle size distribution percentage of the RI soils was 24% gravel, 75% sand and 1.6% silt/ clay (Chang et al. 2007). The complete physical and chemical analyses for soils received summer 2005 (HC), pristine soil received Oct. 2005 (PSB) and replicate samples of soil received Oct. 2005 (HCB1 and HCB2) are presented in Table 3.1. Initial gravimetric water contents of the site soils ranged from 6% to 11%. Nutrient deficiency, typical of Arctic soils (Tarnocai and Campbell 2002) was seen in the site soils, which had only trace amounts of inorganic nitrogen as nitrate, nitrite and ammonia and phosphorus. Heavy metals, sodium, chloride and total organic carbon were measured, the latter of which was found to be 11000 mg kg^{-1} in pristine soil and more than double that amount in hydrocarbon contaminated soils. The soil pH of the RI soils was found to be naturally acidic, with pristine soil pH ~4.8, and contaminated soil pH ~4.6 (Chang et al. 2007). TPH fractions were determined for the hydrocarbon contaminated soils and are presented in Table 3.2. Volatile fractions (F1), ranging from nC6 to nC10, were not detected in the petroleum hydrocarbon contaminated soils. The most abundant fraction in the contaminated site soil was F2 (nC10 to nC16) of which the concentration ranged from 800 to 1400 mg kg^{-1}. The F3 (nC16 – nC34) and F4 (nC34 – nC50) concentrations ranged from 650 to 840 mg kg^{-1} and from 12 to 43 mg kg^{-1}, respectively. The measured TPH, therefore, ranged from 1464 to 2303 mg kg^{-1}. The results from the lettuce seed germination test, earthworm lethality, and microtox test, conducted at BRI, were negative for the presence of toxic components in the RI soil.

Total community DNA was extracted from HC, HCB and PS soils and amplified by PCR for the presence of four hydrocarbon catabolic genes.

alkB, *phnAc*, *ndoB* and *xylE* were detected in HC soil, while HCB was only positive for *alkB* and *phnAc* and PS was negative for all four.

3.2. Phase one: Biotreatability assessment

3.2.1. ^{14}C-Hydrocarbon mineralization assays

The results of the mineralization assays at 5°C of hexadecane, naphthalene and phenanthrene in the HC, PS and HCB soils are presented in Fig. 3.1. The sterile control microcosms never showed greater %$^{14}CO_2$ recovery levels, or ^{14}C-mineralization activity, than the unsterile, experimental microcosm in all mineralization assays. Overall, phenanthrene showed the lowest amount of mineralization activity, with the maximum levels of ~3.5% and ~3.6%, observed from HC soil supplemented with potassium phosphate and HCB soil supplemented with 20:20:20, respectively. Hexadecane mineralization assays showed ~8.0% ^{14}C-mineralization activity from HC and PS soils, both supplemented with 20:20:20. Slightly lower ^{14}C-mineralization activity levels were observed in assays with hexadecane from HCB soil supplemented with 20:20:20 at ~7.0%, MAP supplemented HC soil at ~6.8%, and $CaCO_3$ supplemented HC and HCB soils, both at ~6.8%. Maximum ^{14}C-mineralization activity levels from the assays with naphthalene were observed from the HCB soil, supplemented with 20:20:20 alone, or 20:20:20 and $CaCO_3$, both reaching levels of ~26.1%. The naphthalene mineralization assays also displayed two results not seen in any other assays. The first were the higher %$^{14}CO_2$ recovery levels seen from both the untreated HC soil at ~20.0% and untreated HCB soil at ~8.0%, and second, the relatively high %$^{14}CO_2$ levels of the sterilized controls, both at ~2.5%. Mineralization assays, at -5°C, ˉ10°C and ˉ15°C, with radiolabeled hexadecane and naphthalene showed activity levels barely above that of the background sterilized control microcosm mineralization assays, after three months (data not shown). These sub-zero microcosm assays were then spiked with 250

ppm 20:20:20 and after three additional months still showed no increase in activity (data not shown).

3.2.2. Microbial enumerations

Microbial viable plate enumerations from phase one (biotreatability assessment) used two different types of media to test for aerobic cells from HC, HCB and PS soils. At 5°C, cell counts ranged from 10^2 to 10^4 CFU g^{-1} soil while at ambient room temperature (~24°C) cell counts ranged from 10^3 to 10^5 CFU g^{-1} soil (Table 3.3). Hydrocarbon degrading microorganisms, cultured on MSM + Arctic diesel fuel, resulted with cell counts of 10^2 to 10^5, while heterotrophic organisms, cultured on R2A, ranged from 10^3 to 10^4 CFU g^{-1} soil (Table 3.3). Additionally, at each temperature, each media was adjusted to pH ~7.0 and pH ~4.5. Cell counts ranged from 10^2 – 10^5 CFU g^{-1} soil (Table 3.3). Extensive investigation into the PS soil was not conducted because focus was directed to the contaminated soils.

The membrane binding DTAF dye was used for direct epifluorescent microscopic total microbial enumerations, the result of which, for the HCB soil, was 5.1×10^8 cells g^{-1} soil.

3.2.3. DGGE analyses

Initial DGGE analyses of the DNA extracted from the RI soil and PCR-amplified for the bacterial 16S rRNA gene resulted in multiple DGGE gels with different banding patterns that allowed some bands to be cut for subsequent sequencing analysis. Visualization limitations prevented all bands that could be viewed digitally from being physically cut and the success of sequencing analysis of individually cut bands depended on the precision of band cuts, productive re-amplification and the sequencing center employed. As a result of these multiple variables, not all bands that appear in gel photos were cut and not all cut bands returned reliable sequence information. Attempts were made to construct DGGE profiles of

the eukaryotic soil community, using 18S rRNA gene primers with a GC-clamp, but sufficient quantities of PCR-product could not be obtained.

HC and PS soils were analyzed by DGGE before any treatment addition or incubation time (T0) and after two months at 5°C without treatment (N/T) or with 20:20:20 commercial fertilizer (20) or potassium phosphate (PO_4) supplementation, as in Fig. 3.2. In the HC soil, after the two month incubation period, fewer bands were observed in comparison to the sample before any treatment (Fig. 3.2). Additionally, one band (indicated in box in Fig. 3.2) that appeared in time 0 enhanced after the two month incubation period, in all treatments. This common band was cut, purified and after successful sequencing analysis, compared to online databases and showed a 94 - 97% homology with the hydrocarbonoclastic genus *Alkanindiges*. After the two month incubation period, the banding patterns from the PS soil were more complex than was observed at time 0, and more complex than any of the HC banding patterns (Fig. 3.2.). A single, clear predominant band did not appear in the PS soil bands, like the one observed in the HC soil, and unfortunately sequencing of cut bands was unsuccessful.

3.3. Phase two: Mesocosom trials

3.3.1. ^{14}C-Hexadecane mineralization assays with mesocosm soils

Microcosm mineralization assays were conducted at 5°C with radiolabeled hexadecane to detect and monitor the hydrocarbon mineralization capacity of the P1 and P2 mesocosm soils. Soils samples tested included all three layers (top, middle and bottom) at all four time points (days 0, 20, 40 and 60) from the P1-treated and P2-untreated mesocosm trials. Hexadecane was chosen over the other radiolabeled substrates because the physical-chemical analyses showed that mid-length alkanes to be the greatest hydrocarbon contingent present in the contaminated RI soil (Table 3.2). The consistent results from these assays indicated, as in the biotreatability assessment mineralization assays (section 3.2.1) that the

sterilized controls had the lowest $\%^{14}CO_2$ recovery, not reaching above 1% (Figs. 3.3, 3.4, 3.5). ^{14}C-hexadecane mineralization in P1 soils from all depths at sample Day 0 and from the P2 soils at all depths and all sample days, remained at less than 1% (Figs. 3.3, 3.4, 3.5). Soils from P1 showed higher levels of mineralization activity as the mesocosm trial progressed, with mild variations between depths (Figs. 3.3, 3.4, 3.5). In the bottom depth, both day 20 and 40 showed ~18.0% ^{14}C-hexadecane mineralization, then increasing to ~52.0% ^{14}C-hexadecane mineralization in the day 60 soil sample (Fig. 3.3). The soils from the middle depth showed ^{14}C-hexadecane mineralization levels of ~25.0% and 18.0% from day 20 and 40, respectively, while the day 60 sample showed ~50.0% ^{14}C-hexadecane mineralization (Fig. 3.4). Soils sampled from the top depth on days 20 and 40 showed ~18.0% ^{14}C-hexadecane mineralization, which increased to about 62.0% from the day 60 soil sample (Fig. 3.5).

3.3.2. Microbial viable plate enumerations

Microbial enumerations for the P1 and P2 mesocosm soils were monitored on MSM-hexadecane plates incubated at 5°C, for all three soil layers at days 0, 20, 40 and 60, as presented in Fig. 3.6. Soil sampled from top, middle and bottom depths, at day 0 from the P1-treated mesocosm resulted in $2.63 \times 10^5 - 3.83 \times 10^5$ CFU g^{-1} soil for all three layers. Additionally, all three layers from P1 from the successive sampling days, 20, 40 and 60 showed counts from 8.92×10^6 - 1.49×10^7 CFU g^{-1} soil. The exception was P1 M40, which resulted in 5.5×10^5 CFU g^{-1} soil, similar to that of day 0. Samples from the P2-untreated mesocosm samples displayed similar results from the top, middle and bottom layers, throughout the 60 day mesocosm trial period, with a range of ~1.0×10^5 CFU g^{-1} soil to ~6.0×10^5 CFU g^{-1} soil.

3.3.3. Total direct microbial microscopic enumerations

A summary of the results for the direct microscopic enumerations for the mesocosm soils is presented in Fig. 3.7. Day 0 samples from both P1 and

P2 mesocosms, at all three sample depths, showed a range of 1 x 10^8 - 2 x 10^8 cells g^{-1} soil from all three sample depths. At day 20, the counts for P1 had increased to 3.7 – 6.2 x 10^8 cells g^{-1} soil for the three layers, while the P2 soils showed a range of 1.8 – 3.8 x 10^8 cells g^{-1} soil. The samples from P1 day 40 ranged from 2.7 – 4.0 x10^8 cells g^{-1} soil and those of P2 ranged from 5.6 x 10^7 – 2.3 x 10^8 cells g^{-1} soil. The results from day 60 were similar to those of day 20 and 40, with an overall higher P1 range of 4.9 – 5.8 x 10^8 cells g^{-1} soil than that of the P2 range of 1.2 – 2.3 x 10^8 cells g^{-1} soil.

3.3.4. DGGE analyses of mesocosm soils

The DGGE results from DNA extracted from the P1 and P2 mesocosms consisted of lanes from all three layers and at days 0, 20, 40 and 60 (Fig. 3.8). The P2 DGGE gel displayed no noticeable change in banding pattern; that is, no bands were observed to appear or disappear for all three layers for the duration of the mesocosm trial. This “P2 banding pattern” was similar to that observed from the P1 top, middle and bottom samples from day 0 (Fig. 3.8). Of note was the appearance of a constant band (labeled B in Fig. 3.8) after day 0 in all layers in the P1-treated gel. Subsequent sequence analysis revealed this band to be 94 – 97% homologous to the genus *Alkanindiges.* Sequencing results from various other bands from the P1 gel were homologous to the genera *Aeromicrobium* (band A), at 95-100%, and *Paenibacillus* (band C), at 98%. Sequenced band D was found to be 95% homologous to the genus *Blastococcus* in both the P1 and P2 DGGE lanes (Fig. 3.8).

The DGGE results from DNA extracted from the composite P3, P4 and P5 mesocosm soils are presented in Fig. 3.9. Overall, it seemed that the level of banding pattern complexity for the P3, P4 and P5 gel decreased after day 0. Again, sequence results from isolated bands produced 95 – 100% homologous matches to the genus *Aeromicrobium* (band A in Fig. 3.9). Sequence results from other bands (labelled E in Fig. 3.9) produced 95%

homologous matches to the genus *Rhodanobacter*. The P4 gel also contained a band (labelled F in Fig. 3.9) that sequence analysis showed to be 95% homologous to the genus *Acidobacteria.* Complete sequence results for all DGGE analyses from all five mesocosms are presented in Table 3.4. Direct comparisons of the P1 and P2 DGGE gel and the P3, P4, P5 DGGE gel could not be reliably analyzed due to poor migration of reference samples in the gels.

3.3.5. Clone libraries

To further investigate the bacterial biodiversity and change in community structure due to mesocosm treatments, bacterial 16S rRNA gene clone libraries were constructed from P1 B0 soil and P1 B60 soil and compared. The P1 B0 soil clone library, which consisted of 74 clones, represented the bacterial community initially present in the untreated RI soil, while the P1 B60 soil clone library, which consisted of 72 clones, represented the shift in bacterial community structure after P1 mesocosm treatment. Sequences were grouped into operational taxonomic units (OTUs) of greater than 97% sequence similarity, the generally accepted cutoff for bacterial species differentiation (Stackebrandt & Goebel 1994); the complete results are listed in Table 3.5. The coverage (Good 1953) and the number of OTUs for the P1 B0 and P1 B60 soil clone libraries were similar in size (Table 3.5). Also calculated for each library were the Chao1 and ACE species richness estimates, and the Shannon-Weaver and Simpson diversity indices (Table 3.5). The richness estimates are also presented as a rarefaction curve in Fig. 3.10. Analysis of the two libraries using the computer program webLIBSHUFF showed the P1 B0 and P1 B60 libraries to be significantly different ($p<0.025$).

Library sequences were compared and closest matches determined by the online databases that showed both libraries were dominated by *Proteobacteria*, which increased 13% from the initial soil library to make up 63% of the mesocosm treated soil library (Fig. 3.11). Added to the

proportional increase of this phylum from one library to the next, was the change of the classes within it. Both the *alpha*- and *gamma*-*Proteobacteria* proportions were reduced after mesocosm treatment, from 30% and 46% in the P1 B0 library to 11% and 29% in the P1 B60 library, respectively (Fig. 3.11). However, the proportion of *beta*-*Proteobacteria* increased from 24% initially to 60% after treatment (Fig. 3.11). Furthermore, the libraries displayed a reduction in the proportions from the phyla *Actinobacteria*, *Firmicutes* and *Acidobacteria*, from 27% - 18%, 20% - 4%, and 3% - 1% respectively. The phylum *Bacteroidetes*, which was not present in the P1 B0 library, did emerge to constitute 14% of the P1 B60 library (Fig. 3.11).

Analysis of the closest BLAST (Altschul et al., 1990) matches for individual sequences from the clone libraries mostly resulted in matches related to uncultured bacterial clones. Of interest were sequences related to hydrocarbon-degrading, cold adapted and acid-tolerant organisms. Also, sequences similar to those from DGGE analyses (*Aeromicrobium, Alkanindiges, Paenibacillus, Blastococcus, Rhodanobacter,* or *Acidobacterium*) were of interest. The P1 B0 library (74 sequences) had, in total, three sequences with BLAST matches from hydrocarbon contaminated environments including a *Nocardioides* sp. 43/14 that was from an investigation into hydrocarbon contaminated soil at Scott Base in the Antarctic (Saul et al. 2005), an *alpha-proteobacterium* from Michigan, USA (Allen et al. 2007) and an unclassified bacterial clone from Rancho La Brea Tar Pits, California, USA (Kim and Crowley 2007). Nine sequences were related to an *Aeromicrobium* from Spitsbergen high Arctic permafrost soil (unpublished) and four were related to uncultured *alpha*-proteobacterial clones from Antarctic terrestrial habitats (Yergeau et al. 2007). Four sequences were related to matches from acidic environments; two uncultured *Acidobacterium* from Southern Piedmont, USA (Kamlesh et al. 2006), an uncultured *gamma*-proteobacterial clone from an acidic uranium contaminated aquifer (Reardon et al. 2004) and an unclassified

bacterial clone from forest wetland impacted with acidic, metal rich, saline runoff (Brofft et al. 2002). Twelve P1 B0 sequences were related to *Paenibacillus*; 10 from marine sediments (Zhao et al. 2007) and one from Mediterranean sapropels (Süß et al. 2004). and one was related to a *Rhodanobacter* isolated from ginseng fields (unpublished).

The P1 B60 library (72 sequences) had one sequence related to an uncultured soil bacterium from Romanian oil-polluted soil (unpublished) and four (all *Sphingobacterium* sp. 44/35) from the hydrocarbon contaminated soil of the Scott Base study (Saul et al. 2005). As for other sequences related to matches from cold environments, three were related to unclassified bacterioplankton from Antarctic freshwater (unpublished), one to an unclassified cold tolerant bacterial clone from Finnish Lapland soil (Männistö and Häggblom 2006), one to an unclassified bacterial clone from Arctic saline springs (Perreault et al. 2007), one to an *alpha*-proteobacterial clone from glacial meltwaters of Mount Everest (Liu et al. 2006), a psychrophilic *Arthrobacter* from a cyanobacterial mat in Lake Vestal located near the Miers and Adams glaciers in Antarctica (Loveland-Curtze et al. 1999), a *Spingobacterium* from the clouds of Puy de Dôme, France (Amato et al. 2007). The 13 clones related to sequences from acidic environments were from acid mine drainage systems from China, England; Sweden and the USA. *Aeromicrobium* or *Rhodanobacter* –related sequences were not found in the P1 B60 library and the numbers of *Paenibacillus* and *Acidobacteria* –related sequences were reduced from 12 and two to three and one, respectively.

3.4. Microbial isolates and enrichment cultures

3.4.1. Biotreatability assessment isolates and enrichment cultures

To gain a basic understanding of the aerobic, viable and culturable microbial population, morphologically distinct colonies from the viable plates were isolated and subjected to DNA extraction and PCR amplification of the 16S or 18S rRNA gene, as previously described. The

ten bacterial and three eukaryotic strains isolated were simply identified by comparison of the 16S or 18S rRNA gene fragments to online databases, the results of which are presented in Table 3.5. Biotreatability assessment DGGE analysis (Fig. 3.2) indicated an organism present in the RI soil with high 16S rRNA gene sequence homology to the hydrocarbonclastic genus *Alkanindiges* and enrichment cultures were started in an attempt to isolate this organism. The single bacterial strain, designated LB.1, isolated from these enrichment cultures had a 96% 16S rRNA gene homology to the genus *Pseudomonas* (Table 3.5). Isolated strains were also not represented in the clone libraries.

3.4.2. Mesocosm isolates and enrichment cultures

Bacterial isolation and enrichment cultures from the mesocosm soil were conducted to attempt recovery of an organism with high 16S rRNA gene homology to the genus *Alkanindiges*, due to molecular evidence of a highly related organism in mesocosm soil (Fig. 3.8). Results indicated that the P1 B20 soil sample was best suited for both isolation and enrichment because of the possible dominance of the *Alkanindiges*-like organism in this sample. In total, eight bacterial isolates were recovered, based on the criteria previously mentioned. The enrichment cultures initially yielded six bacterial isolates, but DGGE analysis showed a single band from these isolates that migrated to the same position in the DGGE gel (not shown), and subsequent sequencing and analysis of these six bands revealed identical DNA sequences. A single representative isolate was designated MD.1. The seven additional isolates, designated MD.2, MD.3, MD.4, MD.5, MD.6, MD.7 and MD.9, were isolated from spread plating of diluted P1 B20 soil sample onto MSM-Arctic diesel plates, as previously described. These isolates were additionally tested for sub-zero growth, using modified media (section 2.5). All isolates grew on all media types at room temperature (~24°C). Isolate MD.2 displayed growth at -5°C on MSM-Arctic diesel (1.35 M NaCl). Unfortunately all plates incubated at -10°C and -15°C froze, and growth could not be scored. Isolates MD.1,

MD.2, MD.3, MD.7 and MD.9 grew on MSM pH 4.5-Arctic diesel plates at room temperature. Approximately 800 bp of 16S rRNA gene sequence were recovered from each isolate and used to determine the closest BLAST match results (Table 3.6), and these sequences were not represented in the clone libraries. Phylogenetic relationships show isolates MD.1, MD.2 and MD.9 to cluster closely together with *R. erythropolis, R. erythreus* type strains and the cold-adapted hydrocarbon degrader *Rhodococcus* Q15 (Whyte et al. 1998) (Fig 3.12). Isolate MD.4 clustered closely with *R. corynebacteriodes* type strain and isolates MD.6 and MD.7 clustered with *Arthrobacter globiformis* type strain and *Mycobacterium cosmeticum* type strain, respectively. Isolate MD.5 did not branch closely with any type strain, but clustered with type strains from the genera *Afipia, Rhodopseudomonas, Agromonas, Bradyrhizobium,* and *Nitrobacter*, all members of the *Bradyrhizobiaceae* family (Fig. 3.13).

Table 3.1. Physio-chemical analyses of RI soil.

Test	Unit	HC	PSB	HCB1	HCB2	DL
Moisture	%	11	6	10	10	N/A
pH	pH	4.59	4.83	4.63	4.62	N/A
Cadmium (Cd)	mg/kg	ND	ND	ND	ND	0.5
Chrome (Cr)	mg/kg	27	22	23	24	2
Copper (Cu)	mg/kg	67	80	62	64	2
Lead (Pb)	mg/kg	ND	ND	6	6	5
Nickel (Ni)	mg/kg	72	74	54	56	1
Zinc (Zn)	mg/kg	43	50	32	33	10
Nitrate & Nitrite	mg/kg	ND	0.5	ND	ND	0.2
Ammonia (NH_3)	mg/kg	ND	ND	ND	ND	5
Organic Carbon	mg/kg	23000	11000	27000	24000	500
Phosphorus (P)	mg/kg	200	170	210	210	10
Sodium (Na)	mg/kg	180	170	120	130	10
Chlorine (Cl)	mg/kg	15	5.3	5	5.2	0.5

Soil received summer 2005 (HC), pristine RI soil received Oct. 2005 (PSB), hydrocarbon contaminated soil received Oct. 2005 (HCB1 and HCB2 are replicate samples). DL = Detection limit; N/A = Not applicable; ND = Not detected. (Chang et al. 2007).

Table 3.2. Hydrocarbon constituent analysis of HCB RI soil.

TPH Fractions	**mg kg^{-1}**
F1 (C6 – C10)	ND
F2 (C10 – C16)	800 - 1400
F3 (C16 – C34)	650 - 860
F4 (C34 – C50)	12 – 43
PAH 2- and 3-ring	Less than 0.1

Total petroleum hydrocarbons (TPH) divided into four fractions (F1, F2, F3, F4) and corresponding alkane chain lengths indicated. Polycyclic aromatic hydrocarbons (PAH). ND = Not detected. (Chang et al. 2007.).

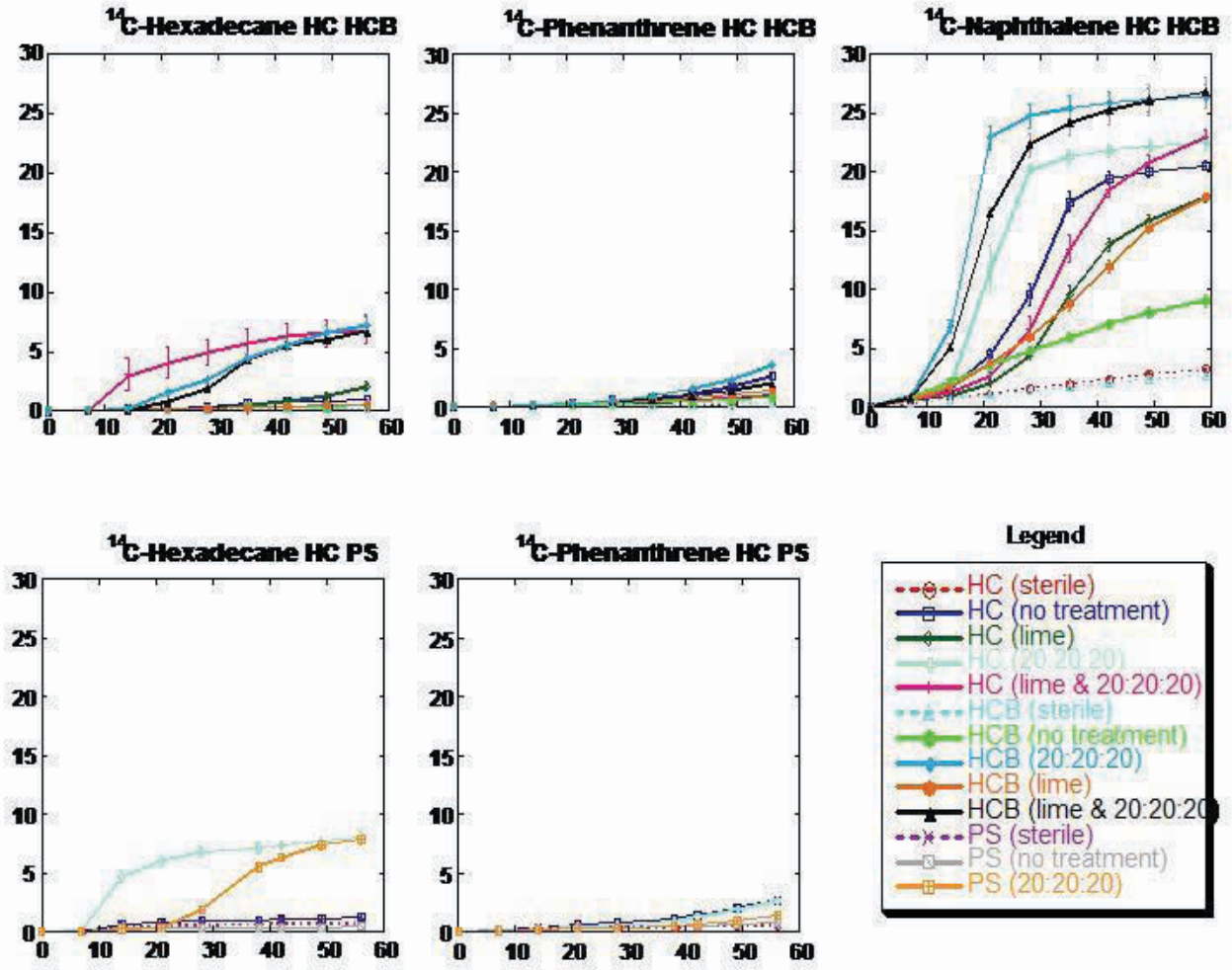

Figure 3.1. Biotreatability assessment mineralization assays.

Mineralization assays are represented in individual graphs with the radiolabeled substrates (^{14}C-hexadecane, ^{14}C-phenanthrene and ^{14}C-naphthalene) and soils (HC, HCB or PS) indicated above. All assays incubated at 5°C for two months. Specific soil treatments, as indicated in the Legend, were prepared as in section 2.3. Each point represents the mean cumulative percent $^{14}CO_2$ from triplicate assays, and the standard error is presented as bars. PS spiked with ^{14}C-naphthalene was not conducted.

Table 3.3. Enumerations of viable aerobic, heterotrophic bacteria from RI soils.

Media (Temp.)	HCB	HC	PS
MSM + diesel (5°C)	1.1×10^4	9.5×10^3	2.1×10^2
MSM + diesel (24°C)	5.3×10^4	2.7×10^4	-
MSM pH 4.5 + diesel (5°C)	7.3×10^3	2.4×10^2	-
MSM pH 4.5 + diesel (24°C)	1.0×10^5	4.1×10^4	-
R2A (5°C)	5.0×10^4	1.2×10^4	7.0×10^3
R2A (24°C)	9.8×10^4	7.3×10^4	-
R2A pH 4.5 (5°C)	2.4×10^3	1.6×10^3	-
R2A pH 4.5 (24°C)	8.6×10^4	4.1×10^3	-

Soil received summer 2005 (HC), pristine RI soil received summer 2005 (PS), and hydrocarbon contaminated soil received Oct. 2005 (HCB). All enumeration values are presented in CFU g^{-1} soil. After appropriate dilution series, cells were plated on mineral salts medium with neutral pH (MSM) or pH ≈ 4.5 (MSM pH 4.5) and supplemented with Arctic diesel as the sole carbon source. R2A culturing media at neutral pH or pH ≈ 4.5 were also used. Incubation temperature as indicated (Temp.).

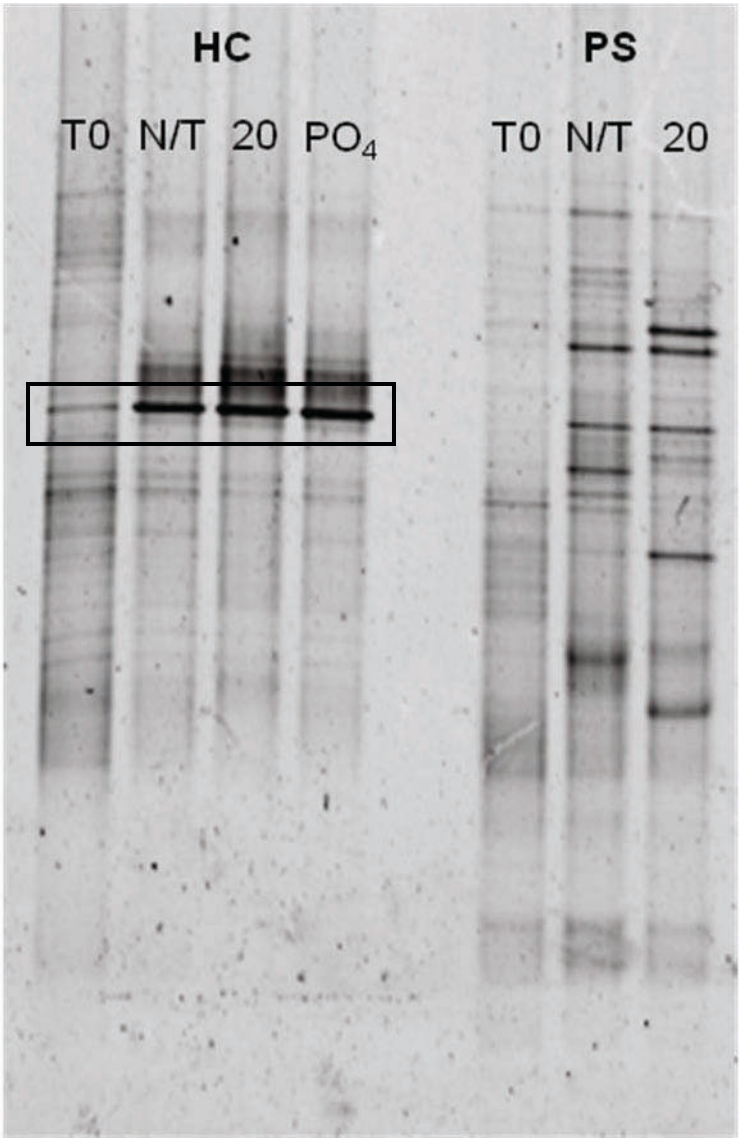

Figure 3.2. Biotreatability assessment DGGE analysis of HC and PS soils.

DNA extracted from soil, and PCR-amplified for 16S rRNA gene, without treatment or incubation (T0), and after eight weeks of incubation at 5°C without supplementation (N/T) or supplemented with commercial fertilizer 20:20:20 (20) or potassium phosphate (PO_4) (see text). Box indicates bands isolated from gel, re-amplified by PCR and successfully sequenced, whose sequences matched to the genus *Alkanindiges* with 94 - 97% homology from online databases. No bands from PS soil were successfully re-amplified. Approximately 500 ng of DNA was loaded into each lane. Denaturant gradient and electrophoresis conditions as described in section 2.3.5.

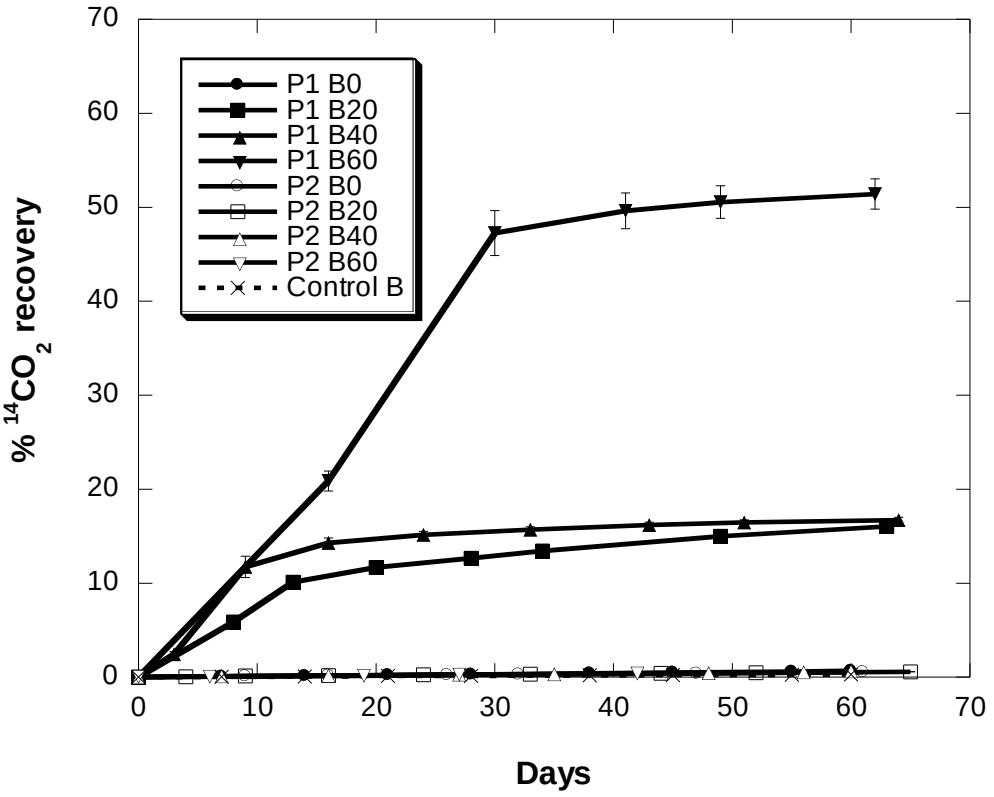

Figure 3.3. ^{14}C-hexadecane microcosm assays from the P1 and P2 mesocosm trials from the bottom (B) layer.

Values for P1 B0 (closed circle), P2 B0 (open circle), P2 M20 (open square), P2 B40 (open up-triangle), P2 B60 (open down-triangle) and the sterilized Control B (x) assays had <1% $^{14}CO_2$ recovery. P1-treated and P2-untreated mesocosms. Error bars are the standard error of the mean of triplicate assays.

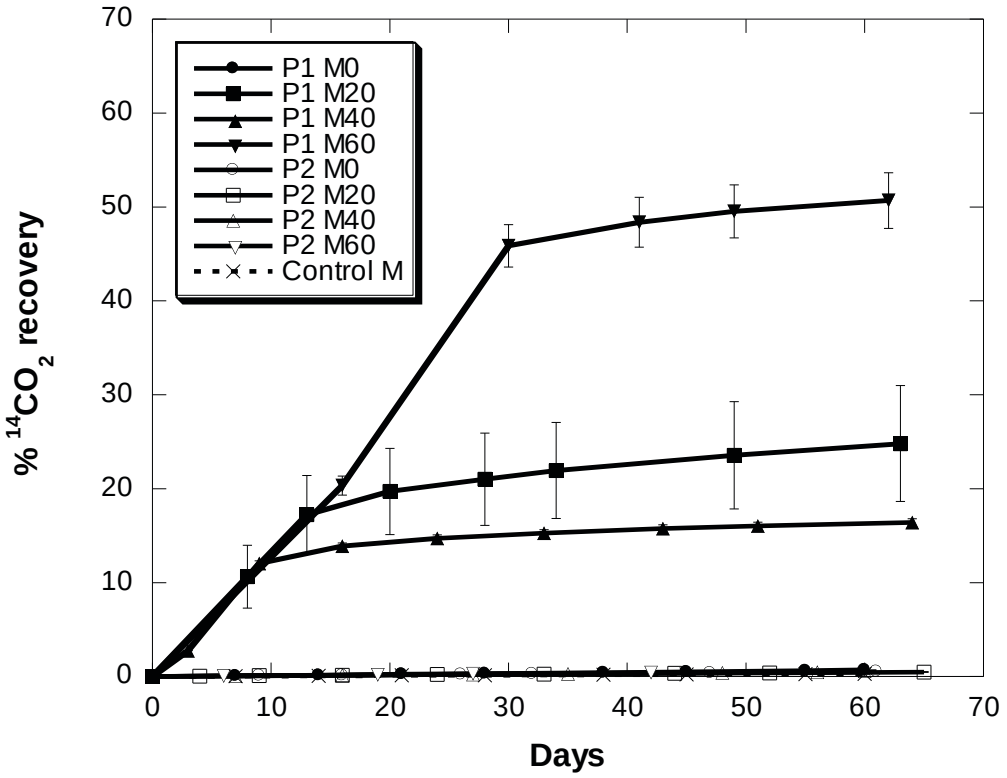

Figure 3.4. ^{14}C-hexadecane microcosm assays from the P1 and P2 mesocosm trials from the middle (M) layer.

Values for P1 M0 (closed circle), P2 M0 (open circle), P2 M20 (open square), P2 M40 (open up-triangle), P2 M60 (open down-triangle) and the sterilized Control M (x) assays had <1% $^{14}CO_2$ recovery. P1-treated and P2-untreated mesocosms. Error bars are the standard error of the mean of triplicate assays.

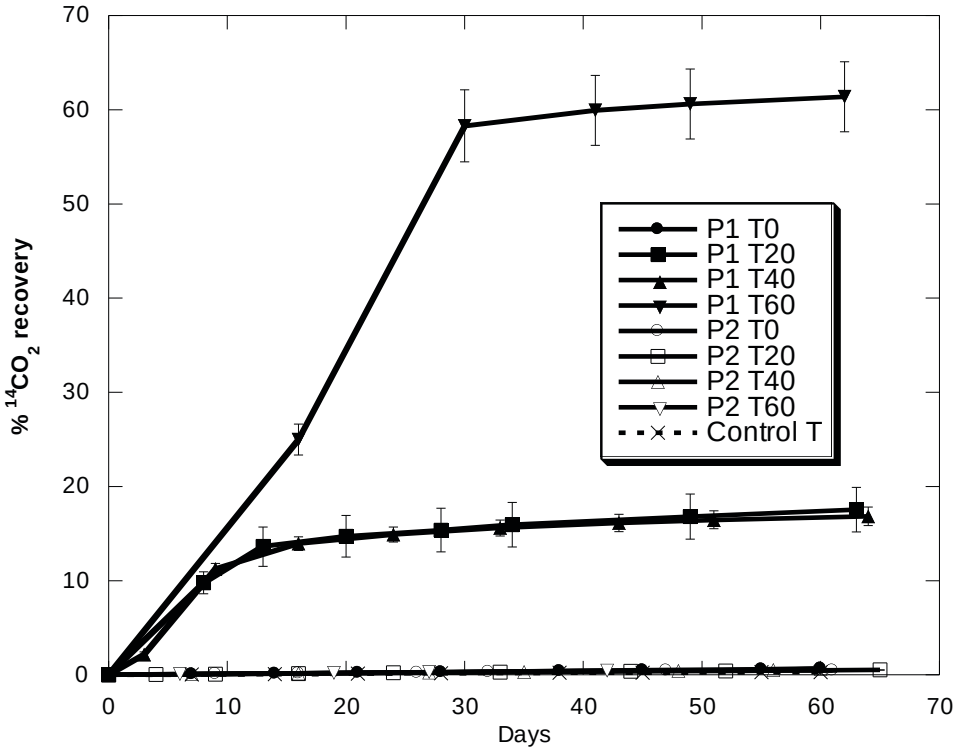

Figure 3.5. ^{14}C-hexadecane microcosm mineralization assays from the P1 and P2 mesocosm trials from the top (T) layer.

Values for P1 T0 (closed circle), P2 T0 (open circle), P2 T20 (open square), P2 T40 (open up-triangle), P2 T60 (open down-triangle) and the sterilized Control T (x) assays had <1% $^{14}CO_2$ recovery. P1-treated and P2-untreated mesocosms. Error bars are the standard error of the mean of triplicate assays.

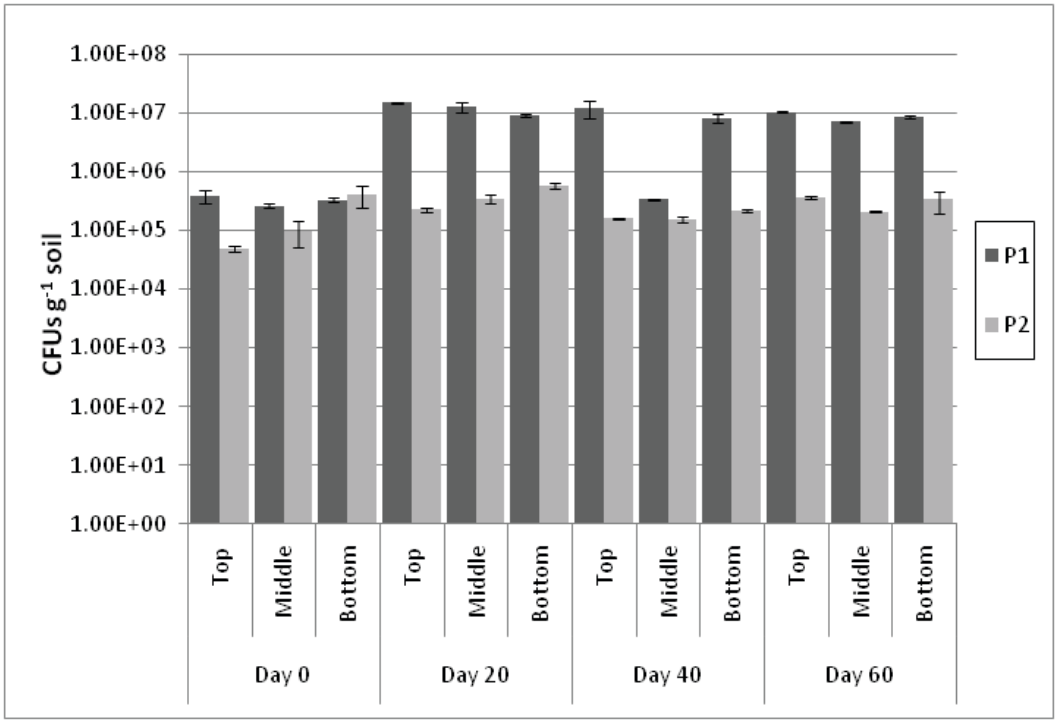

Figure 3.6. Microbial viable plate enumerations from mesocosm P1 and P2.

Diluted soil from the treated-P1 or untreated-P2 mesocosm was diluted and spread on MSM-Arctic diesel plates, and incubated for two months at 5°C (section 2.3.6). Error bars represent the standard error of the mean for triplicate assays.

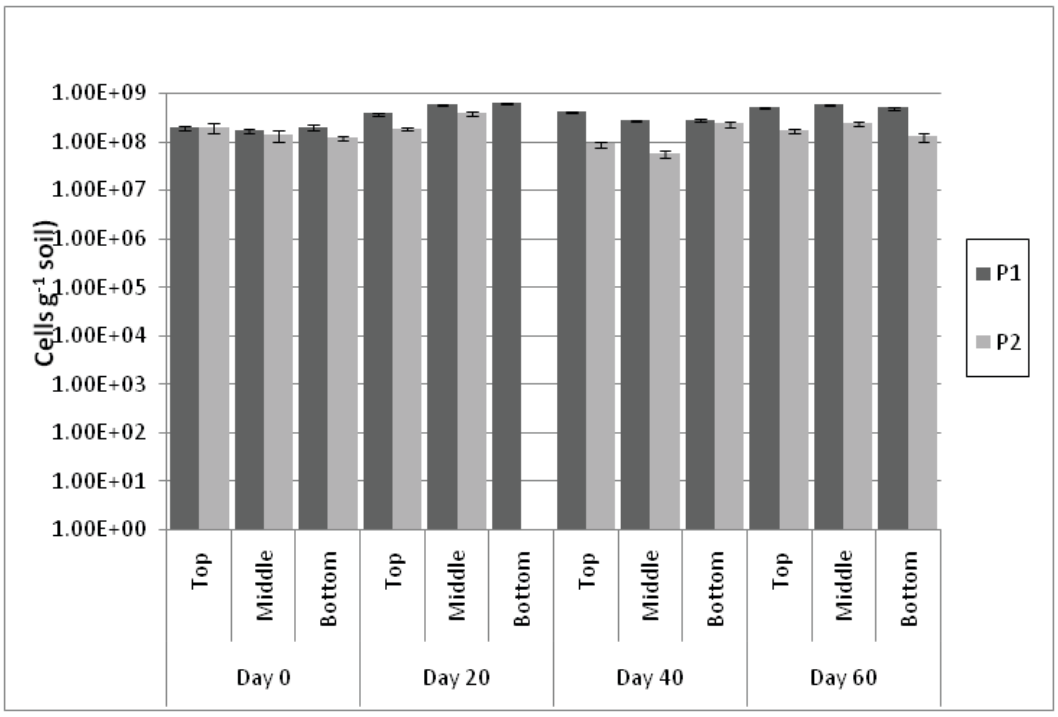

Figure 3.7. Total direct microbial microscope enumerations from mesocosms P1 and P2.

The membrane binding dye DTAF was used for staining of live and dead microbial cells in soil samples (section 2.3.2). Error bars represent the standard error of the mean of 10 microscope fields counted. No value for P2 B20 is presented due to technical difficulties encountered with that sample.

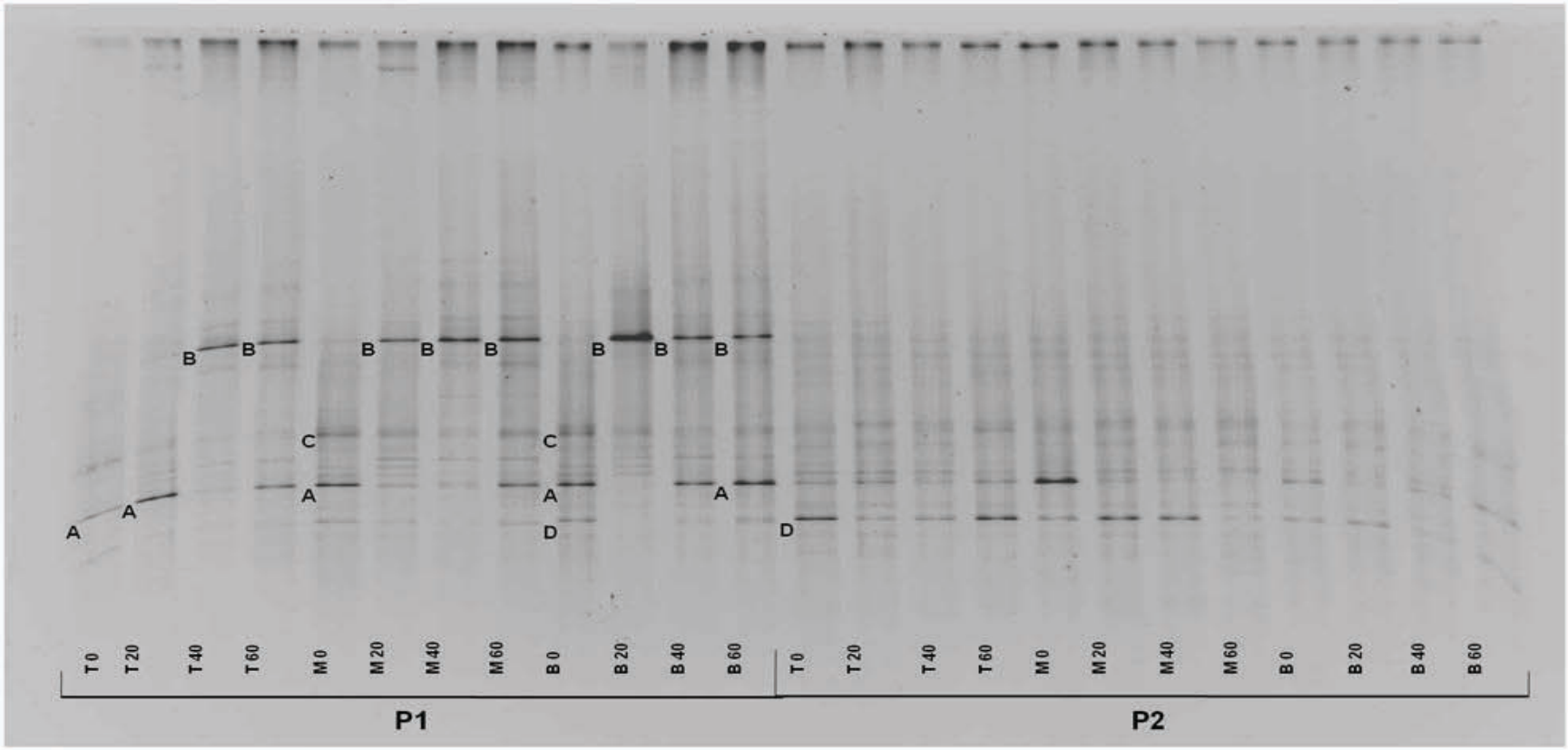

Figure 3.8. DGGE analysis of P1 and P2 mesocosm soils.

DNA extracted from all four sample days (0, 20, 40, 60) and all three layers (Top, T; Middle, M; Bottom, B) were loaded into individual wells, as indicated. Letters (A, B, C, D) indicate bands isolated from gel, re-amplified by PCR and successfully sequenced. Closest sequence matches are presented in Table 3.5. Approximately 500 ng of DNA was loaded into each lane.

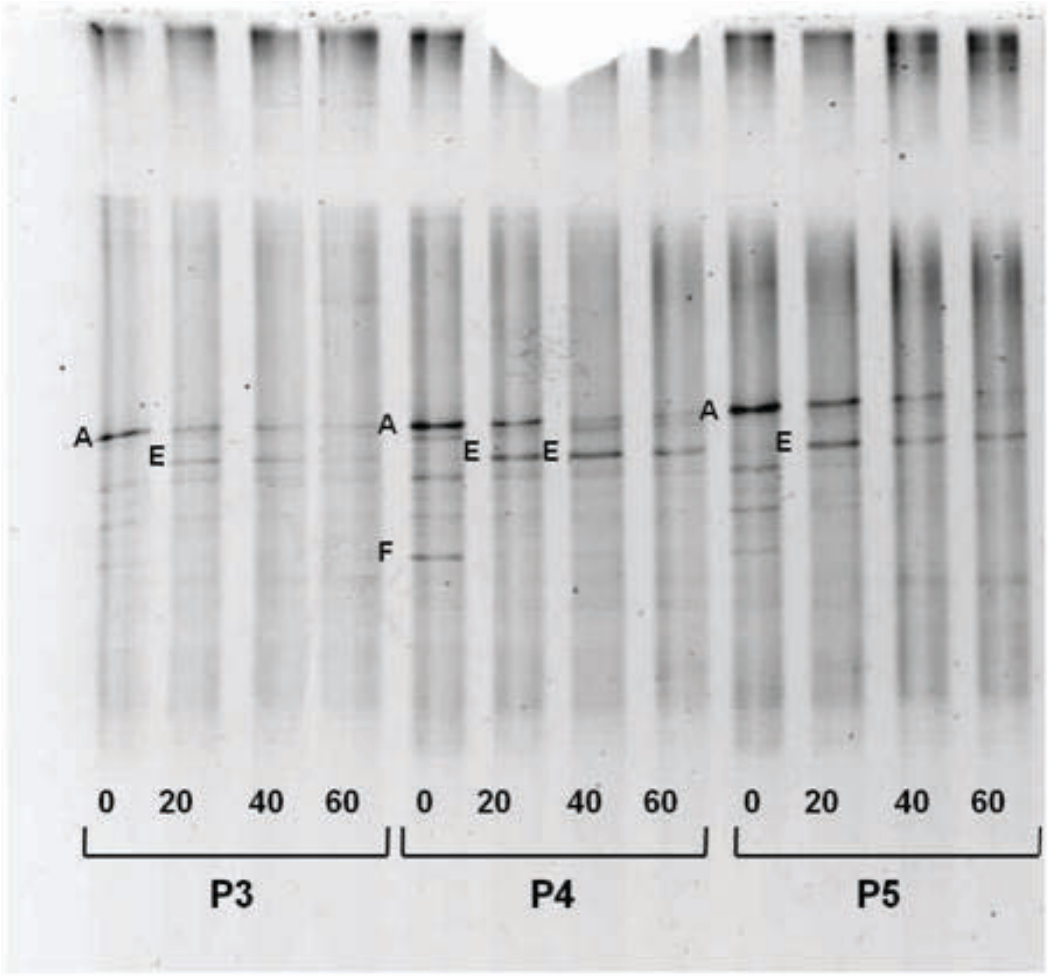

Figure 3.9. DGGE analysis of composite soils sampled from mesocosms P3, P4 and P5 from sample days 0, 20, 40, 60.

Composite samples composed of 4 g of soil from each layer (Top, Middle and Bottom), from which DNA extraction was conducted. Letters (A, E, F) indicate bands isolated from gel, re-amplified by PCR and successfully sequenced. Closest sequence matches are presented in Table 3.5. Approximately 500 ng of DNA were loaded into each lane.

Table 3.4. Sequence information for mesocosm DGGE analyses (Figs. 3.8, 3.9).

DGGE Band	Closest Match (% similarity)	Hydrocarbon Substrates	References
A	*Aeromicrobium* (95% -100%)	crude oil	Chaillan *et al.* (2004)
B	*Alkanindiges* (94 – 97%)	hexadecane, heptadecane, pristane, squalane	Bogan *et al.* (2003)
C	*Paenibacillus* (98%)	naphthalene, phenanthrene	Daane *et al.* (2002)
D	*Blastococcus* (95%)	-	-
E	*Rhodanobacter* (95%)	concomitant growth in consortium grown on diesel	Kanaly *et al.* (2002)
F	*Acidobacteria* (95%)	-	-

Successfully sequenced DGGE bands, labelled A – F from Figs. 3.8 & 3.9, and the closest genus level match from online databases. Hydrocarbons substrates listed correspond to information from the listed references as those utilized by the respective organism.

Table 3.5. Statistical tests for the P1 B0 and P2 B60 clone libraries.

Test	P1 B0	P1 B60
Library Coverage (%)	85.14	86.11
OTUs	24	22
Chao1 Richness Estimate	54.33	29.00
ACE Richness Estimate	52.11	29.57
Shannon's Diversity Index (H')	2.72	2.76
Simpson's Diversity Index (1/D)	13.18	14.12

Library coverage calculated as defined by Good et al. (1953). OTUs, Chao1, ACE, Shannon and Simpson tests calculated by the DOTUR computer program. All values presented were calculated for greater than 97% homology between sequences.

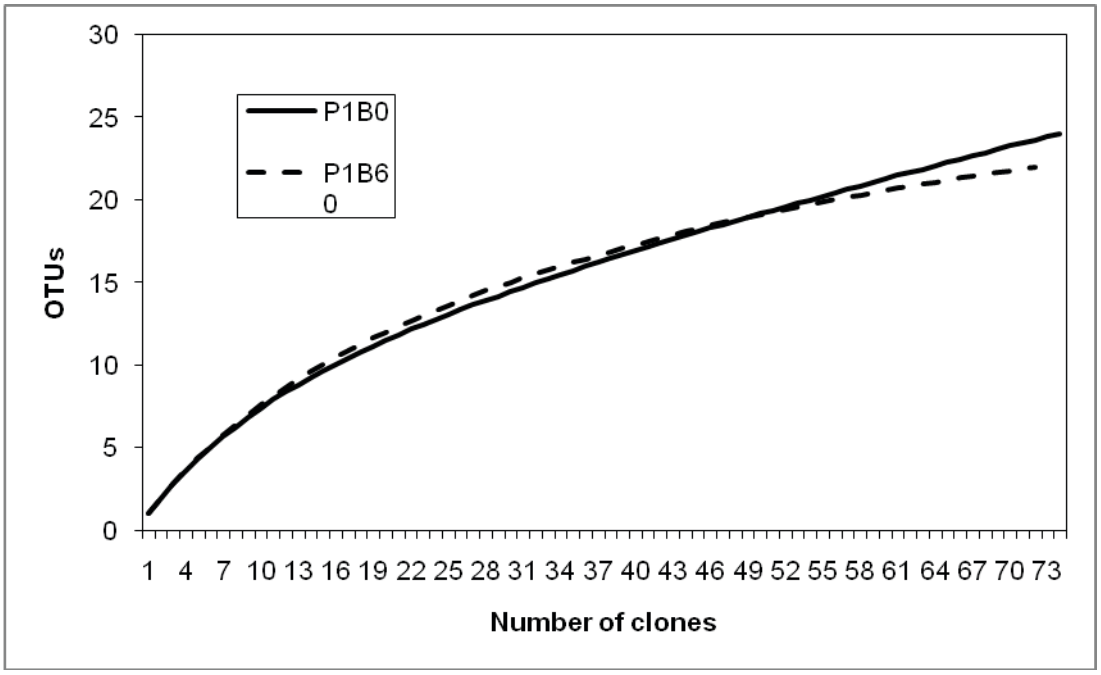

Figure 3.10. Rarefaction curve of clone libraries from P1 mesocosm.

Operational taxonomic units (OTUs) were defined as having ≥97% DNA sequence homology and rarefaction curves calculated by the computer program DOTUR (section 2.4.3).

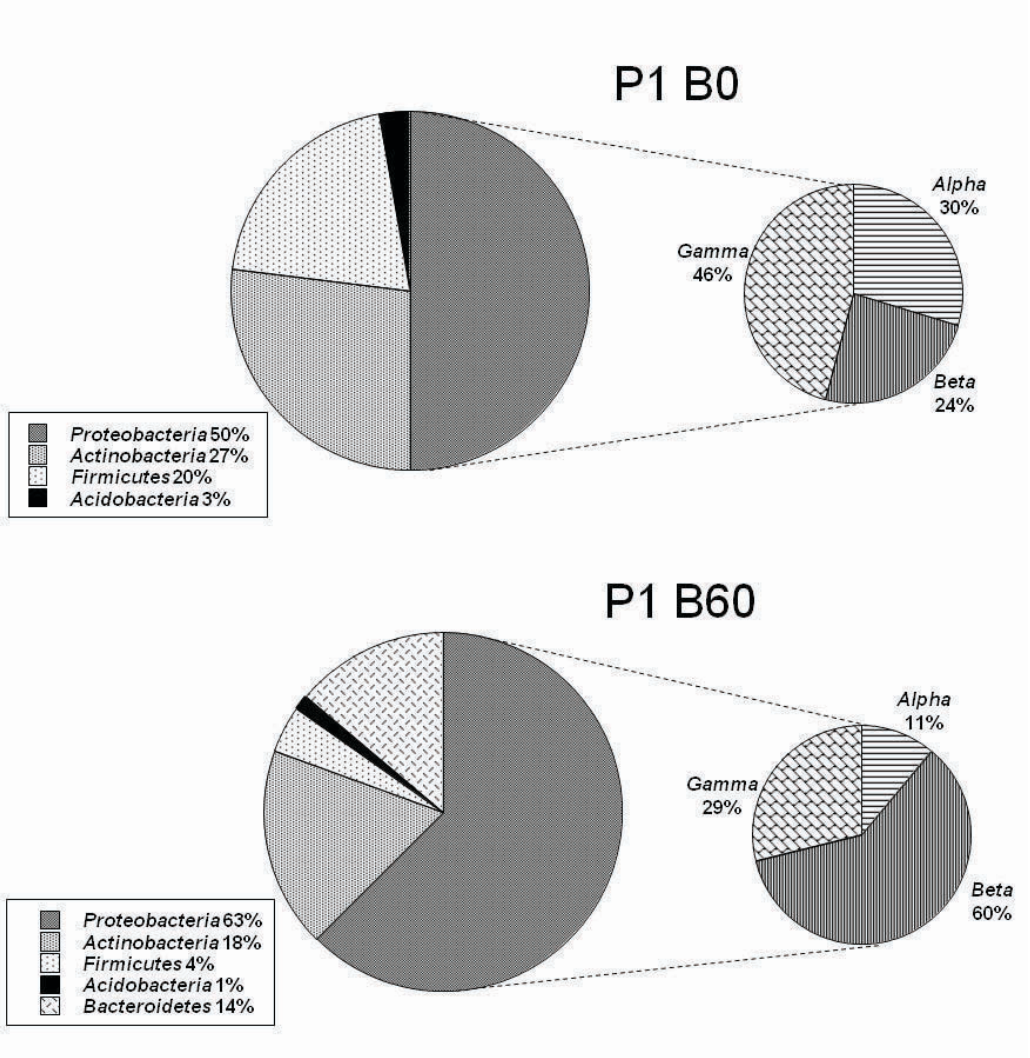

Figure 3.11. Phylotype composition of the two clone libraries from P1 treated mesocosm.

For each library, the larger circle represents the proportion of phylotypes as a percentage of the total library while the smaller inset circle represents the proportion of *Proteobacteria* classes as a percentage of *Proteobacteria* related clones.

Table 3.6. Microbial strains isolated during the present study.

Isolate	Closest BLAST matches (% homology; class)	BLAST Match Notes	Accession
MD.1	*Rhodococcus erythropolis* strain 5 (100%; *Actinobacteria*)	petroleum-degrading strain	EF362636
MD.2	*Rhodococcus erythropolis* strain 5 (96%; *Actinobacteria*)	petroleum-degrading strain	EF362636
MD.3	*Rhodococcus* sp. OS-20 (99%; *Actinobacteria*)	Isolated from lead-zinc mine tailings site	EF612316
MD.4	*Rhodococcus* sp. OS-11 (97%; *Actinobacteria*)	Isolated from lead-zinc mine tailings site	EF612310
MD.5	*Rhodopseudomonas* sp. ORS 1416ri (98%; *alpha-proteobacteria*)	Isolated from root nodule of *O. natrix* subsp. *Falcata*	AJ968691
MD.6	*Arthrobacter* sp. KFC-78 (99%; *Actinobacteria*)	Isolated from soil sample from Kafni Glacier in the Himalayas	EF459540
MD.7	*Mycobacterium* sp. RODSPM7 (99%; *Actinobacteria*)	Isolated from high Arctic permafrost soil in Spitsbergen, Norway	EF451723
MD.9	*Rhodococcus erythropolis* strain 5 (100%; *Actinobacteria*)	petroleum-degrading strain	EF362636
isoHC1	*Arthrobacter* sp. OS-31 (100%; *Actinobacteria*)	Isolated from lead-zinc mine tailings site	EF612321
isoHC2	*Cryobacterium* sp. RODSPM5 (98%; *Actinobacteria*)	Isolated from high Arctic permafrost soil in Spitsbergen, Norway	EF451721
isoHC3	*Burkholderia glathei* isolate Hg 5 (98%; *beta-proteobacteria*)	Naphthalene-degrader isolated from soil	AY154370
isoHCB1	*Pseudomonas* sp. WR7#2 (86%; *gamma-proteobacteria*)	Isolated from alpine tundra soil, CO, USA	AY263480
isoPS1.1	*Oxalobacter* sp. HI-D2 (96%; *beta-proteobacteria*)	Isolated from limestone cave rock surface, AZ, USA	DQ196473
isoPS1.2	*Oxalobacter* sp. HI-D2 (98%; *beta-proteobacteria*)	Isolated from limestone cave rock surface, AZ, USA	DQ196473
isoPS4.2	*Beta proteobacterium* KIT2S2K (97%; *beta-proteobacteria*)	Isolated from alpine stream water, Lapland Finland	DQ234470
isoPS6	*Streptomyces* sp. A00099 (97%; *Actinobacteria*)	Endophyte from pharmaceutical plants	EF690224
isoPS7	*Polaromonas* sp. P6E3 (97%; *beta-proteobacteria*)	Isolated from wheat rhizosphere	AM492164
isoPS8	*Sphingomonas* sp. P5-21 (100%; *alpha-proteobacteria*)	Isolated from acid mine drainage, Keumsan, Korea	AB288314
LB.1	*Pseudomonas* sp. CL16 (96%; *gamma-proteobacteria*)	strain can produce cold active lipase	AY342005
HCB1pH	*Cystofilobasidium infirmominiatum* isolate AFTOL-ID 1888 (100%; Dikarya)	Yeast	DQ645524
HCB2pH	*Cryptococcus gastricus* isolate AFTOL-ID 1887 (98%; Dikarya)	Yeast	DQ645513
HCB3pH	Uncultured *Tremellaceae* clone Amb_18S_1097 (97%; Dikarya)	Yeast	EF023503

Isolated strains were subjected to DNA sequencing for bacterial 16S or eukaryal 18S partial genes and compared to online databases (see text). Closest BLAST matches are presented and available information provided by NCBI GenBank on the closest matches, and the GenBank accession numbers are presented.

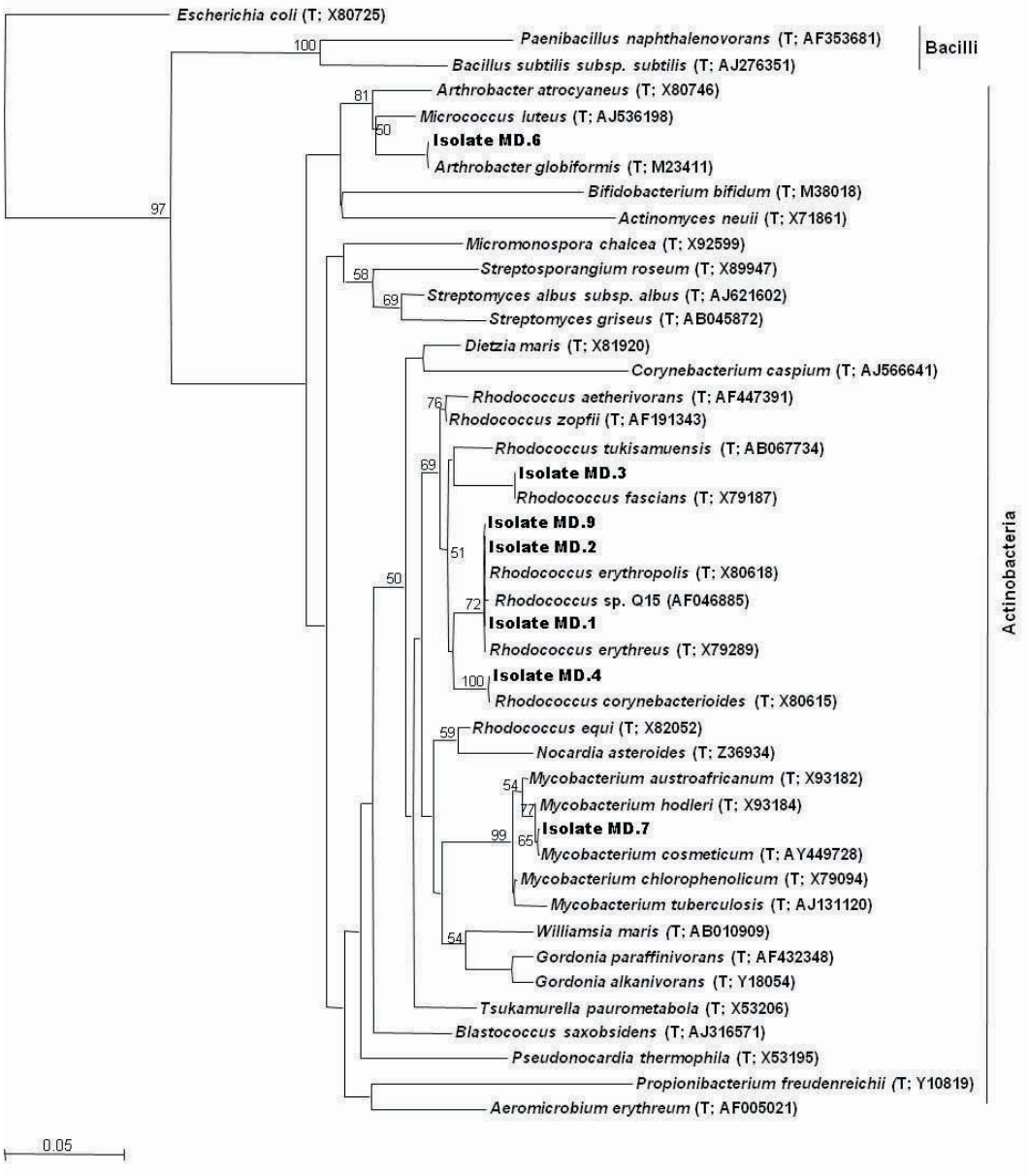

Figure 3.12. Phylogenetic tree of *Actinobacteria*-related isolated strains.

Isolates MD.1, MD.2, MD.3, MD.4, MD.6, MD.7 and MD.9 are presented with sequences from the RDP database. Type species (T) and GenBank accession numbers are indicated. Trees constructed by neighbor joining, best fit trees with Jukes-Cantor correction and bootstrap values, of >50, from 1000 replicates, are indicated at nodes. The scale bar represents the expected number of changes per nucleotide position.

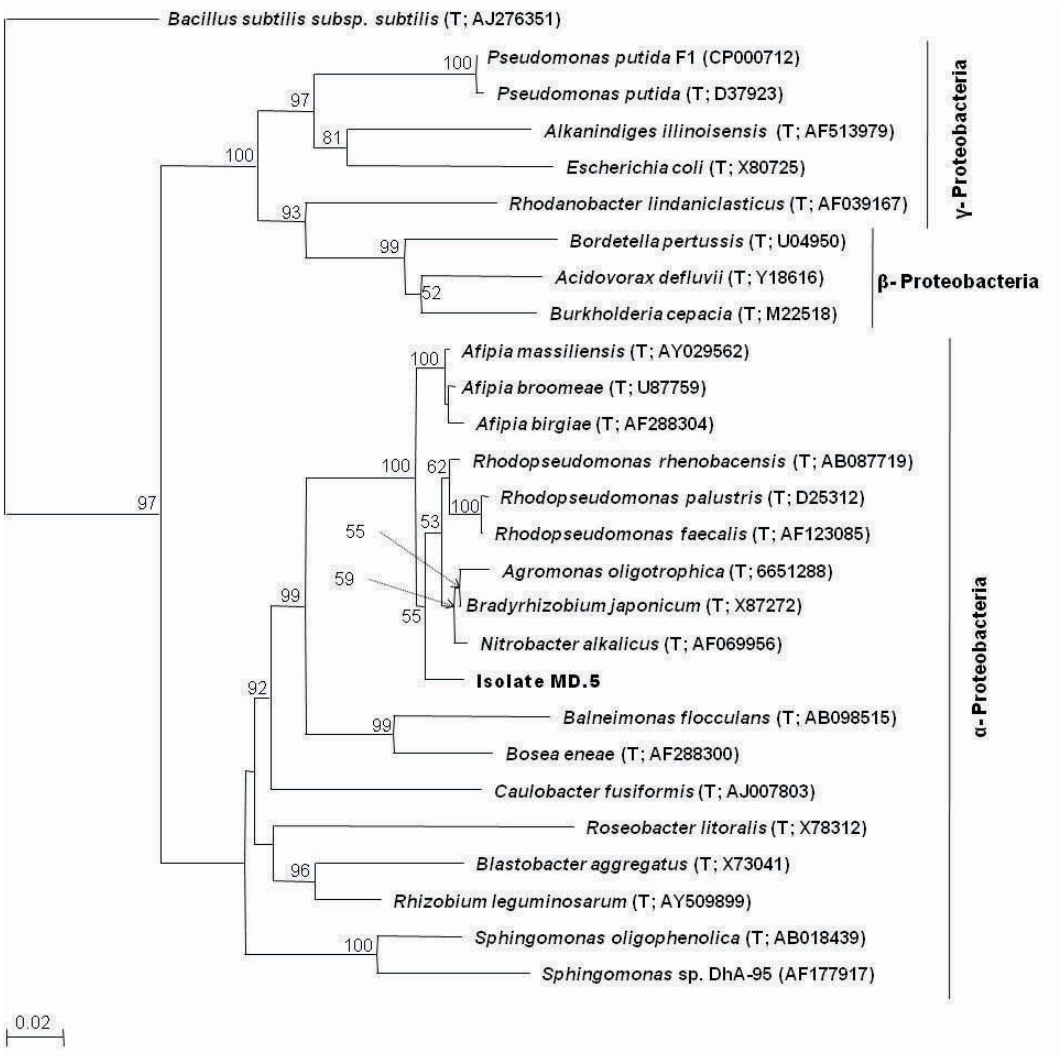

Figure 3.13. Phylogenetic tree of *Proteobacteria*-related isolated strain.

Isolates MD.5 is presented with sequences from the RDP database. Type species (T) and GenBank accession numbers are indicated. Trees constructed by neighbor joining, best fit trees with Jukes-Cantor correction and bootstrap values, of >50, from 1000 replicates, are indicated at nodes. The scale bar represents the expected number of changes per nucleotide position.

Chapter Four. Discussion and Conclusions

This research shows the potential for the bioremediation of petroleum hydrocarbon contaminated soil around the former military radar base on RI, Nunavut. Different soil treatments were tested at the microcosm scale and, the most favorable conditions were then successfully used at the mesocosm scale. This scale up process yielded practical information that can later be translated for more efficient and effective field implementation at RI. Additionally, insight into the contaminated RI soil microbial community was gained through culture-dependent and –independent analyses.

4.1. Phase one: Biotreatability assessment

The initial small scale experiments were conducted to test the biotreatability potential of the RI soil at 5°C. The physical and chemical characteristics of the RI soil indicated two areas of concern for biodegradation; the low nutrient level and the acidic pH of the RI soil. TPH fraction analysis of the RI soil showed that it was contaminated with F2 and F3 alkanes, and an absence of detectable PAH in the system. The total community DNA extracted from the RI soils was analyzed for bacterial genes known to be involved in hydrocarbon biodegradation. However, the catabolic gene detection results did not completely predict mineralization ability as some genes were not amplified from soils that showed mineralization in the microcosm assays. This could have been due to one, or a combination of the reasons mentioned in section 1.3.1, and could include insufficient DNA extraction and purification from soil, primer bias or sub-optimal PCR conditions. Also, the four catabolic genes tested in this study represent a small sample of the numerous hydrocarbon degradation genetic pathways that exist in the environment, as mentioned in section 1.3.2. Ultimately, it was the end mineralization results that would predict bioremediation potential.

The mineralization assays with HC, HCB and PS soils, that addressed the nutrient level and pH, showed enhanced ^{14}C-hydrocarbon mineralization activity, when compared to the sterilized control assays. Additionally, these treated mineralization assays showed enhanced ^{14}C-hydrocarbon mineralization activity when compared to non-treated assays, with %$^{14}CO_2$ levels similar to the sterilized control assays. ^{14}C-Phenanthrene mineralization activity was the lowest of the radiolabeled substrates tested, roughly half of the maximum ^{14}C-hexadecane mineralization activity, from all three soils after addition of 20:20:20, lime or both. Microcosms spiked with ^{14}C-naphthalene showed the greatest mineralization activity of any of the substrates, reaching >20% for multiple treatments in HC and HCB soils. These results were all relatively encouraging; soil treated with fertilizer and lime improved the hydrocarbon biodegradation activity of the RI soils. However, these results were overall less than expected in comparison to similar studies that have found greater hydrocarbon mineralization activity, especially for hexadecane and naphthalene (Whyte et al. 1999; Børresen et al. 2003; Børresen et al. 2007). For example, in a mineralization assay with three hydrocarbon contaminated Arctic soils, each amended with 20:20:20 fertilizer and incubated at 5°C for ~45 days, ^{14}C-hexadecane mineralization activity levels ranged from ~15 - 30% and ^{14}C-naphthalene mineralization levels ranged from ~50 - 65% (Whyte et al. 2001). The low mineralization levels of ^{14}C-phenanthrene may have been due to the increasing difficulty microorganisms generally have with breaking down larger PAH molecules (Huesemann 1995) or some hindrance in the bioavailability of phenanthrene in the soil matrix (Ping et al. 2006).

An explanation for the relatively low hydrocarbon mineralization activities for the small scale biotreatability assays, even when amended with nutrients, could have been because of the relatively low viable microbial populations, ranging from 10^2 – 10^5 CFU g^{-1} soil. Other studies of microbial viable counts from hydrocarbon impacted Arctic soils have

shown a range of 10^5 – 10^8 CFU g^{-1} soil (Berthelet et al. 1996; Braddock et al. 1997; Juck et al. 2000). Additionally, investigation of the possible presence of toxins in the RI soils, which could inhibit microbial growth or metabolism, was conducted. The soil toxicity results showed an absence of toxicity in the RI soil. This may be due to the high total organic carbon (TOC) content (>20,000 ppm) in the hydrocarbon impacted soils where the TPH concentration was only 10% of the TOC. Humic acids are likely a large constituent of the soil TOC and could be inhibitory for microbes, as Loffredo et al. (2007) found with two fungal strains. Anesio et al. (2005) observed inhibition of bacterial growth in lake water due to photodegradation of humic substances into H_2O_2, though it remains unclear if this would occur in a soil system. Nonetheless, these assays established that nutrient and lime addition stimulated a cold-adapted microbial population in the RI soils capable of hydrocarbon biodegradation.

4.2. Phase two: Mesocosm trials

The information gleaned from the biotreatablity assessment studies was then applied to the large scale tanks and the microbiology with the system was analyzed. The mesocosm mineralization assays represented a snap-shot of the alkane biodegradation potential for the P1-treated and P2-untreated mesocosm trials as treatment progressed. As in the biotreatablity assays, mesocosm mineralization assays showed a clear improvement in ^{14}C-hexadecane mineralization from the untreated to the treated system. Moreover, the $\%^{14}CO_2$ recovered increased as the P1 mesocosm trial progressed, therefore, not only did the nutrient and lime amendments have positive impacts on microbial activity, but the mesocosm system also enhanced hydrocarbon mineralization activity. This “enhancement” was lost in the biotreatablity assessment mineralization assays, and was encouraging because the mesocosms were designed to be more representative of the natural RI site. Also, by the end of the P1 mesocosm trial, the $\%^{14}CO_2$ recovered was much

greater than levels seen in the biotreatability mineralization assays, and more akin to other bioremediation studies already mentioned. The shift from the low-mineralization activity phase one results to the high-mineralization activity results from phase two was contrary to other soil hydrocarbon bioremediation studies, in which the rates and extent of contaminant biodegradation decreased with increasing scale of the test system (Korus et al. 2001; Davies et al. 2003). Interconnected with the mesocosm hexadecane mineralization results were the results from the microbial enumerations and DGGE molecular fingerprinting. During the P1-treated mesocosm trial, greater numbers of viable hydrocarbon-degrading microorganisms and total microorganisms were observed, the former increasing ~27x - 48x, and the latter increasing ~2.5x – 3.5x. DGGE profiles and the nucleotide sequences of the bands therein, indicated the emergence of a synergistic hydrocarbon-degrading community during the mesocosm trials. Also, results obtained at CEAM by W. Chang showed steady TPH reduction in the P1-treated mesocosm, from >2000 ppm TPH at day 0 to <1000 ppm TPH at day 60, while no such reduction was observed in the P2-untreated mesocosm (Chang et al. 2007). Similarly in P1, the concentration of the F2 and F3 hydrocarbon fractions were reduced from ~1000 – 1200 ppm at day 0 to ~300 – 500 ppm at day 60 (Chang et al. 2007). Cumulative net CO_2 production and O_2 consumption also increased by the end of the P1 trial, but not in the P2 (Chang et al. 2007).

The specific reason for the difference between the $\%^{14}CO_2$ recoveries from the biotreatability mineralization assays, and those that used the mesocosm soil remains unclear. One possible explanation may concern the addition of essential nutrients to the RI soil. Both the viable enumerations and total direct microscopic enumerations increased after the beginning of the P1 trial, but did not continue to increase, or accelerate. Instead, the levels remained relatively similar from sample days 20, 40 and 60, which is contrary to the accelerating ^{14}C-hexadecane

mineralization activity results. The added nutrients may have enabled the microbial community to metabolize other preferred carbon sources in the soil matrix besides hydrocarbons, switching to hydrocarbons after other sources had become less abundant. This could partly explain the time difference between the population increase and hexadecane mineralization, since, for example, the alkane catabolic pathway of *P. oleovorans* is subject to carbon catabolite repression (Staijen et al. 1999). It is also possible that the tilling of the soil, while eliminating micro-anaerobic environments, volatilized inhibitory compounds from the soil (though no distinct smells were detected during the tilling process) or the fluctuating temperature stimulated biodegradation, as has been noted in other temperature fluctuation experiments (Eriksson et al. 2001, Børresen et al. 2007).

4.2.1. Molecular analyses

The DGGE bands only consisted of a ~400 bp section of the bacterial 16S rRNA gene thus prudence must be observed when discussing closest BLAST matches. That being acknowledged, the DGGE results for the four treated mesocosms did indicate changes in community structure and sequence analysis did imply the presence and emergence of bacteria related to known hydrocarbon degraders. The P2 DGGE results showed no change in banding pattern at all, which coincided with the lack of ^{14}C-hexadecane mineralization and static microbial population results of this untreated mesocosm. DGGE bands isolated and sequenced from all four of the treated mesocosms contained sequences related to known members of the hydrocarbon-degrading bacterial community (Chaillan et al. 2004; Daane et al. 2002; Kanaly et al. 2002; Bogan et al. 2003). This indicated that the community was sufficiently stimulated in the treated mesocosm systems to become detectable. Further evidence that the hydrocarbon degrading community was especially being stimulated were the repeated sequence results from bands extracted from the different soil samples related to known hydrocarbon degraders; for example the

Aeromicrobium-related bands from all four treated mesocosms and the *Alkanindiges*-related bands from the P1 soils and the HC soils.

Since DGGE is considered, at best, semi-quatitative, the specific nature of band intensification could not be determined. For example, whether band B (Fig. 3.9), which corresponded to the *Alkanindiges*-related 16S rRNA gene sequence in the P1 DGGE gel, increased as the cell enumerations did, initially after the beginning of the mesocosm trial and subsequently stabilizing, or in an accelerating fashion resembling the mineralization assay results, remains unclear. Correlation between when, and what members of the hydrocarbon degrading community commenced hydrocarbon biodegradation activity could be useful for designing a specific regime of nutrient supplementation tailored for active members of the community. Though the nature of DGGE band intensification could not be determined, the DGGE analyses did indicate important members of the bacterial hydrocarbon degrading community.

To add further depth to the RI bacterial community findings and the change in community structure due to mesocosm treatment, 16S rRNA gene clone libraries were constructed, using different PCR primers than those used for DGGE analysis to alleviate the possible inherent primer bias of a single primer pair. To directly discern the effect of the mesocosm treatment on the soil bacterial community, the two clone libraries were constructed from the P1 B0 soil and the P1 B60 soil, thus the initial bacterial community could be compared to the post-mesocosm treated bacterial community. Clone libraries were not constructed for samples from the P2-untreated mesocosm because DGGE analyses did not indicated a shift in bacterial community. The libraries were comparable in size and coverage (Good 1953); P1 B0 consisted of 74 clone sequences, which gave coverage of ~85% (at 97% homology cutoff), and P1 B60 consisted of 72 clone sequences, which gave coverage of ~86% (at 97% homology cutoff). Statistical analysis determined the libraries to have a

significant difference ($p<0.025$), when compared using the program webLIBSHUFF, making it clear that a change in the bacterial population had occurred. This difference is also evident from the Chao1 and ACE richness estimators, which both simply count the number of different OTUs in a given library. Chao1 and ACE showed a 46.62% and 43.25% reduction, respectively, from the P1 B0 to P1 B60 libraries. The reduction of the richness estimators could be a result of the mesocosm optimizing conditions for a select sub-population of the initial soil bacteria. The diversity indices of Shannon (1949) and Simpson (1949), which account for abundance and evenness of the OTUs present in a given library, were also calculated. The Shannon values of 2.72 and 2.76 and Simpson values of 13.18 and 14.12 were similar to diversity values from an investigation by Perreault et al. (2007) of two unrelated Arctic bacterial communities, which found Shannon's diversity index of 2.16 and 3.17 and Simpson's diversity index of 4.25 and 14.82. As the two libraries in this study were fundamentally coupled, direct comparison of the Shannon and Simpson indices showed an increase of 1.45% and 6.66%, respectively, from the P1 B0 to P1 B60 library. DGGE results of Hamamura et al. (2006) showed an associated increase in soil bacterial community diversity after reduction of oil contamination during a bioremediation study. Röling et al. (2002) found that after oiling and nutrient amendment of test soils, an initial decrease in biodiversity occurred, followed by recovery to nearly the original levels of soil biodiversity. The authors did note, however, that though the soil biodiversity nearly reached the same Shannon diversity value after bioremediation, the bacterial community structure that had initially been 73.1% *gamma-proteobacteria* changed to 63.3% *alpha-proteobacteria* (Röling et al. 2002). The individual BLAST matches for the clone sequences revealed some relatedness to other known hydrocarbon degraders, cold adapted bacteria or acid tolerant organisms. There was also some overlap between the sequences recovered in the clone libraries and those of the DGGEs, though not of the

cultured organisms, simply reiterating the need for a multifaceted approach to community characterization.

It seems reasonable to assume that given the results from the mesocosm mineralization assays and hydrocarbon-degrading viable cell enumerations, that there is a general increase in the hydrocarbon-degrading microbial community in the P1 soil at the conclusion of the trial. The change in phyla representation from the two clone libraries would then suggest the predominant composition of the hydrocarbon-degrading bacterial community. The change in bacterial community structure from the P1 B0 to P1 B60 clone libraries showed the representation of *Actinobacteria* and *Firmicutes* drop 80.00% and 33.33%, respectively, signifying they were less important in hydrocarbon biodegradation. The *Bacteroidetes*, on the other hand, were not detected in the first clone library but came to represent 14% of the P1 B60 library, while the *Proteobacteria* increased representation by 20.63%. Within the *Proteobacteria,* there is a marked shift in the proportionality of the classes; *alpha*- and *beta*- classes combined to make up 76% in the P1 B0 library, but were reduced to 40% in the second library. Different members of the *beta-proteobacteria* have been shown capable of hydrocarbon degradation (Viñas et al. 2005) and it may be that the indigenous RI soil members of this class are important constituents in the bioremediation of hydrocarbon contaminated RI soil. Likewise, members from the *Bacteroidetes* class have been shown to degrade hydrocarbons at low temperatures (Margesin et al. 2003) and are seemingly central hydrocarbon degraders in the RI system. Culture dependent analyses, however, failed to cultivate members from the *beta-proteobacteria* or *Bacteroidetes*, though the organisms isolated were an interesting facet to information on the general microbial community structure.

4.2.2. Culture-based analyses

It is not too surprising that the indigenous soil microbial community from an island of the Canadian Arctic archipelago would contain cold-adapted microorganisms. The viable aerobes isolated at 5°C on MSM-Arctic diesel plates from the different soils, HC, PS and HCB, represent a diverse group, including bacteria and eukarya. Virtually no information was available specifically for the closest eukaryal BLAST matches; however Brizzio et al. (2007) isolated members of the *Cystofilobasidium* and *Cryptococcus* from glacial and sub-glacial waters in search of cold-adapted yeasts as sources of cold-adapted enzymes for biotechnological applications. The closest BLAST match to isolate HCB3pH was an uncultured *Tremellaceae* clone and the *Tremellaceae* are part of the same class as *Cystofilobasidium* and *Cryptococcus*. An ecological study of fungal populations by López-Archilla et al. (2004) isolated members from the *Cryptococcus* and *Tremellaceae* families from the acidic Tinto River in Spain. Because the identification of the isolates from the HC, PS and PCB soils was monophasic, commenting extensively on the closest BLAST matches would be imprudent. However, it can be said without doubt that three of the thirteen isolates were eukayotes and these were the only strains recovered on acidic media, at ~4.5. The two bacterial phyla represented were the *Actinobacteria* and *Proteobacteria*, both common soil microorganisms with cold-adapted hydrocarbon-degrading representatives (Whyte et al. 1997; Whyte et al. 1998). The information available by GenBank about the closest BLAST matches to these isolates showed many to have demonstrated hydrocarbon-degrading capability or isolated from cold-temperature environments (Table 3.6). Strains related to *Rhodococcus* were the most represented in of the cultured isolates, accounting for ~26% of all isolates, indicating the importance of *Rhodococcus*-like organisms in hydrocarbon biodegradation. The isolated strains from this study were not represented in the DGGE analysis, most likely due to one of the factors previously mentioned. The reoccurrence

and apparent dominance of the *Alkanindiges* related DGGE band led to more directed cultivation attempts, discussed in greater detail below.

Two enrichment cultures were conducted; the first used HC soil because of the strong *Alkanindiges* related band in the DGGE gel of DNA extracted and PCR amplified for the 16S rRNA gene from the HC soil. The method of Whyte et al. (1999) resulted in isolate LB.1, which had high 16S rRNA gene sequence homology to a *Pseudomonas* strain. This may have been a result of the yeast extract used in the first stage of this method selecting for faster growing heterotrophs; the doubling time of *Pseudomonas* is ~2-3 times that of *Alkanindiges* (Bogan et al. 2003; Palleroni 2005). The second enrichment culture prepared started with P1 B20 soil, which had in essence been prescreened for presence of the desired organism by DGGE analysis. The protocol for the original isolation of the type strain for the *Alkanindiges* genus (Bogan et al. 2003) was followed, but, as before the target organism was not selected. The isolated strain, MD.1, had high 16S rRNA gene sequence homology to the genus *Rhodococcus.* Additionally, P1 B20 soil was plated directly with the idea that less manipulation might increase the chance of recovering the desired organisms. However, none of the seven strains recovered had close sequence homology to *Alkanindiges.* In a study by Stach and Burns (2002) that investigated the microbial PAH degrading community from a PAH impacted soil, the authors found that use of enrichment cultures reduced biodiversity, thereby possibly eliminating organisms of interest. The authors also noted the inherent bias of solid media for isolation of target organisms (Stach and Burns 2002). As previously discussed, organisms detected in molecular analysis often prove to be beyond the ability of researchers to culture even if it appears to dominate a community (Hugenholtz et al. 2001), as was the case here. This simply reiterated the necessity for a multifaceted approach to community analysis for best coverage and reliability, even for a bioremediation project where

examination of the microbial community responsible or effected by the bioremediation treatments was of auxiliary interest.

The isolates cultivated by direct spread plating that started with the P1 B20 soil also did not have high 16S rRNA gene homology to *Alkanindiges*. Seven of the eight strains had partial 16S rRNA gene sequences related to *Actinobacteria* and the eighth was related to the *alpha-proteobacteria*. These isolates could prove interesting in their own right as well. Isolate MD.2, which had 96% 16S rRNA gene sequence homology with a *Rhodococcus* strain and branched closely to the type strain of *R. erythropolis* and the known hydrocarbon-degrader *Rhodococcus* sp. Q15 (Whyte et al. 1999), was able to grow on MSM-Arctic diesel plates at -5°C. This suggests the presence of a population of sub-zero, hydrocarbon-degrading microorganisms and such a population could theoretically expand the season for hydrocarbon biodegradation beyond the summer months. However, no hydrocarbon mineralization activity was observed at sub-zero temperatures, indicating the potential use of isolate MD.2 in bioaugmentation experiments with RI soil. Børresen et al. (2007) noted sub-zero mineralization of hydrocarbons in soil microcosm assays, but did not culture individual sub-zero hydrocarbon degrading organisms.

Isolate MD.5, the only strain isolated whose partial 16S rRNA gene sequence was not closely related to the phylum *Actinobacteria*, branched separately from other genera of *alpha-proteobacteria*. Supplementary phylogenetic analysis showed the only other organism that branched with MD.5 was an environmental isolate, designated *Rhodopseudomonas* sp. ORS 1416ri, which showed only 97% similarity to the type species *R. rhenobacensis* (Zakhia et al. 2006). According to the RDP database (Cole et al. 2007), the MD.5 partial 16S rRNA gene sequence was only 87% similar to this type species, and showed closer homology (89%) to *Afipia massiliensis*, also a type species. The preliminary results indicate that MD.5 may be a novel species or even genus of bacteria.

Detailed identification of the hydrocarbon degrading microbial community in RI soil would, of course require more in-depth molecular and culture dependent analyses, but it is clear from this study that the indigenous microbial RI soil community has hydrocarbon biodegradation potential.

4.3. Major conclusions from this project

- Biostimulation positively affected the hydrocarbon biodegradation potential where otherwise, very little biodegradation activity was observed. The mineralization assays clearly displayed the difference between treated and untreated soils, which became more pronounced in the mesocosm trials. This was, perhaps, the most promising result of this study given that this project was designed as a precursor for eventual implementation of selected bioremediation treatments on RI, and the mesocosm trials mimicked the natural environment of RI.
- Culture-dependent and –independent analyses indicated the presence of a diverse indigenous microbial community capable of hydrocarbon biodegradation at cold temperatures.
- DGGE analyses imply proliferation of the hydrocarbon degrading bacterial community and while the clone libraries indicate a loss of bacterial richness due to mesocosm treatment, they also show a slight increase in bacterial diversity.
- Isolated strains, which included bacteria and yeast, showed hydrocarbon-degrading sub-populations able to use hydrocarbons as the sole carbon source while growing under acidic or sub-zero conditions. The initial identification and classification of some of these isolates hinted at possible novelty, and may warrant further characterization conceivably for use in prospective bioaugmentation treatments.

Overall, this study addressed the unique conditions of the RI hydrocarbon contaminated soil, and provided and supported a feasible procedure for the bioremediation of hydrocarbon contaminated RI soil.

References

Aguilar PS, Lopez P, de Mendoza D. 1999. Transcriptional Control of the Low-Temperature-Inducible des Gene, Encoding the Delta 5 Desaturase of Bacillus subtilis. *J. Bacteriol.* 181: 7028-33

Aguilera A, Gomez F, Lospitao E, Amils R. 2006. A molecular approach to the characterization of the eukaryotic communities of an extreme acidic environment: Methods for DNA extraction and denaturing gradient gel electrophoresis analysis. *Systematic and Applied Microbiology* 29: 593-605

Aiken SG, Dallwitz MJ, Consaul LLM, C.L. Gillespie, L.J. Boles, R.L. Argus, G.W. Gillett, J.M. Scott, P.J. Elven, R. LeBlanc, M.C. Brysting A.K. Solstad H. (1999 onwards). Version: 29th April 2003. Flora of the Canadian Arctic Archipelago: Descriptions, Illustrations, Identification, and Information Retrieval. In *http://www.mun.ca/biology/delta/arcticf/'*

Aislabie J, Saul D, Foght J. 2006. Bioremediation of hydrocarbon-contaminated polar soils. *Extremophiles* 10: 171-9

Aislabie JM, Balks MR, Foght JM, Waterhouse EJ. 2004. Hydrocarbon Spills on Antarctic Soils: Effects and Management. *Environ. Sci. Technol.* 38: 1265-74

Allen JP, Atekwana EA, Atekwana EA, Duris JW, Werkema DD, Rossbach S. 2007. The Microbial Community Structure in Petroleum-Contaminated Sediments Corresponds to Geophysical Signatures. *Appl. Environ. Microbiol.* 73: 2860-70

Amann RI, Ludwig W, Schleifer KH. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59: 143-69

AMAP. 1998. AMAP Assessment Report: Arctic Pollution Issues. Arctic Monitoring and Assessment Programme (AMAP). *Oslo, Norway*

Amato P, Parazols M, Sancelme M, Laj P, Mailhot G, Delort A-M. 2007. Microorganisms isolated from the water phase of tropospheric clouds at the Puy de Dôme: major groups and growth abilities at low temperatures. *FEMS Microbiology Ecology* 59: 242-54

Anesio AM, Graneli W, Aiken GR, Kieber DJ, Mopper K. 2005. Effect of Humic Substance Photodegradation on Bacterial Growth and Respiration in Lake Water. *Appl. Environ. Microbiol.* 71: 6267-75

Atlas RM. 1995. Petroleum biodegradation and oil spill bioremediation. *Marine Pollution Bulletin* 31: 178-82

Barns SM, Fundyga RE, Jeffries MW, Pace NR. 1994. Remarkable Archaeal Diversity Detected in a Yellostone National Park Hot Spring Environment. *PNAS* 91: 1609-13

Battaglia A, Morgan DJ. 1994. *Ex Situ* forced aeration of soil piles: A physical model. *Environmental Progress* 13: 178-87

Berthelet M, Whyte LG, Greer CW. 1996. Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. *FEMS Microbiology Letters* 138: 17-22

Bhadbhade BJ, Sarnaik SS, Kanekar PP. 2002. Bioremediation of an Industrial Effluent Containing Monocrotophos. *Current Microbiology* 45: 346-9

Bogan BW, Sullivan WR, Kayser KJ, Derr KD, Aldrich HC, Paterek JR. 2003. Alkanindiges illinoisensis gen. nov., sp. nov., an obligately hydrocarbonoclastic, aerobic squalane-degrading bacterium isolated from oilfield soils. *Int J Syst Evol Microbiol* 53: 1389-95

Børresen M, Breedveld GD, Rike AG. 2003. Assessment of the biodegradation potential of hydrocarbons in contaminated soil from a permafrost site. *Cold Regions Science and Technology* 37: 137-49

Børresen MH, Barnes DL, Rike AG. 2007. Repeated freeze-thaw cycles and their effects on mineralization of hexadecane and phenanthrene in cold climate soils. *Cold Regions Science and Technology* 49: 215-25

Børresen MH, Rike AG. 2007. Effects of nutrient content, moisture content and salinity on mineralization of hexadecane in an Arctic soil. *Cold Regions Science and Technology* 48: 129-38

Bower SM, Carnegie RB, Goh B, Jones SRM, Lowe GJ, Mak MWS. 2004. Preferential PCR Amplification of Parasitic Protistan Small Subunit rDNA from Metazoan Tissues. *The Journal of Eukaryotic Microbiology* 51: 325-32

Braddock JF, Lindstrom JE, Brown EJ. 1995. Distribution of hydrocarbon-degrading microorganisms in sediments from Prince William Sound, Alaska, following the Exxon Valdez oil spill. *Marine Pollution Bulletin* 30: 125-32

Braddock JF, Ruth ML, Catterall PH, Walworth JL, McCarthy KA. 1997. Enhancement and Inhibition of Microbial Activity in Hydrocarbon-Contaminated Arctic Soils: Implications for Nutrient-Amended Bioremediation. *Environ. Sci. Technol.* 31: 2078-84

Bradley PM, Chapelle FH. 1995. Rapid Toluene Mineralization by Aquifer Microorganisms at Adak, Alaska: Implications for Intrinsic Bioremediation in Cold Environments. *Environ. Sci. Technol.* 29: 2778-81

Brizzio S TB, de García V, Libkind D, Buzzini P, van Broock M. 2007. Extracellular enzymatic activities of basidiomycetous yeasts isolated from glacial and subglacial waters of northwest Patagonia (Argentina). *Can J Microbiol* 53: 519-25

Brofft JE, McArthur JV, Shimkets LJ. 2002. Recovery of novel bacterial diversity from a forested wetland impacted by reject coal. *Environmental Microbiology* 4: 764-9

Button DK, Schut F, Quang P, Martin R, Robertson BR. 1993. Viability and Isolation of Marine Bacteria by Dilution Culture: Theory, Procedures, and Initial Results. *Appl. Environ. Microbiol.* 59: 881-91

Cavicchioli R, Thomas T, Curmi PMG. 2000. Cold stress response in Archaea. *Extremophiles* 4: 321-31

Cerniglia CE. 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3: 351-68

Chaillan F, Le Fleche A, Bury E, Phantavong Yh, Grimont P, et al. 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Research in Microbiology* 155: 587-95

Chang WJ, Dyen M, Spagnuolo L, Simon P, Flaherty H, et al. 2007. Pilot-scale landfarming of petroleum hydrocarbon contaminated from Resolution Island, Nunavut. Presented at Proceedings of the Fifth Biennial Workshop on Assessment and Remediation of Contaminated Sites in Arctic and Cold Climates, Edmonton, AB.

Chao A. 1984. Non-parametric estimation of the number of classes in a population. *Scand J Stat.* 11: 265-70

Chao A, Ma MC, Yang MCK. 1993. Stopping rules and estimation for recapture debugging with unequal failure rates. *Biometrics.* 43: 783-91

Chénier, M.R., D. Beaumier, R. Roy, B.T. Driscoll, J.R. Lawrence and C.W. Greer. 2003. Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of

hexadecane by replicated river biofilms, *Applied and Environmental Microbiology* **69** (2003), pp. 5170–5177.

Chuvilin EN, NS; Miklyaeva, EC; Kozlova, EV; Instanes, A. 2001. Factors affecting spreadability and transportation of oil in regions of frozen ground *Polar Record* 37: 229-38

Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, et al. 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucl. Acids Res.* 35: D169-72

Daane LL, Harjono I, Barns SM, Launen LA, Palleroni NJ, Haggblom MM. 2002. PAH-degradation by *Paenibacillus* spp. and description of *Paenibacillus naphthalenovorans* sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants. *Int J Syst Evol Microbiol* 52: 131-9

Davis C, Cort T, Dai D, Illangasekare TH, Munakata-Marr J. 2003. Effects of Heterogeneity and Experimental Scale on the Biodegradation of Diesel. *Biodegradation* 14: 373-84

de la Torre JR, Goebel BM, Friedmann EI, Pace NR. 2003. Microbial Diversity of Cryptoendolithic Communities from the McMurdo Dry Valleys, Antarctica. *Appl. Environ. Microbiol.* 69: 3858-67

DeLong EF, Pace NR. 2001. Environmental Diversity of Bacteria and Archaea. *Systematic Biology* 50: 470 – 8

Dibble JT, Bartha R. 1979. Effect of environmental parameters on the biodegradation of oil sludge. *Appl. Environ. Microbiol.* 37: 729-39

Dojka MA, Harris JK, Pace NR. 2000. Expanding the Known Diversity and Environmental Distribution of an Uncultured Phylogenetic Division of Bacteria. *Appl. Environ. Microbiol.* 66: 1617-21

Eaton RW, Chapman PJ. 1992. Bacterial metabolism of naphthalene: construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions. *J. Bacteriol.* 174: 7542-54

Environment Canada. 1992. Biological test method: toxicity test using luminescent bacteria (*Photobacterium phosphoreum*). Ottawa, Canada: Environmental Protection Series.

Environment Canada home page: Weather Office http://www.weatheroffice.ec.gc.ca/.

EPA. 1989. Protocols for Short Term Toxicity Screening of Hazardous Waste Sites. EPA. 600/3-88/029. US Environmental Protection Agency. Washington, DC.

EPA. 1995. How to evaluate alternative cleanup technologies for underground storage tank sites: A guide for corrective action plan reviewers. EPA 510-B-95-007. United States Environmental Protection Agency, Washington, DC.

Eriksson M, Ka J-O, Mohn WW. 2001. Effects of Low Temperature and Freeze-Thaw Cycles on Hydrocarbon Biodegradation in Arctic Tundra Soil. *Appl. Environ. Microbiol.* 67: 5107-12

Felsenstein J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. *Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.*

Ferrari BC, Binnerup SJ, Gillings M. 2005. Microcolony Cultivation on a Soil Substrate Membrane System Selects for Previously Uncultured Soil Bacteria. *Appl. Environ. Microbiol.* 71: 8714-20

Foght JM, Fedorak PM, Westlake DWS. 1990. Mineralization of ^{14}C-hexadecane and ^{14}C-phenanthrene in crude oil: specificity among bacterial isolates. *Can J Microbiol* 36: 169-75

Fritsche W. and Hofichter M. 2000. Aerobic degradation by microorganisms. In *Biotechnology, 2nd edition; Environmental Processes II – Soil decontamination.* Vol. 11b. pp 146-64. Ed. Klein J. Wiley-VCH Press.

Gans J, Wolinsky M, Dunbar J. 2005. Computational Improvements Reveal Great Bacterial Diversity and High Metal Toxicity in Soil. *Science* 309: 1387-90

Gewin V. 2006. Genomics: Discovery in the dirt. *Nature* 439: 384-6

Ghoshal S, Luthy RG. 1996. Bioavailability of Hydrophobic Organic Compounds from Nonaqueous Phase Liquids: The Biodegradation of Naphthalene from Coal Tar. *Environmental Toxicology and Chemistry* 15: 1894-900

Good I. 1953. The population frequencies of species and the estimation of population parameters. *Biometrika.* 40: 237-64

Greene JC, Bartels, C. L., Warren-Hicks, W. J., Parkhurst, B. R.,, Linder GL, Peterson, S. A. and Miller, W. E. 1989. Protocols for short term toxicity screening of hazardous waste sites. Corvallis, OR, U.S.: Environmental Protection Agency.

Hamamura N, Olson SH, Ward DM, Inskeep WP. 2005. Diversity and Functional Analysis of Bacterial Communities Associated with Natural Hydrocarbon Seeps in Acidic Soils at Rainbow Springs, Yellowstone National Park. *Appl. Environ. Microbiol.* 71: 5943-50

Hamamura N, Olson SH, Ward DM, Inskeep WP. 2006. Microbial Population Dynamics Associated with Crude-Oil Biodegradation in Diverse Soils. *Appl. Environ. Microbiol.* 72: 6316-24

Handelsman J. 2004. Metagenomics: Application of Genomics to Uncultured Microorganisms. *Microbiol. Mol. Biol. Rev.* 68: 669-85

Harbhajan S. 2006. Fungal Metabolism of Petroleum Hydrocarbons. In *Mycoremediation*, pp. 115-48

Head IM, Saunders JR, Pickup RW. 1998. Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. *Microb Ecol* 35: 1-21

Henriksen JR. 2004. webLIBSHUFF (http://libshuff.mib.uga.edu).

Hong S-H, Bunge J, Jeon S-O, Epstein SS. 2006. Predicting microbial species richness. *PNAS* 103: 117-22

Huesemann MH. 1995. Predictive Model for Estimating the Extent of Petroleum Hydrocarbon Biodegradation in Contaminated Soils. *Environ. Sci. Technol.* 29: 7-18

Hugenholtz P, Goebel BM, Pace NR. 1998. Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *J. Bacteriol.* 180: 4765-74

Hugenholtz P, Tyson GW, Webb RI, Wagner AM, Blackall LL. 2001. Investigation of Candidate Division TM7, a Recently Recognized Major Lineage of the Domain Bacteria with No Known Pure-Culture Representatives. *Appl. Environ. Microbiol.* 67: 411-9

Indian and Northern Affairs Canada (INAC) website: Resolution Island Remediation Project http://www.ainc-inac.gc.ca/nu/nuv/zxca_e.html.

Jorgenson MT. 1995. Clean-up, bioremediation and tundra restoration after a crude-oil spill, S.E. Eileen exploratory well site, Prudhoe Bay, Alaska,1993. *Alaska Biological Research* Fairbanks.

Juck D, Charles T, Whyte LG, Greer CW. 2000. Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. *FEMS Microbiology Ecology* 33: 241-9

Jukes TH, Cantor RR. 1969. *Evolution of protein molecules.* New York: Academic Press.

Kaeberlein T, Lewis K, Epstein SS. 2002. Isolating "Uncultivable" Microorganisms in Pure Culture in a Simulated Natural Environment. *Science* 296: 1127-9

Kamlesh J, Williams, M., Franzluebbers, A.J., Jenkins, M., Endale, D.M., Coleman, D.C., Whitman, W.B. 2006. Soil bacterial community composition and diversity as affected by animal manure application in pasture and cropping systems of the Southern Piedmont USA [abstract]. . In *Academy of the Environment Conference*. January 28-February 1, 2007: Savannah, Georgia.

Kanaly RA, Harayama S, Watanabe K. 2002. Rhodanobacter sp. Strain BPC1 in a Benzo[a]pyrene-Mineralizing Bacterial Consortium. *Appl. Environ. Microbiol.* 68: 5826-33

Kastner M, Breuer-Jammali M, Mahro B. 1998. Impact of Inoculation Protocols, Salinity, and pH on the Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) and Survival of PAH-Degrading Bacteria Introduced into Soil. *Appl. Environ. Microbiol.* 64: 359-62

Kepner RL, Jr., Pratt JR. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiol. Mol. Biol. Rev.* 58: 603-15

Killham K. 1994. *Soil Ecology*. Cambridge University Press, Cambridge.

Kim J-S, Crowley DE. 2007. Microbial Diversity in Natural Asphalts of the Rancho La Brea Tar Pits. *Appl. Environ. Microbiol.* 73: 4579-91

Kiyohara H, Torigoe S, Kaida N, Asaki T, Iida T, et al. 1994. Cloning and characterization of a chromosomal gene cluster, pah, that encodes the upper pathway for phenanthrene and naphthalene utilization by Pseudomonas putida OUS82. *J. Bacteriol.* 176: 2439-43

Korus RA. 2001. Scale-up of processes for bioremediation. In *Manual of environmental microbiology, 2nd ed.* Washington D.C.: ASM Press

Krsek M, Wellington EMH. 1999. Comparison of different methods for the isolation and purification of total community DNA from soil. *Journal of Microbiological Methods* 39: 1-16

Labbé D, Margesin R, Schinner F, Whyte LG, Greer CW. 2007. Comparative phylogenetic analysis of microbial communities in pristine and hydrocarbon-contaminated Alpine soils. *FEMS Microbiology Ecology* 59: 466-75

Larsen KS, Jonasson S, Michelsen A. 2002. Repeated freeze-thaw cycles and their effects on biological processes in two arctic ecosystem types. *Applied Soil Ecology* 21: 187-95

Laurie AD, Lloyd-Jones G. 1999. The phn Genes of *Burkholderia* sp. Strain RP007 Constitute a Divergent Gene Cluster for Polycyclic Aromatic Hydrocarbon Catabolism. J. Bacteriol. 181: 531-40

Leahy JG, Colwell RR. 1990. Microbial degradation of hydrocarbons in the environment. *Microbiol. Mol. Biol. Rev.* 54: 305-15

Lesage S, Brown S, Millar K, Novakowski K. 2001. Humic Acids Enhanced Removal of Aromatic Hydrocarbons from Contaminated Aquifers: Developing a Sustainable Technology. *Journal of Environmental Science and Health, Part A* 36: 1515 – 33

Lin Q, Mendelssohn IA, Henry CB, Roberts PO, Walsh MM, et al. 1999. Effects of Bioremediation Agents on Oil Degradation in Mineral and Sandy Salt Marsh Sediments. *Environmental Technology* 20: 825-37

Liu Y, Yao T, Jiao N, Kang S, Zeng Y, Huang S. 2006. Microbial community structure in moraine lakes and glacial meltwaters, Mount Everest. *FEMS Microbiology Letters* 265: 98-105

Loffredo E, Berloco M, Casulli F, Senesi N. 2007. In vitro assessment of the inhibition of humic substances on the growth of two strains of *Fusarium oxysporum*. *Biology and Fertility of Soils* 43: 759-69

López-Archilla AI GA, Terrón MC, Amils R. 2004. Ecological study of the fungal populations of the acidic Tinto River in southwestern Spain. *Can J Microbiol* 50: 923-34

Löser C, Seidel H, Hoffmann P, Zehnsdorf A. 1999. Bioavailability of hydrocarbons during microbial remediation of a sandy soil. *Applied Microbiology and Biotechnology* 51: 105-11

Loveland-Curtze J, Sheridan PP, Gutshall KR, Brenchley JE. 1999. Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the Arthrobacter subgroup and description of Arthrobacter psychrolactophilus, sp. nov. *Archives of Microbiology* 171: 355-63

Luo P, Hu C, Zhang L, Ren C, Shen Q. 2007. Effects of DNA extraction and universal primers on 16S rRNA gene-based DGGE analysis of a bacterial community from fish farming water. *Chinese Journal of Oceanology and Limnology* 25: 310-6

Maltseva O, McGowan C, Fulthorpe R, Oriel P. 1996. Degradation of 2,4-dichlorophenoxyacetic acid by haloalkaliphilic bacteria. *Microbiology* 142: 1115-22

Männistö MK, Häggblom MM. 2006. Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. *Systematic and Applied Microbiology* 29: 229-43

Margesin R, Schinner F. 2001. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Appl Microbiol Biotechnol* 56: 650-63

Margesin R, Sproer C, Schumann P, Schinner F. 2003. *Pedobacter cryoconitis* sp. nov., a facultative psychrophile from alpine glacier cryoconite. *Int J Syst Evol Microbiol* 53: 1291-6

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, et al. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437: 376-80

Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon JC, et al. 2001. DNA Extraction from Soils: Old Bias for New Microbial Diversity Analysis Methods. *Appl. Environ. Microbiol.* 67: 2354-9

McCauley CA, White DM, Lilly MR, Nyman DM. 2002. A comparison of hydraulic conductivities, permeabilities and infiltration rates in frozen and unfrozen soils. *Cold Regions Science and Technology* 34: 117-25

Mohn WW, Stewart GR. 2000. Limiting factors for hydrocarbon biodegradation at low temperature in Arctic soils. *Soil Biology and Biochemistry* 32: 1161-72

Muyzer G, S. Hottentrager, A. Teske, and C. Wawer. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA. A new molecular approach to analyze the genetic diversity of mixed microbial communities. *In A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular Microbial Ecology Manual. Kluwer Academic Publishing, Dordrecht.*: p. 3.4..1-3.4..22

Muyzer G, Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* 73: 127-41

Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59: 695-700

Neufeld JD, Mohn WW. 2005. Unexpectedly High Bacterial Diversity in Arctic Tundra Relative to Boreal Forest Soils, Revealed by Serial Analysis of Ribosomal Sequence Tags. *Appl. Environ. Microbiol.* 71: 5710-8

Olah GA, Molnár Á. 2003. General Aspects. In *Hydrocarbon Chemistry (Second Edition)*, pp. 1-29

Pace NR. 1997. A Molecular View of Microbial Diversity and the Biosphere. *Science* 276: 734-40

Palleroni NJ. 2005. Genus I. *Pseudomonas*. In *Bergey's Manual of Systematic Bacteriology 2nd edn*, ed. DJK Brenner, N. R. Staley J. T. GarrityG. M. , pp. 323-79. East Lansing: Springer

Perreault NN, Andersen DT, Pollard WH, Greer CW, Whyte LG. 2007. Characterization of the Prokaryotic Diversity in Cold Saline Perennial Springs of the Canadian High Arctic. *Appl. Environ. Microbiol.* 73: 1532-43

Pinetreeline website: http://www.pinetreeline.org/site8.html.

Ping L, Luo Y, Wu L, Qian W, Song J, Christie P. 2006. Phenanthrene adsorption by soils treated with humic substances under different pH and temperature conditions. *Environmental Geochemistry and Health* 28: 189-95

Reardon CL, Cummings DE, Petzke LM, Kinsall BL, Watson DB, et al. 2004. Composition and Diversity of Microbial Communities Recovered from Surrogate Minerals Incubated in an Acidic Uranium-Contaminated Aquifer. *Appl. Environ. Microbiol.* 70: 6037-46

Rhykerd RL, Weaver RW, McInnes KJ. 1995. Influence of salinity on bioremediation of oil in soil. *Environmental Pollution* 90: 127-30

Rike AG, Haugen KB, Borresen M, Engene B, Kolstad P. 2003. *In situ* biodegradation of petroleum hydrocarbons in frozen arctic soils. *Cold Regions Science and Technology* 37: 97-120

Rike AG, Haugen KB, Engene B. 2005. In situ biodegradation of hydrocarbons in arctic soil at sub-zero temperatures-field monitoring and theoretical. *Cold Regions Science and Technology* 41: 189-209

Roesch LFW, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, et al. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME J* 1: 283-90

Röling WFM, Milner MG, Jones DM, Lee K, Daniel F, et al. 2002. Robust Hydrocarbon Degradation and Dynamics of Bacterial Communities during Nutrient-Enhanced Oil Spill Bioremediation. *Appl. Environ. Microbiol.* 68: 5537-48

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, et al. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-91

Saitou N, Nei M. 1987. The neighbor joining method: a new method for reconstructing phylogenetic trees. *Mol Biol. Evol.* 4: 406-25

Sambrook J, Russell DW. 2001. *Molecular Cloning: A Laboratory Manual. 3rd Edition*. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Saul DJ, Aislabie JM, Brown CE, Harris L, Foght JM. 2005. Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. *FEMS Microbiology Ecology* 53: 141-55

Schloss PD, Handelsman J. 2005. Introducing DOTUR, a Computer Program for Defining Operational Taxonomic Units and Estimating Species Richness. *Appl. Environ. Microbiol.* 71: 1501-6

Schmidt SK, Scow KM. 2001. Use of soil bioreactors and microcosms in bioremediation research. In *Manual of environmental microbiology, 2nd ed.* Washington D.C.: ASM Press.

Schumann R, Rentsch D. 1998. Staining particulate organic matter with DTAF--a fluorescence dye for carbohydrates and protein: a new approach and application of a 2D image analysis system. *Mar. Ecol. Progr. Ser.* 163: 77-88

Shannon CE, Weaver W. 1949. The mathematical theory of communication. *Illinois Press, Urbana, Illinois.*

Simpson EH. 1949. Measurement of diversity. *Nature* 163: 688

Singleton DR, Furlong MA, Rathbun SL, Whitman WB. 2001. Quantitative Comparisons of 16S rRNA Gene Sequence Libraries from Environmental Samples. *Appl. Environ. Microbiol.* 67: 4374-6

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, et al. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". *PNAS* 103: 12115-20

Spiegelman D, Whissell G, Greer CW. 2005. A survey of the methods for the characterization of microbial consortia and communities. *Can. J. Microbiol.* 51: 355–86

Stach JEM, Burns RG. 2002. Enrichment versus biofilm culture: a functional and phylogenetic comparison of polycyclic aromatic hydrocarbon-degrading microbial communities. *Environmental Microbiology* 4: 169-82

Stackebrandt E, Goebel BM. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44: 846-9

Staijen IE, Marcionelli R, Witholt B. 1999. The $P_{alkBFGHJKL}$ Promoter Is under Carbon Catabolite Repression Control in *Pseudomonas oleovorans* but Not in Escherichia coli *alk*+ Recombinants. *J. Bacteriol.* 181: 1610-6

Stallwood B, Shears J, Williams PA, Hughes KA. 2005. Low temperature bioremediation of oil-contaminated soil using biostimulation and bioaugmentation with a *Pseudomonas* sp. from maritime Antarctica. *Journal of Applied Microbiology* 99: 794-802

Stapleton RD, Savage DC, Sayler GS, Stacey G. 1998. Biodegradation of Aromatic Hydrocarbons in an Extremely Acidic Environment. *Appl. Environ. Microbiol.* 64: 4180-4

Steven B, Briggs G, McKay CP, Pollard WH, Greer CW, Whyte LG. 2007. Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. FEMS Microbiology Ecology 59: 513-23

Steven B, Niederberger TD, Bottos EM, Dyen MR, Whyte LG. July 2007. Development of a Sensitive Radiorespiration Method for Detecting Microbial Activity at Subzero Temperatures. Submitted for publication in the *Journal of Microbiological Methods*.

Süß J, Engelen B, Cypionka H, Sass H. 2004. Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods. *FEMS Microbiology Ecology* 51: 109-21

Takahata Y, Kasai Y, Hoaki T, Watanabe K. 2006. Rapid intrinsic biodegradation of benzene, toluene, and xylenes at the boundary of a gasoline-contaminated plume under natural attenuation. *Applied Microbiology and Biotechnology* 73: 713-22

Tarnocai C, Campbell IB. 2002. Soils of the polar regions. In *Lal R (ed) Encyclopedia of soil science*, pp. 1018–21. New York: Marcel Dekker

Thomassin L, Thomassin-Lacroix E, Eriksson, Eriksson M, Reimer, et al. 2002. Biostimulation and bioaugmentation for on-site treatment of weathered diesel fuel in Arctic soil. *Applied Microbiology and Biotechnology* 59: 551-6

Thompson IP, van der Gast CJ, Ciric L, Singer AC. 2005. Bioaugmentation for bioremediation: the challenge of strain selection. *Environmental Microbiology* 7: 909-15

Torrance JK, Schellekens FJ. 2006. Chemical factors in soil freezing and frost heave. *Polar Record* 42: 33-42

Torsvik V, Goksoyr J, Daae FL. 1990. High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.* 56: 782-7

Trindade PVO, Sobral LG, Rizzo ACL, Leite SGF, Soriano AU. 2005. Bioremediation of a weathered and a recently oil-contaminated soils from Brazil: a comparison study. *Chemosphere* 58: 515-22

Uyttebroek M, Vermeir S, Wattiau P, Ryngaert A, Springael D. 2007. Characterization of Cultures Enriched from Acidic Polycyclic Aromatic Hydrocarbon-Contaminated Soil for Growth on Pyrene at Low pH. *Appl. Environ. Microbiol.* 73: 3159-64

van Beilen J, Li Z, Duetz W, Smits T, Witholt B. 2003. Diversity of alkane hydroxylase systems in the environment. *Oil & Gas Science and Technol.* 58: 427-40

van Beilen J, Wubbolts M, Witholt B. 1994. Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation* 5: 161-74.

van Beilen JB, Panke S, Lucchini S, Franchini AG, Rothlisberger M, Witholt B. 2001. Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. *Microbiology* 147: 1621-30

Van Hamme JD, Singh A, Ward OP. 2003. Recent Advances in Petroleum Microbiology. *Microbiol. Mol. Biol. Rev.* 67: 503-49

Viñas M, Sabate J, Espuny MJ, Solanas AM. 2005. Bacterial Community Dynamics and Polycyclic Aromatic Hydrocarbon Degradation during Bioremediation of Heavily Creosote-Contaminated Soil. *Appl. Environ. Microbiol.* 71: 7008-18

Walworth J, Braddock JF, Woolard C. 2001. Nutrient and temperature interactions in bioremediation of cryic soils. *Cold Regions Science and Technology* 32: 85-91

Weissenfels WD, Klewer H-J, Langhoff J. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. *Applied Microbiology and Biotechnology* 36: 689-96

White JC, Qui, ones-Rivera A, Alexander M. 1998. Effect of Wetting and Drying on the Bioavailability of Organic Compounds Sequestered in Soil. *Environmental Toxicology and Chemistry* 17: 2378-82

Whyte LG, Bourbonniere L, Bellerose C, Greer CW. 1999. Bioremediation Assessment of Hydrocarbon-Contaminated Soils from the High Arctic. Bioremediation Journal 3: 69 – 80

Whyte LG, Bourbonniere L, Greer CW. 1997. Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (*alk*) and naphthalene (*nah*) catabolic pathways. *Appl. Environ. Microbiol.* 63: 3719-23

Whyte LG, Goalen B, Hawari J, Labbe D, Greer CW, Nahir M. 2001. Bioremediation treatability assessment of hydrocarbon-contaminated soils from Eureka, Nunavut. Cold Regions Science and Technology 32: 121-32

Whyte LG, Greer CW. 2005. Molecular Techniques for Monitoring and Assessing Soil Bioremediation. In *Soil Biology*, ed. R Margesin, F Schinner. Berlin Heidelberg: Springer-Verlag.

Whyte LG, Greer CW, Inniss WE. 1996. Assessment of the biodegradation potential of psychrotrophic microorganisms. *Can J Microbiol* 42: 99-106

Whyte LG, Hawari J, Zhou E, Bourbonniere L, Inniss WE, Greer CW. 1998. Biodegradation of Variable-Chain-Length Alkanes at Low Temperatures by a Psychrotrophic *Rhodococcus* sp. *Appl. Environ. Microbiol.* 64: 2578-84

Whyte LG, Slagman SJ, Pietrantonio F, Bourbonniere L, Koval SF, et al. 1999. Physiological Adaptations Involved in Alkane Assimilation at a Low Temperature by *Rhodococcus* sp. Strain Q15. *Appl. Environ. Microbiol.* 65: 2961-8

Whyte LG, Smits THM, Labbe D, Witholt B, Greer CW, van Beilen JB. 2002. Gene Cloning and Characterization of Multiple Alkane Hydroxylase Systems in *Rhodococcus* Strains Q15 and NRRL B-16531. Appl. Environ. Microbiol. 68: 5933-42

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T. 2002. Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. *Applied Microbiology and Biotechnology* 58: 202-9

Widdel F, Rabus R. 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Current Opinion in Biotechnology* 12: 259-76

Woese CR. 1987. Bacterial evolution. *Microbiol. Rev.* 51: 221-71

Woese CR, Fox GE. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc Natl Acad Sci* 74: 5088–90

Woese CR, Kandler O, Wheelis ML. 1990. Towards a Natural System of Organisms: Proposal for the Domains Archaea, Bacteria, and Eucarya. *PNAS* 87: 4576-9

Yergeau E, Newsham KK, Pearce DA, Kowalchuk GA. Patterns of bacterial diversity across a range of Antarctic terrestrial habitats. *Environmental Microbiology* (OnlineEarly Articles). doi:10.1111/j.1462-2920.2007.01379.x

Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, de Lajudie P. 2006. Diverse Bacteria Associated with Root Nodules of Spontaneous Legumes in Tunisia and First Report for *nifH*-like Gene within the Genera *Microbacterium* and *Starkeya*. *Microbial Ecology* 51: 375-93

Zengler K, Toledo G, Rappé M, Elkins J, Mathur EJ, et al. 2002. Cultivating the uncultured. *PNAS* 99: 15681-6

Zhao J-S, Manno D, Hawari J. 2007. Abundance and diversity of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)-metabolizing bacteria in UXO-contaminated marine sediments. *FEMS Microbiology Ecology* 59: 706-17

Zucchi M, Angiolini L, Borin S, Brusetti L, Dietrich N, et al. 2003. Response of bacterial community during bioremediation of an oil-polluted soil. *Journal of Applied Microbiology* 94: 248-57

Appendix

A1. Basic Safety Course Certificate

(on following page)