IDENTIFYING THE USEFULNESS OF MICROBIAL ENUMERATION, DIVERSITY, AND RESPIRATION FOR IMPLEMENTING STRATEGIES FOR INTRINSIC REMEDIATION

BY

LÉO JR. NICOLAS

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Soil Science
University of Manitoba
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Nicolas, Leo Jr. M.Sc., The University of Manitoba, August 2001. Identifying the usefulness of microbial enumeration, diversity, and respiration for implementing strategies for intrinsic remediation. Major Professor; Dr. David L. Burton.

Hydrocarbon utilizing bacteria have been found widely distributed in natural environments, their proportions affected by many factors, such as level of previous hydrocarbon exposure and from variations in soil conditions. This research compares microbial enumeration, diversity, and respiration between contaminated and adjacent uncontaminated soils and three native agricultural soils from Manitoba at a variety of depths. The purpose is to understand the effects of hydrocarbon contamination on these microbial parameters and the usefulness of the parameters for implementing intrinsic remediation as a remedial option. Four contaminated soils were examined, varying in the type of previous hydrocarbon exposure (two diesel fuel, one coal tar, and one crude oil).

The enumeration of aliphatic and aromatic degrading microorganisms from contaminated versus adjacent uncontaminated soils and agricultural soils were examined using a most-probable number method. Results indicated that both populations of degraders were present in all uncontaminated soils, including the agricultural soils, at a variety of depths. The effect of prior hydrocarbon exposure produced greater numbers of degraders in the contaminated soils when compared to adjacent uncontaminated material, indicating adaptation of the microbial populations to the presence of the contaminants through an increase in numbers.

The effect of previous contamination on the microbial diversity of soils was examined in a second experiment. Some contaminated soils had greater percentages of utilized substrates than their respective uncontaminated soils, indicating possible selection of microorganisms with increased metabolic versatilities due to the previous contamination event. Principle component analysis and cluster analysis were used to observe possible shifts in substrate utilization patterns caused by prior hydrocarbon exposure when comparing contaminated and uncontaminated soils. Both statistical tests did not reveal any consistent trends.

The final experiment was to determine differences in degradation rates in contaminated versus uncontaminated soils and agricultural soils upon the addition of ^{14}C-labelled phenanthrene. All surface and most subsurface agricultural soils were able to degrade phenanthrene, indicating the presence of degraders capable of significant levels of hydrocarbon degradation, ranging from 42% to 51% $^{14}CO_2$ production for surface soils and 6% to 30% $^{14}CO_2$ production for subsurface soils. In most cases, contaminated soils yielded higher percent $^{14}CO_2$ production than adjacent uncontaminated soils. Metabolic activity was also determined between contaminated versus adjacent uncontaminated soils, by measuring total CO_2 production, but did not reveal any consistent differences in activity. The presence of a plateau toward the end of the incubation period for some soils seemed to indicate that contaminant availability might be a controlling factor in degradation.

Comparison of results obtained from the three experiments revealed that in six of the ten contaminated soils studied, the contaminated soils produced greater numbers of hydrocarbon degraders that had increased metabolic capabilities with higher rate of $^{14}CO_2$ production. In these cases, previous hydrocarbon exposure resulted in the selection of

tolerant microbes able to utilize numerous substrates, including hydrocarbons, as sources of carbon and energy, resulting in quicker response to the addition of ^{14}C-labelled phenanthrene yielding higher $^{14}CO_2$ production. Naturally occurring hydrocarbon (HC) degraders have the ability to adapt and increase in numbers in facilitating intrinsic remediation. Efforts to enhance the availability of these hydrophobic contaminants may be necessary if the metabolic capability shown to exist in this study and others is to be used in intrinsic remediation strategies.

FOREWARD

The following thesis was prepared using the manuscript format outlined in the Guide to Thesis Preparation for Graduate Students in the Department of Soil Science. All of the manuscripts presented in the thesis (Chapters 4,5, and 6) will be submitted for publication to referred journals. The manuscripts will also include a co-author, Dr. David L. Burton, who is also the major professor and advisor.

CHAPTER 1

INTRODUCTION

Many spills of petroleum hydrocarbons occur onto soils, causing hazards to both humans and the environment. The removal of hydrocarbon-contaminated soils through excavation is costly, and off-site treatment increases the risk of spreading the contaminants to surrounding areas. Intrinsic remediation, a cost effective approach, attempts to facilitate remediation without the removal of the contaminated material from the site. The understanding of the effect on soil microbial communities in the presence of these contaminants becomes important, especially when assessing strategies for implementing intrinsic remediation. Intrinsic remediation attempts to facilitate remediation without the removal of the contaminated material from the site. For successful intrinsic remediation, the soil microbial community should include the presence of a naturally occurring hydrocarbon degrading population with the potential to respond to hydrocarbon (HC) contaminants. As every contamination event is different, it is difficult to predict the effect of hydrocarbons on the soil microbial populations. The immense diversity of soil microorganisms, varying in response to many environmental factors, increases the difficulty. The evaluation of the usefulness of microbial enumeration, diversity, and respiration as parameters for gathering information on the hydrocarbon degrading potential of the microbial communities in soils, has been the focus of some study. We hypothesise that these microbial parameters are increased in

previously contaminated soils and would help indicate their usefulness as tools for intrinsic remediation strategies.

Soils consist of heterogeneous microorganisms that include naturally occurring populations with the ability to degrade petroleum products (Atlas, 1978; Bossert and Bartha, 1984; Dragun, 1988), including polycyclic aromatic hydrocarbons such as phenanthrene (Manilal and Alexander, 1991). In soils, microorganisms that degrade aromatic HCs are frequently distinct from those that attack aliphatic HC (Foght *et al*, 1990; Atlas, 1991; Wrenn and Venosa, 1996). The number of HC-degrading organisms in a microbial community, determined by enumeration techniques, has been shown to be a decisive criterion for efficient bioremediation of contaminated sites (Atlas, 1981; Wilson, 1999). Observations of the metabolic diversity of the soil communities using Gram-negative BIOLOG plates can provide information on the substrate utilization patterns of microbial communities. Some researchers have observed microbes within contaminated environments with enhanced substrate utilization capabilities (Atlas, 1991). Shifts in utilized substrates have also been seen in soils previously contaminated when compared to pristine soils (Wünsche *et al.*, 1995). In most cases, degradation studies using ^{14}C-labelled compounds have shown that higher rates of HC degradation occurred in previously contaminated soils compared to uncontaminated soils (McGill *et al.*, 1981; Wilson and Jones, 1993; Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997). Nyman (1999) found that microbial activity (total CO_2) was not inhibited by addition of crude oil and concluded that tolerant microorganisms maintained activity. Results from enumeration, diversity, and respiration were compared to examine possible relationships and to evaluate their usefulness as potential tools for implementing strategies for intrinsic remediation.

CHAPTER 2

LITERATURE REVIEW

2.1 Petroleum Hydrocarbon Contamination of Soils

The contamination of soils by petroleum hydrocarbons can occur from various crude oil and oil products via many pathways. Soils can become contaminated by accidental release by storage tanks and tankers. Diesel fuel, coal tar oil, and bunker C fuel oil are a few examples of the many commercially available oil products. Release of these products into the soil environment is an on-going problem due their increase usage in our society. The presence of toxic petroleum products in the soil can result in adverse effects on the environment including risks to human health.

Numerous approaches to the remediation of contaminated soil have been developed, some of which are costly and/or require a high degree of site disturbance. The goal of every remediation strategy is to lower the concentration of the contaminant to acceptable levels. Many remediation strategies are very costly and intensive, such as soil washing, excavation, and soil flushing. A more cost effective non-invasive method based on intrinsic remediation, attempts to facilitate remediation without the removal of the contaminated material from the site. This remediation strategy attempts the proper management of several environmental factors and a microbial community capable of hydrocarbon degradation, to ensure that they are not limiting contaminant degradation

(Atlas, 1991; Atlas and Cerniglia, 1995; Huesemann, 1995; Margesin and Schinner, 1997). These factors can vary dramatically with soils of different textures. The knowledge of the variation of these factors among soils and how they might restrict the degradation of target contaminants is an important tool in intrinsic remediation strategies.

For efficient bioremediation of a contaminant, the number of contaminant degrading organisms in a microbial community is an important factor (Becker and Dott, 1995). The increase in numbers of hydrocarbon utilizing bacteria and their relative abundance in bacterial communities due to the presence of bioavailable hydrocarbons is well known among many researchers (Atlas, 1981; McGill *et al.*, 1981; Atlas, 1991; Wünsche et al., 1997; Wilson, 1999). Reported increases in bacterial numbers have ranged from slight to several orders of magnitude (McGill *et al.*, 1981).

2.2 Soil quality guidelines

Guidelines have been developed by the Canadian Council of Ministers of the Environment (CCME) to guide regulators and minimise the impact of petroleum contaminants on human health and the environment. The CCME had established the Recommended Canadian Soil Quality Guidelines for Contaminated Sites, which provide guidelines for acceptable levels of individual PAHs found in soil and water (Table 2.1; CCME, 1991). These levels serve as benchmarks in assessing the degree of contamination at a site and provide direction on the need for remediation, the setting of remediation goals and strategies, and verification of the adequacy of remedial actions (CCME, 1991). The importance of these levels is that they constitute a common scientific basis for the establishment of remedial objectives for specific sites (CCME,

1991). A detailed consideration of site specific factors, such as variations in local conditions and existing guidelines and standards, should be considered in the application of these values before remedial actions can be finalized (CCME, 1997).

Table 2.1 CCME guidelines for naphthalene, anthracene, phenanthrene, pyrene, and benzo[a]pyrene (μg/g).

	Naphthalene	Phenanthrene	Pyrene	Benzo[a]pyrene
Land Use				
Agricultural	*0.1	0.1	0.1	*0.1
Residential-parkland	*0.6	5	10	*0.7
Commercial	*22	50	100	*0.7
Industrial	*22	50	100	*0.7

* Indicates criteria taken from the CCME Recommended Canadian Soil Quality Guidelines (March 1997). All other criteria shown are from the CCME Interim Canadian Environmental Quality Criteria for Contaminated Soils (CCME, 1991).

Regulation of contaminated sites and the enforcement to reduce contaminant levels to acceptable limits are a provincial jurisdiction. The provincial department of Conservation also had established recommended guidelines for acceptable concentrations of total extractable hydrocarbons (TEH) in contaminated sites (Manitoba Environment, 1993). The concentrations have been divided into three levels and are outlined in Table 2.2.

Table 2.2 Manitoba Conservation Remediation Criteria for TEH (mg/kg) (Manitoba Environment, 1993).

	Concentration (mg/kg)
Level 1 (residential)	500
Level 2 (commercial)	2000
Level 3 (industrial)	2000

2.3 Hydrocarbon Degradation

2.3.1 Composition of diesel fuel

Diesel fuel is a complex mixture made up of hundreds of different compounds including aliphatic, polycyclic and aromatic compounds (Widrig and Manning, 1995). Diesel is composed primarily of carbon numbers in the C_{10} to C_{20} range, with boiling points ranging between 160°C and 360°C (Millner *et al.*, 1992). The composition of diesel fuel is dependent on the source of the oil, fractionation cracking, formulation, and the climate for which it was prepared (Gillespie *et al.*, 1989).

The description of diesel fuel can be simplified by grouping the compounds into the following broader categories: alkanes, monoaromatics, and polynuclear aromatics (McGill *et al.*, 1981; Atlas and Bartha, 1993). Aliphatic hydrocarbons (paraffin) including normal, branched and cyclic alkanes (cycloparaffin) dominate diesel fuel. Normal (n-) alkanes are saturated straight chain hydrocarbons that can vary in length from one carbon to over forty. Carbon chain lengths of C_{12} to C_{24} dominate in middle distillates such as diesel. Simple monoaromatic rings such as benzene and substituted benzene and polyaromatics such as phenanthrene and naphthalene comprise a significant portion of diesel fuel ranging from 10-30% (Block *et al.*, 1991). Sulphur, olefins, nitrogen, and oxygen containing compounds are also present as impurities (Ruth *et al.*, 1993).

2.3.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs include a group of organic priority pollutants of critical environmental and public health concern due to the following characteristics: (1) chronic health effects (carcinogenicity); (2) microbial recalcitrance; (3) high bioaccumulation potential; and (4)

low removal efficiencies in traditional wastewater treatment processes (Park *et al.*, 1990). The Canadian Environmental Protection Act (CEPA) required the Minister of the Environment and the Minister of Health to publish a Priority Substances List that included many PAHs, classified as toxic and harmful compounds to both human health and the environment (Government of Canada, 1994). In the USA, sixteen PAHs have been labelled as priority pollutants (Wilson and Jones, 1993).

PAHs are organic substances that make up a family of benzene derivatives, composed of adjoined six membered rings of carbon atoms. The structures can vary in complexity from simple two ringed structures like naphthalene to condensed multi-ringed substances such as benzo[a]pyrene, Figure 2.1 (Pothuluri and Cerniglia, 1994).

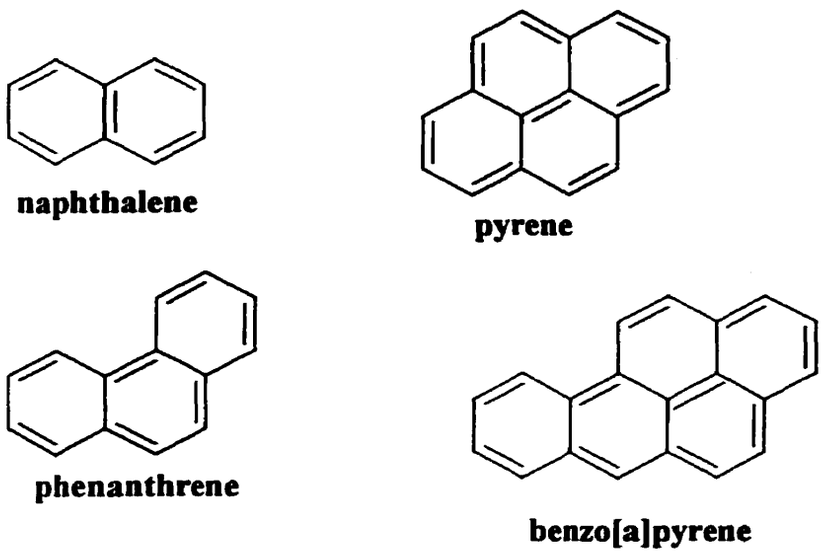


Figure 2.1 Chemical structures of PAHs (Government of Canada, 1994).

PAHs having less than four rings are considered to be low molecular weight compounds, while high molecular weight PAHs have four or more rings (Government of Canada, 1994). The arrangement of these ringed compounds can be linear, cluster, or

angular, with increasing stability from linear to angular (Sims and Overcash, 1983). As the number of rings in the structure of the compound is increased, the stability of the PAHs is greatly increased (Bumpus, 1989). PAHs are in general insoluble in aqueous environments, becoming more insoluble as the number of rings increases (Wilson and Jones, 1993). An increase in the number of rings results in an increase of the octanol-water partition coefficient (K_{ow}) and the melting point of PAHs, while vapour pressure and Henry's law constant decreases (Table 2.3).

Table 2.3 Physical Properties of some PAHs (Government of Canada, 1994).

Compound	Molecular weight	Log K_{ow}	Water solubility at 25°C (mg/L)	Melting point (°C)	Vapour pressure at 25°C (mPA)
Naphthalene	128.16	3.5	31.7	80.5	11 960
Phenanthrene	178.24	4.5	3.42	95	594
Anthracene	178.24	4.5	0.045	216	25
Pyrene	202.26	4.9	0.135	156	91.3×10^{-6}
Benzo[a]pyrene	252.32	6.0	0.0038	179	0.37×10^{-6}
Benzo[k]fluoranthene	276	6.06	0.0043	217	2.8×10^{-9}

2.3.3 Aliphatic hydrocarbons

Aliphatic hydrocarbons are often described as alkanes or saturated hydrocarbons-hydrocarbons because they contain only carbon and hydrogen; saturated because they have only C-C and C-H single bonds. They have a general formula of C_nH_{2n+n}, where n is any integer. Aliphatic hydrocarbons constitute the bulk of all diesels and may be further subdivided into n-alkanes, branched and cyclic. Normal (n)-alkanes are straight chain hydrocarbons that may vary in length from one carbon to over forty. Increase in chain length results in an increase in molecular weight and melting point, and a decrease in water solubility and vapour pressure.

2.3.4 Hydrocarbon metabolism

The metabolism of hydrocarbons by microorganisms was first characterised by Zobell in 1946. From this work, researchers realised that these organisms could be used for industrial purposes as well as for *in situ* bioremediation (Rainwater *et al.*, 1993). The popularity for using *in situ* bioremediation as a remedial option increased in the mid-1980s, when natural attenuation was considered for the remediation of oil washed ashore along the Alaskan coast as a result of the Exxon Valdez oil spill (Atlas and Cerniglia, 1995). The levels of petroleum were monitored along the shorelines and were found to decrease in concentration over time. The Exxon Valdez spill emphasized the potential for, and practicality of, *in situ* remediation. Rapid growth in the use of *in situ* remediation has resulted from these observations and now represents a billion to trillion-dollar industry (Rainwater *et al.*, 1993; Widrig and Manning, 1995).

2.3.5 Metabolism of N-Alkanes

N-alkanes are the most rapidly degraded diesel fuel compounds in soil. Their degradation is done aerobically and involves the incorporation of oxygen atoms into the structure of the chemical (McGill *et al.*, 1981). Degradation times are increased with increases in chain length and branching.

The enzymes responsible for initial introduction of oxygen into the molecule are called monooxygenases and dioxygenase. Monooxygenases (Figure 2.2) merges one oxygen atom into the alkane forming a primary alcohol and water. Dioxygenase (Figure 2.3) incorporates two atoms of oxygen into the structure initially forming a hydroperoxide, which is reduced to form an alcohol and water (Atlas and Bartha, 1993). The terminal methyl group is attacked in either case, resulting in the formation of an

alcohol functional group. The subsequent conversion of the primary alcohol to aldehydes and fatty acids allows β-oxidation to occur (Figure 2.4). Conversion of the fatty acid to an acyl coenzymeA through β-oxidation, leads to the formation of acetyl CoA that can enter the TCA cycle (McGill *et al.*, 1981).

$$R\text{-}CH_2\text{-}CH_3 + O_2 + NADPH_2 \rightarrow R\text{-}CH_2\text{-}CH_2\text{-}OH + NADP + H_2O$$

Figure 2.2 Conversion of an alkane molecule to a primary alcohol catalyzed by monooxygenases (Atlas and Bartha, 1993).

$$R\text{-}CH_2\text{-}CH_3 + O_2 \rightarrow R\text{-}CH_2\text{-}CH_2\text{-}COOH$$
$$R\text{-}CH_2\text{-}CH_2\text{-}COOH + NADPH_2 \rightarrow R\text{-}CH_2\text{-}CH_2\text{-}OH + NADP + H_2O$$

Figure 2.3 Conversion of and alkane molecule eventually forming a primary alcohol catalyzed by dioxygenase (Atlas and Bartha, 1993).

$$R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}OH \rightarrow R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COCoA \xrightarrow[+CoA]{} R\text{-}CH_2\text{-}CH{=}CH\text{-}COCoA$$
$$R\text{-}CH_2\text{-}CH{=}CH\text{-}COCoA \xrightarrow[+H_2O]{} R\text{-}CH_2\text{-}CHOH\text{-}CH_2\text{-}COCoA \xrightarrow[-2H^+]{} R\text{-}CH_2\text{-}CO\text{-}CH_2\text{-}COCoA$$
$$R\text{-}CH_2\text{-}CO\text{-}CH_2\text{-}COCoA \xrightarrow[+CoA]{} R\text{-}CH_2\text{-}COCoA + CH_3\text{-}COCoA$$

Figure 2.4 Transformation of a primary alcohol to acetyl CoA through a β-oxidation sequence (Atlas and Bartha, 1993).

2.3.6 PAHs Degradation

The degradation of PAHs requires a complex reaction due to the presence of fused ring structures. The complexity of the reactions for PAH degradation increases with increasing number of fused ring structures. Increasing number of ring structures and degradation complexity for the following PAHs are as follows: naphthalane, phenanthrene, pyrene, and benzo[a]pyrene.

Initial oxygenation of the aromatic ring by bacteria occurs via the action of dioxygenase, an enzyme that incorporates two oxygen atoms, forming a dihydrodiol. Monooxygenase also catalyzes oxygenation of aromatic rings and is present mostly in fungi (eukaryotes). Monooxygenase incorporates one oxygen atom to form an arene oxide during initial oxygenation.

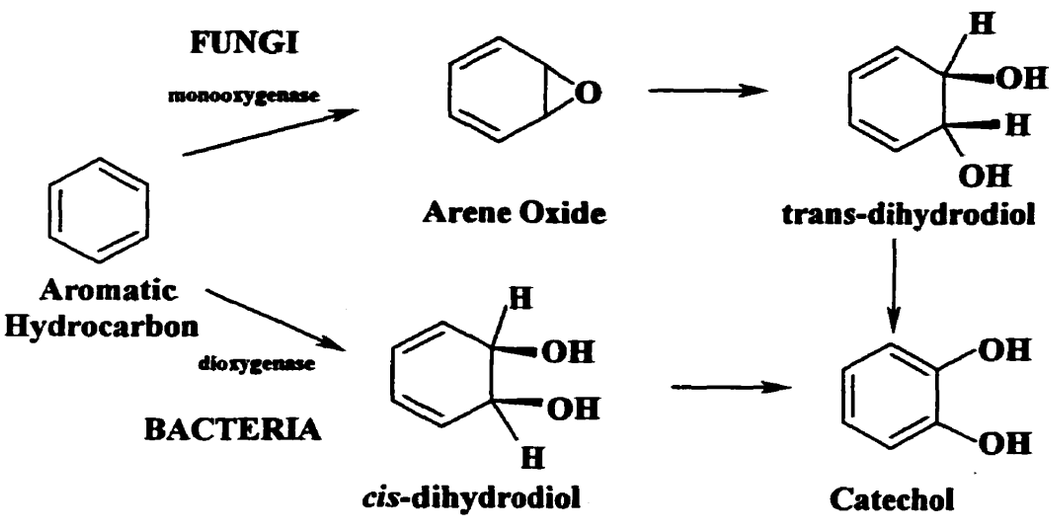


Figure 2.5 Initial oxygenation of aromatic hydrocarbon by bacteria and fungi (Atlas and Bartha, 1993).

Compounds resembling the simpler PAHs are formed during the breakdown of the complex structures. The understanding of the degradation of simpler PAH compounds are important since the structures of simpler molecules are contained in the more complex PAHs, such as benzo[a]pyrene. Rogoff (1962) suggested that the more similar PAHs are structurally, the more likely the oxygenases for initial hydroxylation are also similar.

PAHs can be microbially degraded in two ways; either as sole source of carbon and energy to the microbes or by co-metabolism (co-oxidation). Co-metabolism involves the production of an enzyme for degradation of a certain growth supporting compound that can also degrade a PAH that is not essential for growth. Low molecular weight PAHs (two and three rings) can be microbially degraded as sole carbon and energy sources while co-metabolism has been observed to enhance degradation of high molecular weight PAHs (four or more rings) (Sims and Overcash, 1983; Bumpus, 1989; Kästner *et al.*, 1994; Pothuluri and Cerniglia, 1994; Wilson and Jones, 1994). Richow *et al.* (1995) reported strong decreases in phenanthrene, fluorene, and anthracene concentrations (90-95%), indicating preferential microbial degradation in tar-oil contaminated soils, while decreases of higher molecular weight PAHs were below 50%. Little is known about the mechanism and factors involved in controlling co-metabolism (Wilson and Jones, 1993). Observation of studies have shown that high molecular weight PAHs were more resistant to degradation when present as pure compounds in soil than in the same soil in complex waste mixtures (Wilson and Jones, 1993). Other studies have reported co-metabolism of complex PAHs such as pyrene and benzo[a]pyrene when combined with simpler PAHs, such as naphthalene and phenanthrene (Herbes and Schwall, 1978).

The complete degradation of organic contaminants to harmless constituents, such as CO_2 and H_2O is the ultimate aim of bioremediation. The formation of intermediate metabolites during degradation includes dihydrodiols, phenols, and arene oxides, many of which have been identified as carcinogenic, mutagenic, and teratogenic (Wilson and Jones, 1993). As well as being as equally or more toxic than the parent compound,

intermediate compounds are often more soluble, leading to an increase in mobility in the environment (Wilson and Jones, 1993).

2.3.7 Factors affecting the rate of hydrocarbon degradation

2.3.7.1 Aeration

Under anoxic conditions, unsubstituted PAHs are extremely resistant to microbial degradation (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). Atlas (1981) stated that rapid biodegradation does not occur in anaerobic environments and that hydrocarbons that enter such conditions are well preserved and persist indefinitely as environmental contaminants. As the initial microbial attack of PAH requires oxygen, its presence is essential for successful degradation with optimal contents ranging from 10-40% (Wilson and Jones, 1993). Soil moisture content should be maintained between 30 and 90% water holding capacity for PAH degradation, as excessive moisture of soils can limit oxygen availability (Wilson and Jones, 1993). Manilal and Alexander (1991) found an increase in phenanthrene degradation by increasing aeration of the soil (48% mineralized in aerated soil vs. 39% mineralized in non-aerated). Field *et al.* (1995) concluded that aeration was an important factor in successful PAH degradation, by finding enhanced degradation of benzo[a]pyrene when the aeration status of the soil was artificially improved by the addition of pumice stones.

2.3.7.2 Soil pH

Generally, pH ranges between seven and eight were found optimal for PAH degradation. The pH will greatly affect the microbial composition of a soil. Most soil bacteria prefer a neutral pH environment as fungi are much more tolerant of acidity and

therefore dominate acidic environments. Consequently, the soil pH will often determine what type of microorganisms can participate in hydrocarbon degradation. There is also evidence that the overall rate of hydrocarbon biodegradation is higher under slightly alkaline than under acidic conditions as alkaline environments are more suitable for developing a more diverse group of microorganisms (Bossert and Bartha, 1984). Margesin and Schinner (1997) concluded that a neutral pH was more advantageous for mineral oil decontamination.

2.3.7.3 Temperature and solubility

Temperature can also affect rates of hydrocarbon degradation. An increase in temperature affects the chemical properties of PAHs by increasing vapour pressure and solubility while decreasing adsorption to soil particles (Maliszewska-Kordybach, 1993). The ability of a microbial community to degrade a compound is related to its solubility and therefore availability (Maliszewska-Kordybach, 1993). Ideal temperatures for PAH degradation is usually between 20° to 30°C (Wilson and Jones, 1993). Rate of degradation are generally observed to decrease with decreasing temperature, which is believed to be a result primarily of decreased rates of enzymatic activities, or the Q_{10} effect (Atlas, 1991). Sims and Overcash (1983) found that phenanthrene degradation increased by approximately 20% for each 5°C temperature, over a range of 15° to 37°C, with a maximum degradation of 90% mineralized at 37°C (at 1000 μg/L). Field *et al.* (1995) found that an increase in temperature from 20° to 30°C increased the rate of benzo[a]pyrene degradation by 75%. Margesin and Schinner (1997) found that diesel-oil decontamination was higher by 10% at 25°C in comparison to the one at 10°C.

The solubility of hydrocarbons has a marked effect on their biodegradation. At very low concentrations hydrocarbons are soluble in water, but most spills release petroleum HCs in concentrations far exceeding the solubility limits (Atlas, 1981). The degree of spreading is reduced in low temperatures because of the decrease in viscosity of the oils (Atlas, 1981). An increase in the number of aromatic rings within a molecule results in a dramatic decrease in solubility of PAHs (Table 2.3), limiting their ability to be biodegraded. Wodzinsky and LaRocca (1977) found that bacteria could utilize liquid aromatic HCs at the water-hydrocarbon interface but that solid aromatic HCs were not metabolized. They also found that naphthalene could not be degraded in the solid state but could be utilized if dissolved in a liquid HC. Atlas (unpublished data but reviewed in Atlas, 1981) similarly found that hexadecane supported little bacterial growth at 5°C when the compound was in the solid form, but if hexadecane was dissolved in another liquid HC, extensive degradation of the liquid hexadecane occurred at 5°C.

2.3.7.4 Nutrients

The availability of nutrients in soils may affect the persistence of HCs in the environment. Significant HC spills onto soils results in an environment with a high ratio of C to P/N. Various types of fertilizers can be added to soils to introduce nutrients to soils that may be absent or limiting for efficient biodegradation (Widrig and Manning, 1995). Manilal and Alexander (1991) found that addition of phosphorus (150 mg P/kg soil) did not increase degradation of phenanthrene (~48% degradation) but did decrease the number of days required to reach maximum degradation (from 11 days for soil with no P addition to 7 days with P addition). Doubling the amount of phosphorus to 300 mg P/kg soil did not significantly enhance or accelerate degradation beyond levels achieved

by previous addition at 150 mg P/kg soil. The addition of nitrogen at 140 mg N/kg soil decreased the amount of degraded phenanthrene (~41% mineralized) and increased the number of days to achieve the maximum rate of degradation (12 days). Margesin and Schinner (1997) found that the addition of fertilizers having a C:N ratio of 10:1 increased the rate of degradation, reducing diesel fuel contamination by 55% at 25°C compared to only 17% at 25°C without nutrient supplementation.

2.3.7.5 Hydrocarbon contaminant concentration

The initial concentration of hydrocarbons in soils has been found to be directly proportional to the rate of degradation. Field *et al.* (1995) found that continued exposure of benzo[a]pyrene to soils from 100 to 1000 mg/kg doubled the initial rate of degradation by an order of magnitude.

Khesina *et al.* (1969) investigated degradation of B[a]P under natural conditions of temperature and soil moisture. They found that B[a]P can be microbially degraded in soils under natural conditions provided that the soils has been acclimatized to the presence of PAHs over a period of several days before introduction of additional PAHs. They concluded that the rate of degradation appeared to be a function of concentration of B[a]P in soil. Sims and Overcash (1983) found that a 100-fold increase in phenanthrene concentration (10 to 1000 μg/L) resulted in a three-fold increase in the rate of phenanthrene degradation (12.1 μg/L-day vs. 43.4 μg/L-day) at 25°C. Sims and Overcash (1983) also summarised many rates of degradation of a variety of PAHs in soil systems (Table 2.4) and concluded from the following general trends that for a given PAH compound, the initial rate of transformation increases with increasing soil concentration.

Table 2.4 Summary of selected results from the effect on initial PAH concentrations on the rates of transformation (Sims and Overcash, 1983).

PAH	Initial concentration (μg/g soil)	Rate of transformation (μg/g day)
Naphthalene	7.0	0.4
	25000	4331
Phenanthrene	2.1	0.056
	25000	6930
Anthracene	3.4	0.714
	25000	4950

2.4 Population distribution of microbial hydrocarbon degraders

Soils consist of heterogeneous microorganisms that include naturally occurring populations with the ability to degrade petroleum products (Atlas, 1978; Bossert and Bartha, 1984; Dragun, 1988). Hydrocarbon utilizing bacteria are ubiquitously distributed in natural environments, their proportion in heterotrophic bacterial soil communities ranging from 0.13% to 50% (Wünsche *et al.*, 1995). The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera, but include a diverse group of bacteria and fungi. Bacteria and fungi are the principal agents of petroleum biodegradation in soil, but the relative contribution of each is not clear (Dragun, 1988). Table 2.5 lists the genera of HC-degrading bacteria and fungi isolated from soil. The degradation of complex hydrocarbons is generally not the result of the activity of a single species but rather represents the action of a community of organisms. Individual organisms have limited capability for degradation relative to the enzymatic capacity of mixed cultures (Miethe *et al.*, 1994). The composition of microbial communities in different soils and their biocatalytic potential differs quantitatively rather than

qualitatively (Miethe *et al.*, 1994). The reporting of numbers of hydrocarbon degraders in literature is hindered by the differences in methodology used to enumerate petroleum-degrading microorganisms. The number of HC-degrading organisms in a microbial community has been shown to be a decisive criterion for efficient bioremediation of contaminated sites (Atlas, 1981; Wilson, 1999).

Many researchers enumerate HC utilizing populations using either agar or silica based plates, in an attempt to enumerate HC degraders more easily and rapidly. The difficulty in preparing silica gel plates has limited their general use even though they would probably give more accurate counts (Randall and Hemmingsen, 1994). The use of plates is important for those who are interested in isolating and characterizing bacterial and fungal species of HC degraders. Comparisons are not possible between numbers found from plate counts to those found from liquid medium using most-probable-number technique. Agar based media may contain organic contaminants which would allow nonselective microbial growth, leading to an overestimation of the hydrocarbon degrading population (Walker and Colwell, 1976; Sextone and Atlas, 1977; Atlas, 1978; Randall and Hemmingsen, 1994; Wrenn and Venosa, 1996). One advantage in studying petroleum degradation by the MPN procedure is that it allows the researchers to extract the HC source from the liquid medium for GC analysis. This can confirm that degradation is actually occurring from a change in HC concentration after GC analysis. Various types of liquid media have been used in MPN experiments, from Bushnell-Haas broth designed specifically for isolating HC degraders (Bushnell and Haas, 1941; Song and Bartha, 1990; Williams *et al.*, 1998) to a variety of minimal salts medium having various concentrations of salts (Mills *et al.*, 1978; Margesin and Schinner, 1997), all of

which provide acceptable results for each specific experiment. Mills *et al.* (1978) evaluated three minimal salts medium, two varying in phosphate concentration (6.2 mM and 32 mM phosphate) and one with no phosphate addition. They found that the medium containing 32 mM phosphate produced the greatest number of HC degraders. Confirmation of growth in the test tubes can be done by simply looking at turbidity (Herbes and Schwall, 1978) or by using a variety of colour indicators such as resazurin (Song and Bartha, 1990; Williams *et al.*, 1998), bromothymol blue (Higashihara *et al.*, 1978), or iodonitrotetrazolium violet (Wrenn and Venosa, 1996). All of these indicators seem to provide a better indication of growth rather than turbidity, which can be difficult to assess when the HC used is very viscous (Atlas, 1979). Some researchers also relied on protein determination for confirmation that turbidity is a reliable indicator of HC degradation (Mills *et al.*, 1978; Wrenn and Venosa, 1996). The type of petroleum contamination will also influence the type of HC used in the MPN experiments. A variety of petroleum products have been used such as crude oil (Higashihara, 1979), kerosene (Higashihara, 1979), light gas oil (Higashihara, 1979), diesel fuel (Wrenn and Venosa, 1996; Margesin and Schinner, 1997), or specific PAH compounds (Herbes and Schwall, 1978; Grosser *et al.*, 1991; Madsen *et al.*, 1992; Kästner *et al.*, 1994; Carmichael and Pfaender, 1997). Isolation of specific HC degraders will also influence the type of HC used, such as aliphatic degraders (hexadecane, diesel fuel) or aromatic degraders (PAH compounds). All researchers did seem to use HC concentrations ranging from 1 to 1.4% (Higashihara, 1979; Mills *et al.*, 1978; Song and Bartha, 1990; Kästner *et al.*, 1994; Wrenn and Venosa, 1996; Williams *et al.*, 1998). Due to these many variations

in procedures of the MPN experiments, comparisons of results from the literature becomes next to impossible.

The ability of soil microbes to produce the enzymes necessary to degrade petroleum components introduced into the soil results from the presence of naturally occurring hydrocarbons and structurally related compounds such as fats, waxes, resins, lignin, and tannins. The microbial population in soil is exposed to a diverse group of substrates and as a result selects for organisms with a broad range of enzymatic capabilities. This selective process also favours organisms with the capabilities to degrade complex organics such as petroleum hydrocarbons.

Table 2.5 Genera of Hydrocarbon-Degrading Bacteria and Fungi Isolated from Soil.

Bacteria		Fungi	
Achromobacter	*Sarcina*	*Acremonium*	*Monilia*
Acinetobacter	*Serratia*	*Aspergillus*	*Mortierella*
Alcaligenes	*Spirillum*	*Aureobasidium*	*Paecilomyces*
Arthrobacter	*Streptomyces*	*Beauveria*	*Penicillium*
Bacillus	*Vibrio*	*Botrytis*	*Phoma*
Brevibacterium	*Xanthomonas*	*Candida*	*Rhodotorula*
Chromobacterium		*Chrysosporium*	*Saccharomyces*
Corynebacterium		*Cladosporium*	*Scolecobasidium*
Cytophaga		*Cochliobolus*	*Sporobolomyces*
Erwinia		*Cylindrocarpon*	*Sprotrichum*
Flavobacterium		*Debaryomyces*	*Spicaria*
Micrococcus		*Fusarium*	*Tolypocladium*
Mycobacterium		*Geotrichum*	*Torulopsis*
Nocardia		*Gliocladium*	*Trichoderma*
Proteus		*Graphium*	*Verticillium*
Pseudomonas		*Humicola*	

Source: Bossert and Bartha, 1984

2.4.1 Effect of hydrocarbon contamination on soil microbial communities

The impacts of hydrocarbon contamination on the soil microbial communities vary from soil to soil and throughout the contamination event. The initial exposure to petroleum products does not seem to affect the entire microbial population in soils (Jerkinson, 1966; Fuller and Warrick, 1985). Some immediate toxic effects are related to the solvent action of certain HCs on lipid components of the cell membranes, resulting in a decrease in microbial diversity. Liquid HCs containing alkanes, cycloalkanes, and aromatic HC with carbon numbers less than ten will dissolve lipid-containing membranes therefore destroying the microorganisms (Riser-Roberts, 1992). The levels at which a specific compound is toxic may to some extent be site specific as the microbial communities have substantial capacity to adapt to a variety of compounds (Norris, 1994). The most toxic components may volatilize or become immobilized by sorption to soil organic matter, therefore reducing the potential toxicity or inhibitory effect of petroleum HCs.

It is difficult to generalize about the microbial response of soils subjected to oil contamination. The great diversity of soils and variety of petroleum products studied in different geographical and climate regions that do not readily lend themselves to comparison have led to numerous reports that at times appear to be contradictory. The most studied microbial parameters of HC contamination will be further discussed, noting the similarities and/or differences found in the literature.

2.4.1.1 Population distribution of HC utilizers

Many researchers have observed numbers of HC-degraders and their relative abundance in microbial communities increase significantly if HC is bioavailable

(reviewed by Atlas, 1981; Kästner *et al.*, 1994; Williams *et al.*, 1998; Duncan *et al.*, 1997; Duncan *et al.*, 1999; Nyman 1999). In some oil-polluted ecosystems, HC-utilizers can constitute up to 100% of the viable organisms (Atlas, 1981). The addition of petroleum products to soils selectively favours and enriches the microbial population that is able to adapt and utilize the new substrate (Bossert and Bartha, 1984), resulting in a change in the dominant microbial population. A number of studies have demonstrated that the distribution of HC-degrading microorganisms reflects the historical exposure of the environment to hydrocarbons. This has been shown in a variety of soils in a wide distribution of climatic zones, including sub arctic and arctic regions (Sextone and Atlas, 1977; Atlas, 1981; Wilson, 1999).

The predominant hydrocarbon type in the contaminated soil matrix has been found to influence microbial HC-degrading population dynamics. Microorganisms that degrade aromatic HCs are frequently distinct from those that attack aliphatic HCs (Foght *et al.*, 1990; Atlas, 1991; Wrenn and Venosa, 1996). Totally different populations of microorganisms may carry out the degradation of different classes of HCs. In uncontaminated soils, alkane degraders appear to be more common in soil microbial communities than PAH degraders (Wrenn and Venosa, 1996). Kästner *et al.* (1994) found alkane and naphthalene degraders in both uncontaminated and contaminated soils from their study. PAH contaminated soils contained a variety of PAH degrading bacteria using three and four-ring structures as sole carbon sources. These microorganisms were also able to grow on more than one PAH except for those selected of naphthalene as sole carbon source. It has been suggested that the more similar PAHs are structurally, the more likely the oxygenases for initial hydroxylation are also similar (Grosser *et al.*,

1991). Both Kästner *et al.* (1994) and Wrenn and Venosa (1996) observed that pure cultures of PAH degraders were unable to grow on diesel fuel or any of its components. They also found that diesel fuel contaminated soils contained alkane and naphthalene degraders but no other PAH degrading microorganisms. These findings emphasize the importance of distinguishing between aliphatic and aromatic degrading bacteria in HC-contaminated soils.

Kästner *et al.* (1994) studied two contaminated sites having PAH levels of 3700 μg PAH/g of soil and 2000 μg PAH/g of soil. Both sites had significant colony forming units of PAH degraders ($>10^7$ organisms/g soil) grown on only one PAH, such as naphthalene, phenanthrene, anthracene, and pyrene. They concluded from their results that PAH contaminated soils probably contain significant number of PAH degrading bacteria although a minimum concentration of PAH must be present in the soil in order to select and stimulate the growth of these organisms. Threshold concentrations of PAH contamination in soils that is required to activate PAH degrading microorganisms is currently not known. Thomas *et al.* (1989) reported threshold concentrations from other work, ranging between 5 and 30 μg/L for naphthalene, phenanthrene, and fluorene in samples of creosote-contaminated ground water. Carmichael and Pfaender (1997) showed that concentration of PAHs greater than background was required for selection and stimulation of HC degraders. For their experiment, background level of PAH in uncontaminated soil was 0.08 μg/g of soil.

The analysis of HC-degrading genes has identified separate enzymes for alkane and aromatic degradation. The dioxygenase responsible for the first step in the aerobic oxidation of lower molecular weight aromatic hydrocarbons have many similarities,

suggesting that they have a common, although distant, evolutionary origin (Hamann *et al.*, 1999). In contrast, little information is known about bacterial genes encoding proteins for the degradation of higher molecular weight PAH, including phenanthrene, anthracene, pyrene, and fluroanthene (Hamann *et al.*, 1999).

2.4.1.2 Microbial diversity and substrate utilization

The addition of oil-products to soils creates an environmental stress or disturbance generally resulting in a decrease in the diversities of the microbial communities by upsetting the ecological balance of population interactions within the community (Atlas *et al.*, 1991). The change in microbial diversity has been observed through shifts in the substrate utilization patterns and changes in dominant bacterial genera (Wünsche *et al.*, 1995). Wünsche *et al.* (1995) examined changes in substrate utilization patterns and hydrocarbon utilizing bacteria content in three soils, before and after a ninety-four day incubation period. The soils were one pristine arable soil contaminated with spindle oil (1.2%), one pristine soil left uncontaminated, and a previously mineral oil-contaminated soil. Using three replicates, bioremediation for the three soils was simulated under aerobic conditions in a chamber during the entire incubation. The community of the pristine soil was dominated by species of the genera *Bacillus* (13% of isolates), *Xanthomonas* (10%), and *Pseudomonas* (10%), as well as *Cytophaga* (9%). In contrast, pseudomonads (24%) dominated in the mineral-oil contaminated soil. During the 94 days of incubation, changes in the composition of the autochthonous microbial community as a consequence of HC utilization were well correlated to an increase of HC-utilizing bacteria (from 3.6% to 55% in the spindle-oil contaminated arable soil and form 2.5% to 54% in the long-term contaminated soil). Pseudomonads clearly became

predominant in the "artificially" contaminated arable soil. The results obtained by comparison of the substrate utilization patterns of the two soil communities were in accordance with these previous findings. After the incubation period of the pristine soil contaminated with spindle oil, the total number of substrates utilized decreased insignificantly (from 86 to 82), but considerable shifts took place within the substrate utilization pattern: 16 substrates could no longer be used while 12 new substrates were utilized under the changed conditions. Similar values were also found for the long term polluted soil sample: 19 substrates no longer used while 11 new substrates utilized. Incubation of the unpolluted arable soil without HC addition significantly reduced the number of substrate utilized by 23 substrates, probably caused by the long-term deficiency of substrates during the incubation period. Analysis of these results using principle component analysis (PCA) and cluster analysis clearly indicated the shifts in substrate utilization patterns in the short and long-term contaminated soils. They concluded that the effect must have resulted from similar adaptation processes caused by selective enrichment of HC-utilizing bacteria. In a similar study, Derry *et al.* (1998) examined soils contaminated for more than three years with creosote. From the analysis of their results using PCA, they found no significant differences in functional microbial diversity between uncontaminated and contaminated soils, possibly reflecting processes of microbial adaptation to creosote contamination. Derry *et al.* (1998) did not comment on the significance of their observation for understanding adaptation of the microbial community, to the contamination. Adaptation of the microbial community to creosote contamination would require a change in population dynamics in the soil environment, favouring growth and dominance of HC degraders. Perhaps the lack of change in

substrate utilization reflects a return to a steady-state level governed by factors other than substrate supply. Most microbial diversity studies have used soils that were examined shortly after contamination. Atlas *et al.* (1991) found that microbes within disturbed environments demonstrated enhanced physiological tolerances and substrate utilization capabilities. The disturbance may be a basis for selecting populations with increased substrate utilization capabilities and enhanced survival under a variety of conditions.

The age of contamination as well as the concentration of HC present in the soil does seem to affect the metabolic diversity of the soil microflora. Knowledge of the contamination history of a site would help in the interpretation of the changes or shifts in substrate utilization patterns, especially when comparing with adjacent uncontaminated soils.

2.4.1.3 Microbial Activity

In most cases, higher rates of hydrocarbon degradation have been shown to occur in previously contaminated soils compared to uncontaminated soils (McGill *et al.*, 1981; Wilson and Jones, 1993; Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997). Since contaminated soils have adapted microbial communities with higher proportions of HC-degraders, they can quickly respond to the presence of HC pollutants. The lack of inhibition at the community level does not necessarily mean an absence of toxicity. Nyman (1999) found that microbial activity was not inhibited by addition of crude oil and concluded that activity was maintained by tolerant species that could metabolize substrates normally used by sensitive species.

Thomas *et al.* (1989) examined the microbial ecology of subsurface pristine soils and slightly creosote contaminated (<0.5 μg PAH/g) and heavily creosote contaminated (~50

μg PAH/g) soils. Slightly contaminated material was taken from the periphery of the spill plume. The depth of soils ranged from 1.2 to 7.7 meters. Except for the heavily contaminated sample, degradation of glucose indicated a metabolically active microflora in all subsurface materials. Toxic levels of organics that were once present in the creosote sludge may have inhibited the microbes from these samples. However, degradation (<40%) of naphthalene, phenanthrene, and 2-methylnaphthalene was observed in both slightly and heavily contaminated materials, but not in pristine material.

Madsen *et al.* (1989) also compared rates of PAH degradation between subsurface soils outside and inside the contaminated (coal tar) zones. The results from their study provided clear qualitative evidence for metabolic adaptation of the subsurface microbial community to PAHs compounds. They found that ^{14}C-naphthalene and ^{14}C-phenanthrene were mineralized (<12%) by samples from inside but not outside the plume of contamination after two weeks incubation at 20°C.

The rate of degradation, especially for PAHs, was found to be directly proportional to the initial concentration in soil (Carmichael and Pfaender, 1996) and upon the presence of lower molecular weight (LMW) aromatics (Atlas and Atlas, 1991). Carmichael and Pfaender (1996) compared percent ^{14}C-phenanthrene recoveries between contaminated (269 mg PAH/kg) and uncontaminated (0.082 mg PAH/kg) surface soils and found significant differences (60% recovery for contaminated soil and >10% recovery for uncontaminated soil) indicating microbial adaptation to previous contamination. The finding that enzymes for degrading at least some PAHs, especially higher molecular weight structures, are not induced by the substrate itself is important and may explain the apparent resistance of these compounds to microbial attack (Atlas and Atlas, 1991).

Higher rates of degraded benzo[a]pyrene were found in the presence of other LMW aromatics (Grosser *et al.*, 1991; Weissenfels *et al.*, 1992). Rogoff (1962) suggested that the more similar PAHs are structurally, the more likely the oxygenases for initial hydroxylation are also similar. At low contaminant concentrations (<100 μg PAH/g soil), degradation may be occurring very slowly, and would increase with continued contaminant application (Field *et al.*, 1995).

2.4.1.4 Correlations between microbial enumeration, diversity, and activity

In some studies, investigators have found that petroleum application to soil increased activity and total microbial counts, as well as shifts in the composition of the microbial community attributed to the abundance of indigenous HC degraders (Atlas, 1981; Bossert and Bartha, 1984; Song and Bartha, 1990; Wünsche *et al.*, 1995). In contrast, some researchers have found inconsistent relationships between the numbers of HC degrading microorganisms and the extent of HC degradation, in some cases soils which usually had the largest amounts of degradation did not have the greatest number of HC degraders (Grosser *et al.*, 1991; Miethe *et al.*, 1994; Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997). Many researchers have found good responses in microbial activity and numbers through the addition of nitrogen and phosphorus fertilizer amendments to the contaminated soils (Atlas, 1991; Widrig and Manning, 1995; Braddock *et al.*, 1997; Duncan *et al.*, 1997; Wilson, 1999). Grosser *et al.* (1991) discovered that continuous inputs of PAHs produced an increased ability within the microbial community to utilize certain PAHs, by selecting PAH degraders. As discussed in the previous sections, the initial concentration of HC contaminants does seem to be an important factor in selecting and stimulating appropriate HC degrading microbes. Concentrations of previous

exposure required to induce increased HC degrading activity are not consistent and may reflect the influence of other environmental constraints.

Comparisons between correlation results obtained in the literature are difficult as no two contamination scenarios are the same. Results of microbial enumeration, diversity, and activity from contaminated and adjacent uncontaminated soils do seem to provide important information on the effects of HC contamination and the possibilities for implementing intrinsic remediation.

CHAPTER 3

DESCRIPTION OF SITES AND SOILS

3.1 Contaminated soils

The objective of this project was designed to examine characteristics of the microbial population in previously contaminated and adjacent uncontaminated soils from Manitoba and not the rate of remediation occurring on site. A survey of historical contaminated sites was done in consultation with Manitoba Hydro, which is responsible for several hydrocarbon contaminated locations across the province. Manitoba Hydro collected soil samples from three locations, Thicket Portage, Pikwitonei, and Brandon. The location of the sites in Manitoba can be found in Figure 3.1. As this study required the use of contaminated soil samples from Manitoba, arrangements were made with Manitoba Hydro for the supply of contaminated samples from some of their contaminated sites. As part of Manitoba Hydro procedure in dealing with their contaminated sites, all soils from the contaminated areas were sampled by their personnel, which recorded detailed descriptions of each sample at every site (see Appendix for drill log data). Site maps, provided by Manitoba Hydro (Figure 3.2 to 3.4), show the locations at which the contaminated and uncontaminated (contaminant levels below detection limit) samples were taken. The site maps were used to provide information of the suitability of uncontaminated soil samples to be used as uncontaminated control samples for the

degradation studies. The information provided by Manitoba Hydro of these sites was sufficient to meet the objectives of this study and did not require visitation of these remote areas. Comparisons between contaminated and adjacent uncontaminated soils were done at similar depths.

Contaminated and adjacent uncontaminated soil samples from Somerset, collected by Enbridge Pipeline personnel, were also used in this study. The contaminated sample was taken by the Enbridge personnel within the contaminated area, while the uncontaminated sample was taken outside of the area. Site map of the area (Figure 3.5) show the location at which the contaminated and uncontaminated (contamination levels below detection limit) samples were taken. A more detailed site description of the spill site can be found in Timmerman (1999).

Due to the lack of contaminated soil samples, only uncontaminated soils from each area were analysed for particle size, field capacity, while all contaminated and uncontaminated soils were analysed for pH, and electrical conductivity (Table 3.1). The determination of all soil properties was done using composite samples ranging in depth to compensate for the lack of soil samples (Table 3.1). Manitoba Soil Survey (Winnipeg, MB) performed the particle size analysis according to the method of Haluschak (1986).

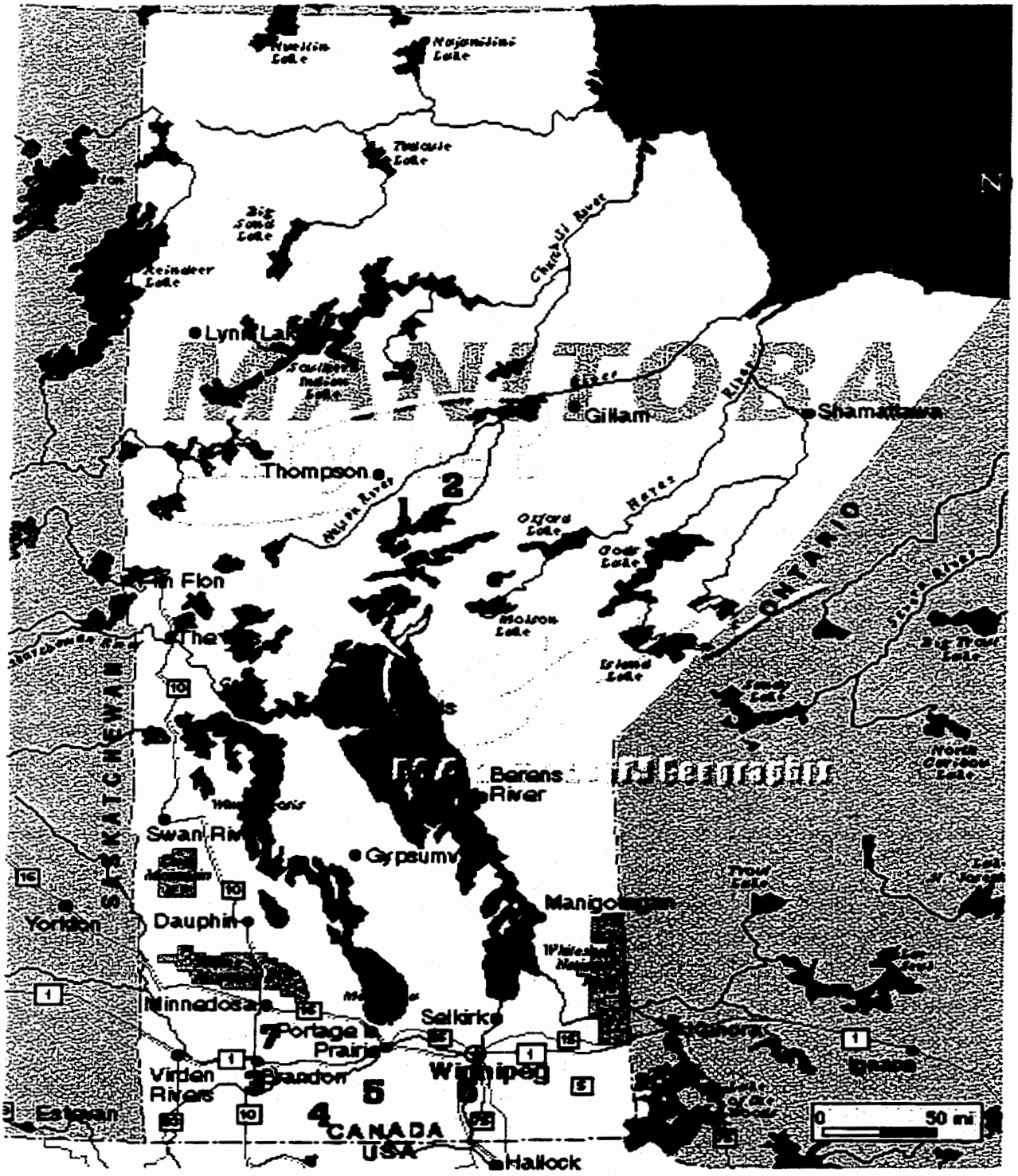


Figure 3.1. Map of Manitoba indicating location from which soils were sampled; (1) Thicket Portage, (2) Pikwitonei, (3) Brandon, (4) Somerset, (5) St.Claude, (6) Glenlea, (7) north of Brandon.

Legal descriptions, soil series, and classification for all the contaminated sites can be found in Table 3.2

Table 3.1 Soil properties for contaminated and adjacent uncontaminated soils.

Site and Depth (meters)	Particle Size Analysis			Texture	Field Capacity (%)	pH	Electrical Conductivity (dS/m)
	%sand	%silt	%clay				
Thicket Portage uncontaminated (0.5-2.0)	0	12	88	heavy clay	37.4±0.3	7.9	0.48
Thicket Portage* (0.5-2.0)	**	**	**	**	**	7.5	0.38
Pikwitonei uncontaminated (0.5-2.5)	0	13	87	heavy clay	37.4±0.3	7.8	0.39
Pikwitonei* (0.5-2.5)	**	**	**	**	**	7.5	0.48
Brandon A uncontaminated (1.5-3.0)	88	7	5	coarse sand	8.5±0.2	8.1	0.40
Brandon A* (1.5-3.0)	**	**	**	**	**	7.5	0.51
Brandon B uncontaminated (1.5-4.5)	20	25	56	silty loam	16.1±0.7	7.3	1.98
Brandon B* (1.5-4.5)	**	**	**	**	**	6.9	1.85
Somerset (0-0.15)	19	47	34	clay loam	39.7±0.8	7.7	0.77

*contaminated soils **values assumed to be similar to uncontaminated soils

Table 3.2 Soil classification and legal description of contaminated sites.

Site	Soil Series	Soil Classification	Site Location
Thicket Portage	Wabowden	N.A.*	5-73-2W
Pikwitonei	Wabowden	N.A.	13-76-2E
Brandon coarse sand	Marringhurst	N.A.	SW 24-10-19W
Brandon silty loam	Assiniboine	N.A.	SW 24-10-19W
Somerset	Joyale	Gleyed Rego Black Chernozem	SW 32-4-9W

* not available

3.1.1 Thicket Portage And Pikwitonei

Manitoba Hydro collected diesel-contaminated and uncontaminated soil samples from Thicket Portage and Pikwitonei, in July of 1998, from storage facilities for diesel-powered electricity generators. Both towns are located along the Hudson Bay Railroad,

south of Thompson, in an area designated as the "Northern Clay Belt" (Ehrlich et al, 1959). These soils were developed on lacustrine clay and form part of the Wabowden soil series. Diesel-powered electricity generation was first provided in September 1965 and July 1966 for Thicket Portage and Pikwitonei, respectively and continued until April 1997. Manitoba Hydro sampled both locations in July 1998. All samples were stored at 4°C until analysis. Log data recorded by Manitoba Hydro personnel of each sample studied for both sites can be found in the Appendix.

For soils from Pikwitonei, two uncontaminated soil samples, uncontaminated 1 and uncontaminated 2, were taken from two different auger holes (Figure 3.2). Uncontaminated 1 samples were taken from three different depth intervals (0.75-1.0 m, 1.25-1.75 m, and 2.0-2.5 m) while uncontaminated 2 was taken from only one depth (2.5-3.0 m). Two contaminated soil samples, contaminated 1 and contaminated 2, were taken from two different auger holes. Contaminated 1 samples were taken from the same depth intervals at uncontaminated 1, while contaminated 2 was taken from the same depth as uncontaminated 2. An aerial map supplied by Manitoba Hydro provides a description of the area from which both uncontaminated and contaminated samples were taken.

For soils from Thicket Portage, uncontaminated samples were taken from the same auger hole at two different depths (0.2-1.0 meters and 1.5-2.0 meters) while the contaminated soil samples were also taken from the same auger hole at the same depths. A drawn aerial map supplied by Manitoba Hydro (Figure 3.3) provides a description of the area from which both uncontaminated and contaminated samples were taken.

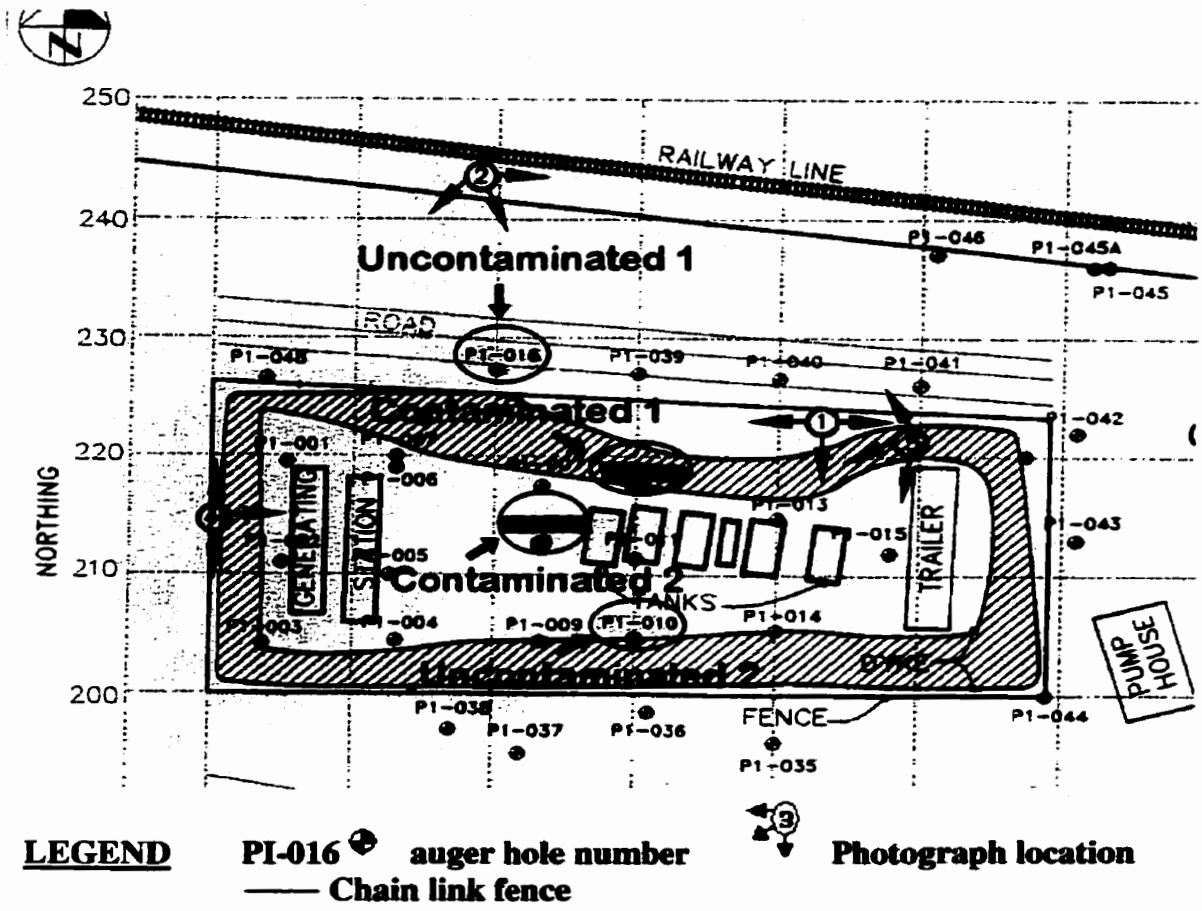
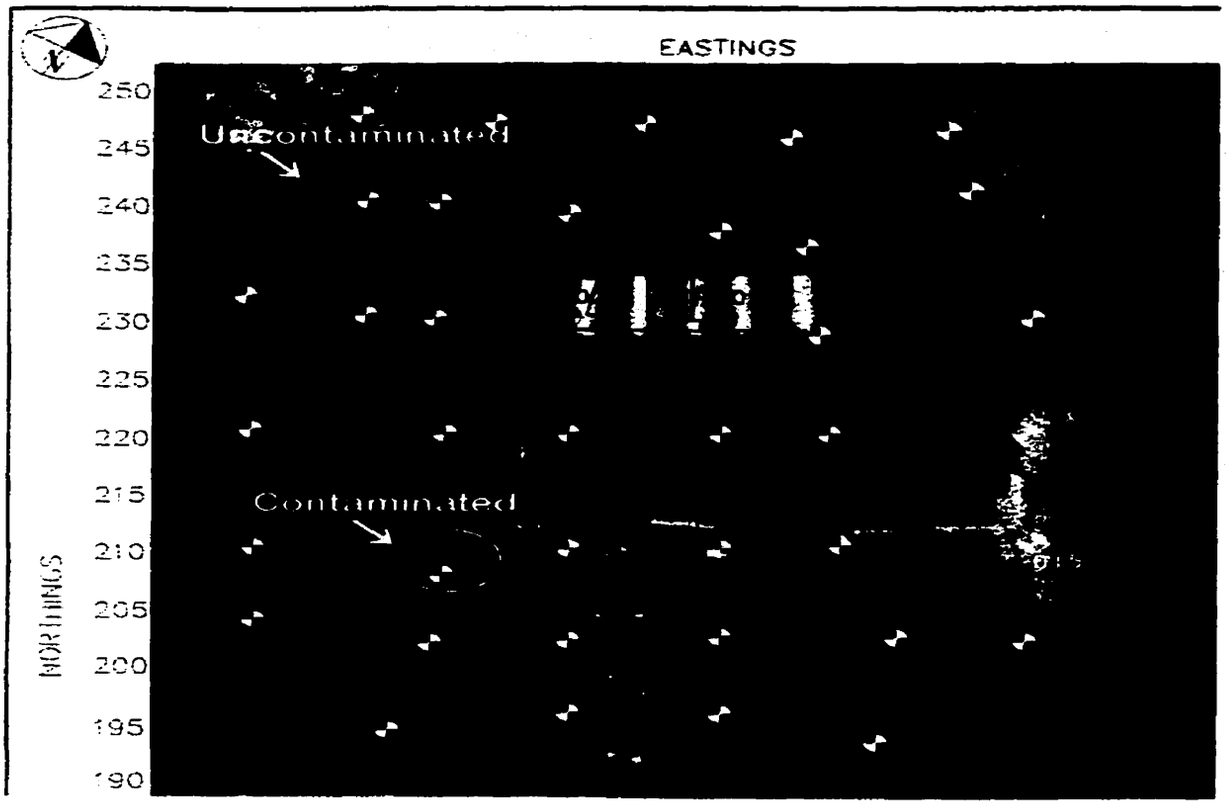


Figure 3.2 Drawn aerial map of Pikwitonei contaminated area including location of auger holes from which soils from this study were sampled.



LEGEND TP-015 auger hole number ——— Chain link fence

Figure 3.3 Aerial map of Thicket Portage contaminated area including location of auger holes from which soils from this study were taken.

3.1.2 Brandon

Soils from this industrial site were primarily contaminated with polycyclic aromatic hydrocarbons. This facility used to produce manufactured gas from coal from 1910 to 1949. The plant was initially operated by Brandon Gas and Power Company and was succeeded in 1922 by Canada Gas and Electrical Corporation. The plant was operated by private enterprise until 1931 after which Manitoba Power Commission took over its operation. The plant was eventually inherited by Manitoba Hydro, which used the property as a warehouse/stores operation, complete with pole yard. Manitoba Hydro collected samples from October 1997 to January 1998. All samples were stored at 4°

until analysis. Log data recorded by Manitoba Hydro personnel of each sample used in this study can be found in the Appendix.

Results of particle size analysis of soils from this area revealed soils of two textural classes, coarse sand and silty loam. A study by Carmichael *et al.* (1997) discovered a relationship between the fraction of silt and clay in contaminated soils and the percent of ^{14}C-PAH mineralized. Based on this finding, microbial experiments would be performed on this site by grouping the soils according to their textural classes. Although the finding by Carmichael *et al.* (1997) was concluded from only the determination of microbial respiration, it was decided that all experiments should be conducted following the same procedure to remain consistent throughout. However, the lack of soil samples varying in depth prevented some comparisons between contaminated and uncontaminated of similar textures. For this reason, some contaminated sand soils were compared to uncontaminated loam soils at three different depths (Table 3.5).

As previously mentioned, soils from this site were divided according to texture. Table 3.3 provides a description of the sand soils from the Brandon contaminated site according to auger hole (Figure 3.6).

Table 3.3 Description of contaminated and uncontaminated sand soils from Brandon.

Texture	CTM[a] or UNCTM[b]	Depth (m)	Auger hole[c]
Sand	UNCTM	1.5-2.0	Uncontaminated Sand 1
Sand	UNCTM	2.6-3.2	Uncontaminated Sand 2
Sand	UNCTM	3.1-3.7	Uncontaminated Sand 1
Sand	CTM	1.5-2.0	Contaminated Sand 1
Sand	CTM	2.6-3.2	Contaminated Sand 1
Sand	CTM	2.6-3.2	Contaminated Sand 2
Sand	CTM	3.1-3.7	Contaminated Sand 1

a = contaminated, b = uncontaminated, c = see Fig 3.4

Table 3.4 provides a summary of the pairings between contaminated and uncontaminated sand soils according to depth used for this study.

Table 3.4 Pairings between contaminated and uncontaminated sand soils from Brandon according to depth used throughout this study.

Depth (m)	Uncontaminated	Contaminated
1.5-2.0	Uncontaminated Sand 1	Contaminated Sand 1
2.6-3.1	Uncontaminated Sand 1	Contaminated Sand 1 and Sand 2
3.1-3.7	Uncontaminated Sand 2	Contaminated Sand 1

Table 3.5 provides a description of the contaminated and uncontaminated loam soils from the Brandon site according to auger hole location. As was previously discussed, due to the lack of samples from the contaminated site from Brandon, some contaminated sand soils were compared to uncontaminated loam soils (italicized).

Table 3.5 Description of contaminated and uncontaminated loam soils from Brandon. Contaminated sand soils used for comparisons with uncontaminated loam soils are italicized.

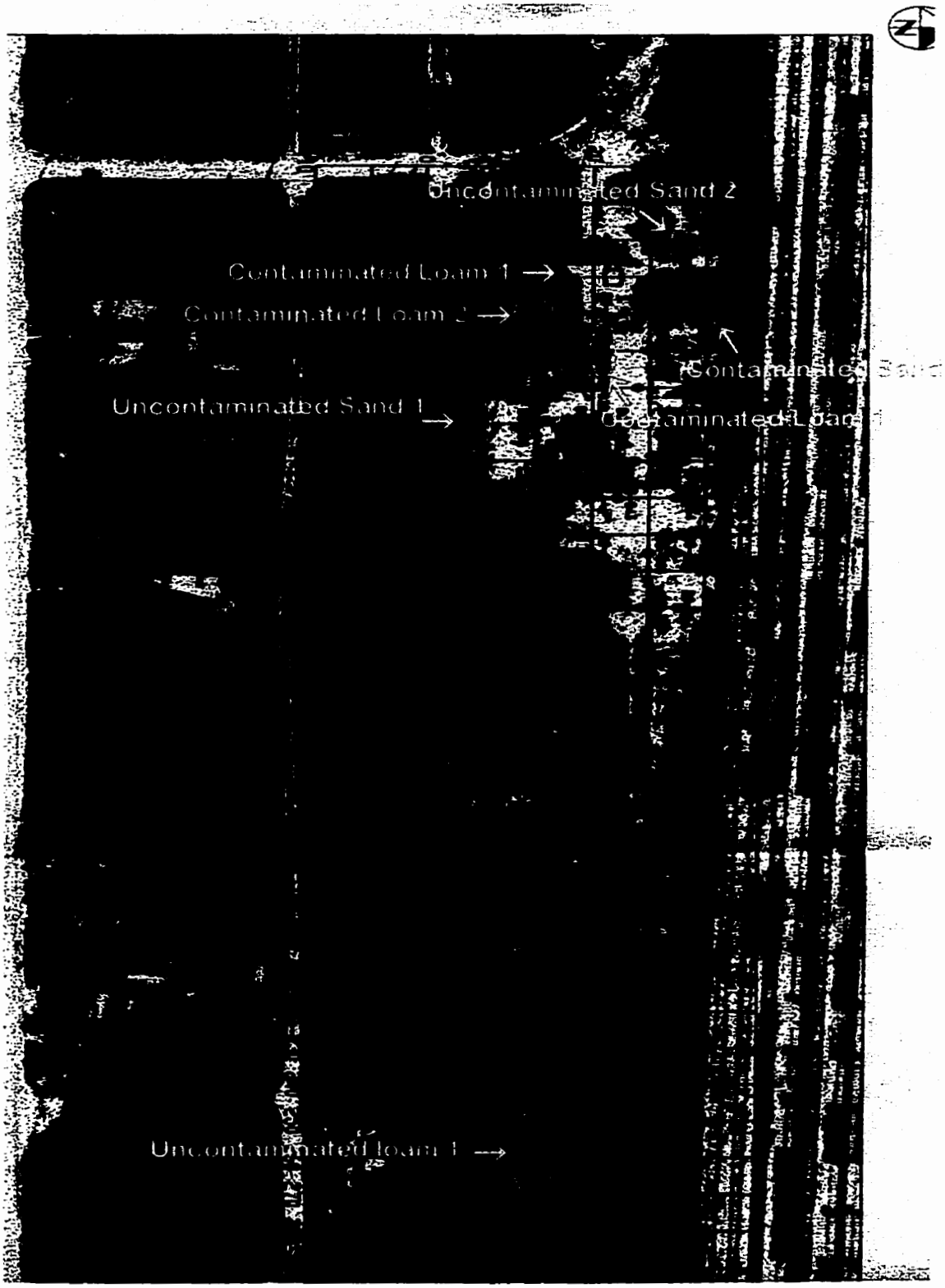
Texture	CTM[a] or UNCTM[b]	Depth (m)	Auger hole[c]
silty loam	UNCTM	1.5-2.0	Uncontaminated loam 1
silty loam	UNCTM	2.0-2.5	Uncontaminated loam 1
silty loam	UNCTM	2.75-3.5	Uncontaminated loam 1
silty loam	UNCTM	3.5-4.0	Uncontaminated loam 1
silty loam	UNCTM	4.0-4.5	Uncontaminated loam 1
silty loam	CTM	1.5-2.0	Contaminated loam 1
silty loam	CTM	2.0-2.5	Contaminated loam 2
silty loam	CTM	2.75-3.5	Contaminated loam 2
sand	*CTM*	*2.75-3.5*	*Contaminated sand 2*
sand	*CTM*	*3.5-4.0*	*Contaminated sand 2*
loam	CTM	4.0-4.5	Contaminated loam 1
sand	*CTM*	*4.0-4.5*	*Contaminated sand 2*

a = contaminated, b = uncontaminated, c = see Fig 3.4

Table 3.6 provides a summary of the pairings between contaminated and uncontaminated loam soils according to depth used for this study. The exceptions being the three contaminated sand soils (in italics) being compared to uncontaminated loam soils.

Table 3.6 Pairings between contaminated and uncontaminated loam soils from Brandon according to depth used throughout this study. Contaminated sand soils used in these pairings with uncontaminated loam soils are italicized.

Depth (m)	Uncontaminated	Contaminated
1.5-2.0	Uncontaminated loam 1	Contaminated loam 1
2.0-2.5	Uncontaminated loam 1	Contaminated loam 2
2.75-3.5	Uncontaminated loam 1	Contaminated loam 2 and *Contaminated sand 2*
3.5-4.0	Uncontaminated loam 1	Contaminated sand 2
4.0-4.5	Uncontaminated loam 1	Contaminated loam 1 and *Contaminated sand 2*



LEGEND BG-043 ◒ auger hole number ⟷ chain link fence

Figure 3.4 Aerial map of Brandon contaminated area including location of auger holes from which soils from this study were taken.

3.1.3 Somerset

A pipeline leak in October 1994 caused the release of crude oil onto agricultural land near the town of Somerset, MB. Soil was collected from the contaminated area as well and adjacent uncontaminated soil. Soils were sampled to a depth of 15 cm and were stored in plastic pails at 4°C prior to the start of the experiments. Enbridge Pipeline personnel sampled the soil in late September 1999. This site was also used in a study of the effects of a crude oil spill on crop productivity and biological quality and the potential for phytoremediation (Timmerman, 1999). Results of particle size analysis for the uncontaminated soil can be found in Table 3.7.

Figure 3.5 Aerial map of Somerset contaminated area including location of auger holes from which soils from this study were taken.

3.2 Agricultural soils

Three soils of varying texture from Glenlea, Brandon, and St. Claude (see map of Manitoba, Fig. 3.1) were sampled to assess the size of the native population of hydrocarbon-degraders in uncontaminated agricultural soils. These soils were chosen for their differences in textures and geographical location, providing a good range of the various soil types found in Manitoba. The soil was collected at intervals of 0-10 cm and 90-100 cm. Replicates were completed in quadruplicate and stored in airtight plastic bags at 4°C until the beginning of the experiments. Sampling of the soils was done in early September (1999) using Dutch augers.

Results of particle size analysis for both surface and subsurface soils can be found in Table 3.7. Description of soil series, classification, as well as legal description can be found in Table 3.8. Determination of field capacity, pH, and electrical conductivity for these agricultural soils can be found in Table 3.9.

Table 3.7 Particle size analyses and texture of agricultural soils selected for enumeration study.

Site	Particle size analysis			Texture
	%sand	%silt	%clay	
Somerset (UNCTM*)	13	44	43	clay loam
Glenlea 0-10 cm	4	19	77	heavy clay
90-100 cm	1	46	83	heavy clay
Brandon 0-10 cm	29	34	37	clay loam
90-100 cm	33	35	32	clay loam
St.Claude 0-10 cm	87	4	9	sand
90-100 cm	91	3	6	sand

*uncontaminated

Table 3.8 Soil classification and location of agricultural sites used in this study.

Site	Soil series	Soil classification	Site location
Glenlea	Osborne	Rego humic Gleysol	SW 3-8-3E
St.Claude	Almasippi	Gleyed rego black Chernozem	SE 10-9-8W
Brandon	Newdale	Orthic Black Chernozem	NE 31-12-18W

Table 3.9 Soil properties of agricultural soils.

Site	Field Capacity (%)	pH	EC (dS/m)
Glenlea 0-10cm	51.3±1.4	6.1	1.03
Glenlea 90-100cm	50.1±0.3	7.9	1.84
St.Claude 0-10cm	27.3±1.5	7.8	0.30
St.Claude 90-100cm	12.6±2.0	8.5	0.12
Brandon 0-10cm	36.6±2.5	7.0	0.73
Brandon 90-100cm	29.5±0.8	8.0	2.30
Somerset Uncontaminated	34.6±1.7	7.6	1.07

3.3 Hydrocarbon extraction

Ultrasonic extraction method (US EPA method 3550B) was used for the extraction of diesel fuel from the contaminated soils from Thicket Portage and Pikwitonei and PAHs from the contaminated soils from Brandon. This was done to get an indication of the contamination level of the various soil samples. This extraction method has proven to be an efficient method for the extraction of organic compounds from soils (Brilis *et al.*, 1990; Chen *et al.*, 1996; Evans *et al.*, 1998). All soils were extracted in duplicate. Previously contaminated and uncontaminated soil samples including agricultural soils were analysed for TEH or PAHs using gas chromatographic techniques with flame ionization detection.

3.3.1 Gas chromatography

All hydrocarbon extraction of the various contaminated soil samples were analysed by Varian Star 3400Cx gas chromatograph with flame ionization detector. A J&W

Scientific column (DB-5MS) with a 30 m length, 250 μm diameter, and 0.25 μm thickness was used to analyse the hydrocarbons. Compressed air (300 mL/min), carrier gas (prepurified helium 1.2 mL/min), and fuel (prepurified hydrogen 3 mL/min) were also used in the analysis. Each injection was run on a preset program using the Star Chromatography Software (Copyright® 1989-1995, Varian Associates, Inc., v. 4.02).

For diesel fuel or total extractable hydrocarbons (TEH) analysis of contaminated sites from Thicket Portage and Pikwitonei, a 2 μL sample was injected into the GC inlet. The injection temperature was 275°C and the detector temperature was 300°C. The oven parameters were: initiation temperature and time, 50°C for 4 minutes; program rate, 10°C/min; final temperature and time were 250°C for 5 min. Total run time was 30 minutes. This procedure was also used for the extraction of diesel fuel in test tubes from MPN experiment (see section 3.4.5.1).

For phenanthrene analysis, a 1 μL sample was injected into the GC inlet. This volume was adequate for a good peak response from the detector. The injection temperature was 275°C and the detector temperature was 275°C. The oven parameters were: initiation temperature and time, 125°C for 2.5 minutes; program rate, 20°C/min; final temperature and time were 275°C for 2 min. Total run time was 12 minutes.

For the determination of PAHs in contaminated soils from Brandon, a 1 μL sample of soil extract was injected into the GC inlet. Both injector and detector temperatures were maintained at 285°C. The temperature gradient consisted of two minute hold time at 50°C, a ramp to 130°C at 30°C/min followed by three minute hold time, a ramp to 240°C at 7°C/min, and a final ramp to 290°C at 12°C/min with a 7.47 minute hold time. Total run time was 35 minutes.

3.3.2 TEH determination

A 15 g soil sample was used for the analysis of total extractable hydrocarbons (TEH). For only heavy clay soils, an equal amount of sodium sulphate was used to make the sample free flowing. A mixture of 1:1 acetone and hexane (30 mL) was used as the extraction solvent. The sample-solvent slurry was sonicated for 2 minutes at 100% output and 50% duty cycle using a Model VibroCell 500 Sonic & Material. Following sonication, the sample was centrifuged at 3000 rpm for 10 minutes and the supernatant was decanted into a screw-capped glass vial. Sonication and centrifugation were performed twice for each sample. Samples with low levels of TEH (<1000μg/g) were concentrated by N_2 bleeding in a heated water bath (40°C). Extracts were analysed by gas chromatographic techniques previously mentioned.

3.3.3 PAH determination

Samples from each PAH-contaminated soil from Brandon were spiked with a 0.5 mL solution of surrogate compounds two hours prior to extraction as a quality control. The surrogate spiking solution contained naphthalene-d_8, spiked at a level of 20 mg/kg into the soils. A 15 g soil sample was used for the analysis of PAHs. For heavy clay soils, an equal amount of sodium sulphate was used to make the sample free flowing by removing excess water within the soil.

A mixture of 3:1 dichloromethane and acetone (30 mL) was used as the extraction solvent. Sonication procedures were similar to those mentioned in the previous section. Soil extracts were analysed for the following four PAHs: naphthalene, phenanthrene, pyrene, and benzo[a]pyrene.

3.4 TEH and PAHs levels

The concentrations of TEH found in soils from Thicket Portage and Pikwitonei are summarised in Table 3.10. Concentrations of naphthalene, phenanthrene, pyrene, and benzo[a]pyrene for the Brandon contaminated site can be found in Table 3.12. All sites were shown to have high concentrations of contaminant present according to the criteria determined and enforced by Manitoba Conservation. Levels of PAHs in the contaminated and uncontaminated soils from Somerset were below detection limit (Table 3.11). Extraction efficiencies for diesel ranged from 75% to 100% with an average recovery of approximately 87%. PAH extraction efficiencies from recovery of naphthalene-d_8 ranged from 68% to 85% with an average recovery of approximately 77%.

Table 3.10 Concentrations of total extractable hydrocarbon in Thicket Portage (TP) and Pikwitonei (Pik) soil samples.

Site and Depths (m)	TEH (μg/g)
TP Contaminated 1 (0.5-1.0)	6500
TP Uncontaminated 1 (0.5-1.0)	<5*
TP Contaminated 1 (1.5-2.0)	5200
TP Uncontaminated (1.5-2.0)	<5
Pik Contaminated 1 (0.75-1.0)	4400
Pik Uncontaminated 1 (0.75-1.0)	<5
Pik Contaminated 1 (1.25-1.75)	5700
Pik Uncontaminated 1 (1.25-1.75)	<5
Pik Contaminated 1 (1.75-2.25)	5600
Pik Uncontaminated 1 (1.75-2.25)	<5
Pik Contaminated 1 (2.0-2.5)	2200
Pik Uncontaminated 1 (2.0-2.5)	<5
Pik Contaminated 2 (2.5-3.0)	700
Pik Uncontaminated 2 (2.5-3.0)	<5
Acceptable Limits Level 1 (residential)	***500***
Level 2 (commercial)	***2000***
Level 3 (industrial)	***2000***

* less than limit of detection (5 μg/g)
**Manitoba Conservation Remediation Criteria (Manitoba Environment, 1993).

Table 3.11 Concentrations of PAHs and TEH in soils from Somerset.

Site and Depth (cm)	CTM[a] or UNCTM[b]	µg PAH/g soil	TEH (µg/g)
Somerset 0-15	UNCTM	<5[c]	<5
Somerset 0-15	CTM	<5	<5

a = contaminated, b = uncontaminated, c = less than limit of detection (5µg/g)

Table 3.12 Concentration of PAHs (µg/g) in soil samples from Brandon contaminated site.

Site and depth	Benzo[a]pyrene	Pyrene	Phenanthrene	Naphthalene
Contaminated Sand 1				
1.5-2.0m	27	34	14	<0.1*
2.6-3.2m	<0.1	0.5	0.6	<0.1
3.1-3.7m	<0.1	0.5	0.3	<0.1
Uncontaminated Sand 1				
1.5-2.0m	<0.1	<0.1	<0.1	<0.1
2.6-3.2m	<0.1	<0.1	<0.1	<0.1
Contaminated Sand 2				
2.6-3.2m	6	13	5	<0.1
3.1-3.5m	145	1500	2000	80
3.5-4.0m	130	1450	1500	40
4.0-4.5m	110	1300	1900	50
Uncontaminated Sand 2				
3.1-3.7m	<0.1	<0.1	<0.1	<0.1
Contaminated Loam 1				
1.5-2.0m	170	1400	1300	120
4.0-4.5m	1.3	4.3	4.0	0.4
Uncontaminated Loam 1				
1.5-2.0m	<0.1	<0.1	<0.1	<0.1
2.0-2.5m	<0.1	<0.1	<0.1	<0.1
2.75-3.5m	<0.1	<0.1	<0.1	<0.1
3.5-4.0m	<0.1	<0.1	<0.1	<0.1
4.0-4.5m	<0.1	<0.1	<0.1	<0.1
Contaminated Loam 2				
2.0-2.5m	120	1300	1700	60
2.75-3.5m	70	780	1700	1200
Remediation Criteria				
Land Use				
Agricultural	**0.1	0.1	0.1	**0.1
Residential/Parkland	**0.7	10	5	**0.6
Commercial	**0.7	100	50	**22
Industrial	**0.7	100	50	**22

*less than limit of detection (0.1 µg/g)

**indicates criteria taken from the CCME Recommended Canadian Soil Quality Guideline (March 1997) otherwise the criteria shown are from the CCME Interim Canadian Environmental Quality Criteria for Contaminated Soils.

The suitability of all the uncontaminated soil samples from all the contaminated sites was confirmed by the finding of contamination levels below detection. Although no contaminants were detected in the uncontaminated soils, the possibility of previous hydrocarbon exposure cannot be ruled out. Based on these findings, the use of these uncontaminated samples for comparisons with adjacent contaminated material is appropriate.

All agricultural surface and subsurface soils were also tested for the presence of both total extractable hydrocarbons and polycyclic aromatic hydrocarbons (Table 3.13). The possibility of previous hydrocarbon exposure onto these soils cannot be ruled out. The suitability of all agricultural soils as uncontaminated soils was confirmed by the results obtained by GC indicating contaminant levels below detection limit.

Table 3.13 Concentration of TEH and PAHs in agricultural surface and subsurface soils.

Site and Depth (cm)	TEH (μg/g)	μg PAH/g soil
Glenlea 0-10	<5*	<0.1**
90-100	<5	<0.1
Brandon 0-10	<5	<0.1
90-100	<5	<0.1
St.Claude 0-10	<5	<0.1
90-100	<5	<0.1

* less than limit of detection (5μg/g)
** less than limit of detection (0.1μg/g)

CHAPTER 4

THE ENUMERATION OF DIESEL AND PHENANTHRENE DEGRADING BACTERIA AS A MANAGEMENT TOOL FOR INTRINSIC REMEDIATION

4.1 Abstract

A most-probable-number (MPN) procedure was used to enumerate the two major groups of hydrocarbon (HC) degraders (aliphatic and aromatic) from soils previously contaminated with diesel fuel, coal tar, and crude oil. Adjacent uncontaminated soils sampled to allow an evaluation of the adaptation of the soil microbial community to the presence of HC-contaminants. In a parallel study, agricultural soils varying in texture were taken from three locations in Manitoba to determine the potential of their microbial populations to degrade hydrocarbons. These samples were collected from 0-10 cm and 90-100 cm depths in order to compare surface and subsurface HC-degrading populations.

All uncontaminated soils, except for two subsurface samples, yielded microbial populations capable of aliphatic and aromatic degradation. In most cases, contaminated soils yielded greater microbial numbers than their respective uncontaminated soils. This study confirms the ubiquitous distribution of both aliphatic and aromatic hydrocarbon degraders in native microbial populations found in uncontaminated and agricultural soils throughout the soil profile. Preexposure to contaminants generally resulted in increased

numbers of HC degraders, reflecting the development and adaptation of the appropriate microbial communities, through either growth or enzyme induction.

4.2 Introduction

The increasing use of petroleum hydrocarbons (HCs) has led to an increase in the probability of spills contaminating terrestrial environments across Canada. Increased environmental awareness of this problem has resulted in efforts to remediate these sites (CCME, 1991). The recognition of oil as a complex but largely biodegradable mixture of HC and the knowledge that microbial degraders can be enriched in many, if not most, types of environments (Atlas, 1981) has contributed greatly in the development of oil bioremediation techniques (Morgan *et al.*, 1989; Margesin and Schinner, 1996). The microbiological decontamination of oil-polluted soils is claimed to be an efficient, economic, versatile, and ecologically acceptable remediation alternative (Bartha, 1986). Petroleum is a complex mixture of many thousands of compounds, which can be divided into four major groups: alkanes, aromatics, resins, and asphaltenes. Alkanes are generally the most biodegradable fraction whereas polycyclic aromatic hydrocarbons (PAHs) are of intermediate biodegradability (Wrenn and Venosa, 1996). Degradation of PAHs in nature proceeds very slowly, resulting in their long-term persistence. PAHs are of most concern due to their toxicity and tendency to bioaccumulate. It has been suggested that the number of bacteria able to metabolize PAHs in the soil is too low to support PAH degradation in the environment (Kästner *et al.*, 1994). Enumeration of hydrocarbon degraders has been found to be accomplished more reliably by MPN

procedures than with agar based media, because they do not utilize media containing impurities found in highly purified agars which may support the growth of non-hydrocarbon degrading bacteria (Walker and Colwell, 1976; Sextone and Atlas, 1977; Randall and Hemmingsen, 1994; Wrenn and Venosa, 1996). Agar based media may contain organic contaminants which would allow nonselective microbial growth, leading to an overestimation of the hydrocarbon degrading population (Atlas, 1978).

Degradation rate of petroleum compounds has been shown to increase as the population adapts to the presence of the contaminant (Atlas, 1991; Wilson and Jones, 1993; Carmichael and Pfaender, 1997). A number of factors relating the environment of degradation including soil texture and extent of previous contamination will influence the degradation of these compounds, in relation to the adaptive response of the soil microbial community (Atlas, 1991; Carmichael and Pfaender, 1997). Knowledge of the potential of the soil microbial population to degrade HC through enumeration procedures may allow an assessment of the suitability of intrinsic remediation and may support its recommendation as a remedial option.

4.3 Objective of Study

The objective of this study was to enumerate the native microbial hydrocarbon (HC) degrading population in a range of Manitoba soils including: agricultural soils varying in texture at surface and subsurface depths; and sites with previous hydrocarbon exposure in relation to adjacent uncontaminated soils, at a variety of depths. The utility of enumeration as a potential management tool for intrinsic remediation will be considered.

4.4 Materials and Methods

4.4.1 Soils

Information pertaining to all soils, contaminated, adjacent uncontaminated, and agricultural, has been described in Chapter 3.

4.4.2 Most Probable Number (MPN) method

The MPN procedure (Alexander, 1982) was used to evaluate the numbers of HC-degrading microorganisms in all the soil samples, contaminated and uncontaminated. Numbers from contaminated sites were compared to their respective uncontaminated sites according to depth. The HC-degrading microbial communities were quantified using Bushnell-Haas (BH) medium with resazurin (Song and Bartha, 1990; Williams *et al.*, 1998). Resazurin is a growth indicator that changes colour, resulting from the reduction of resazurin by microbial oxygen consumption, easily identifying growth. Filter sterilized diesel fuel or phenanthrene was used as the sole carbon and energy sources for the enumeration of aliphatic or aromatic degraders, respectively. As diesel fuel is primarily composed of aliphatic HC, it was used as an indicator of the number of alkane degraders (Wrenn and Venosa, 1996). The numbers of HC degraders were expressed as the means and standard errors of the most probable number (MPN) g^{-1} of dry soil. MPNs were determined by using five test tubes of BH (10 mL), diesel or phenanthrene, and resazurin for each soil dilution. Inocula from serial dilutions of 10 g of soil in 90 mL of sterile water (1:10 dilution), ranging from 10^{-1} to 10^{-8} were taken. A 1 mL sample of phenanthrene was added to the test tubes as a solution in acetone (10 g/L). The acetone was allowed to evaporate under sterile conditions. For MPN-diesel, 50 μL of diesel fuel

filtered through a 0.2 μm-pore size PTFE membrane was added after inoculation. Test tubes were incubated at 20°C for one to two weeks.

Two sets of diluted contaminated soil samples, as well as uncontaminated soil samples were examined. One set contained diesel fuel while the other set did not. For the HC supplemented samples, the procedure determined the MPN of hydrocarbon degraders compared to the MPN of degrading microorganisms using non-hydrocarbon organic matter in the soil

4.4.2.1 Analysis of Hydrocarbons

After incubation for MPN experiment, two culture broths for each soil for the same dilution were shaken with 20 mL of hexane in order to extract residual hydrocarbon. The mixture was then centrifuged at 10000 rpm for 10 minutes at 5°C to remove the cells. This procedure was repeated twice. The extracts were concentrated (from 10 mL to 2 mL) by N_2 bleeding in a heated water bath (40°C) and analysed by gas chromatographic techniques (see Chap 3, section 3.3.1 for details).

Hydrocarbon degradation was calculated on the basis of the residual hydrocarbon in sterile controls run under the same conditions.

4.4.2.2 MPN and TEH time assay

A time course of population growth and hydrocarbon degradation using contaminated and uncontaminated soils from Somerset was conducted using the MPN procedure. Triplicate samples of thirty grams of soil (oven dry basis) were weighed into 60 mL glass vials and wet to field capacity. The soils were incubated at room temperature for approximately two months before the start of the experiment. After the incubation period, the vials containing the soil were placed into screwed capped 500 mL glass jars.

A 15 mL vial containing 10 mL water (pH 3) was included to humidify the air. Each sample of soil was contaminated with 5000 μg/g of diesel fuel in hexane (carrier solvent) and later placed in a fumehood to allow the hexane to volatilize. Time zero samples from both contaminated and uncontaminated soils were analysed in triplicate for both MPN and total extractable hydrocarbons (TEH) to determine initial numbers and concentrations, respectively. The time assay experiment was for a six-week period, with an MPN and TEH determinations done every week. Analysis of TEH remaining in the test tubes after MPN experiment for both contaminated and uncontaminated soils was also conducted to identify the rate of degradation. The MPN and both TEH determinations followed the procedures previously mentioned in this section. Sterile soils from both contaminated and uncontaminated sites were also included to determine the amount of diesel fuel loss from volatilization. Sterilization was achieved by autoclaving the soil at 121°C for 15 minutes.

4.4.3 Statistical Analysis

T-tests were conducted using SAS (Statistical Analysis System, SAS Institute, Cary, NC, USA).

4.5 Results

4.5.1 Most Probable Number of Diesel Degraders

Table 4.4 reports the most probable number assessment of HC degraders obtained using diesel fuel as the sole source of carbon and energy for microbial growth in the MPN procedure.

4.5.1.1 Thicket Portage and Pikwitonei

Significantly ($p \leq 0.05$) greater numbers of diesel degraders were detected in contaminated soils relative to uncontaminated soils at all depths for both Thicket Portage and Pikwitonei (Table 4.4). A decrease in microbial numbers was visible with increasing depth, except for the uncontaminated soil from the Pikwitonei site at depth of 1.25-1.75 metres. Although numbers were greater for the contaminated soils, hydrocarbon-degraders were also found at all depths for both uncontaminated soils. Control samples were used to confirm that the colour change in the test tubes was indicative of diesel degradation. Low background TEH values allowed these tubes to be used to confirm that colour change in the test tubes was indicative of diesel degradation. Confirmation of diesel degradation from decreased levels of diesel fuel (TEH) in uncontaminated soils is summarized in Table 4.5, for Thicket Portage and Pikwitonei.

Table 4.1Average most probable numbers of diesel degraders in contaminated versus uncontaminated soils according to depth (± standard deviation).

Site	Depth (m)	Uncontaminated soils MPN	Contaminated soils MPN	t-test (α=0.05)
Thicket Portage	0.2-1.0	420±160	51000±26000	S[a]
	1.5-2.0	150±80	6000±2900	S
Pikwitonei	0.75-1.0	90±8	57000±34000	S
	1.25-1.75	2±2	9000±3900	S
	1.75-2.25	61±50	6600±3600	S
	2.0-2.5	35±14	2200±1400	S
	2.5-3.0	9±6	340±200	S
Brandon (sand)	1.5-2.15	4300±2000	3300± 2500(sand 1)	NS[b]
	2.6-3.1	65±60	230±100 (sand 1)	S
	2.6-3.1		227000±110000 (sand 2)	S
	3.1-3.7	2700±500	940± 500(sand 1)	S
Brandon (loam)	1.5-2.0	540±380	21400±13000 (loam 1)	S
	2.0-2.5	55±40	14000±9200 (loam 2)	S
	2.75-3.5	2800±1900	150±80 (loam 2)	S
	2.75-3.5		60000±30000 (sand 2)	S
	3.5-4.0	15±7	63000±26000 (sand 2)	S
	4.0-4.5	3±2	200±0 (loam 1)	S
	4.0-4.5		9200±50 (sand 2)	S

a = significantly different, b = not significantly different

Table 4.2Confirmation of diesel fuel degradation from decreased levels of TEH (µg/g) in test tubes from MPN experiment for uncontaminated soils from Thicket Portage, Pikwitonei, and Brandon (± standard deviation).

Site	Depth (m)	TEH in test tubes (sterile)	TEH in test tubes (innoculated)
Thicket Portage	0.2-1.0	5100±70	3600
	1.5-2.0		4500
Pikwitonei	0.75-1.0	5300±50	4800±70
	1.25-1.75		4700±50
	1.75-2.25		5000±70
	2.0-2.5		5000
	2.5-3.0		5100
Brandon (sand)	1.5-2.15	5200±100	4600± 70 (sand 1)
	2.6-3.1		4800±70 (sand 2)
	3.1-3.7		4700±70 (sand 1)
Brandon (loam)	1.5-2.0	5200±100	4600±70 (loam 1)
	2.0-2.5		4700±70 (loam 1)
	2.75-3.5		4800 (loam 1)
	4.0-4.5		4900±100 (loam 1)

4.5.1.2 Brandon

Comparisons of soils were done according to their textural classes determined from particle size analysis (Table 4.4). Due to the limited number samples of the loam textured soils, a contaminated sand sample (sand 2) was compared to uncontaminated and contaminated loam samples at three depths ranges, 2.75-3.5 m, 3.5-4.0 m, and 4.0-4.5 m (see Chapter 3). Most of the contaminated soils from the Brandon site yielded significantly higher numbers than their respective uncontaminated soils. In some cases microbial numbers in contaminated and uncontaminated soils were not significantly different. At two sites, the uncontaminated soil (sand 1 at 3.1-3.7 m and loam 1 at 2.75-3.5 m) had significantly higher numbers than the contaminated soil. The relationship between a decrease in microbial numbers with increasing depth was not visible for the contaminated sand 1 and control loam 1 soil samples. The contaminated sand 2 samples produced high numbers at depths ranging from 3.5 to 4.5 meters when compared to numbers from more surface contaminated sand soils. Even the uncontaminated soils displayed organisms capable of diesel degradation at these great depths.

Uncontaminated soils were again used to confirm the degradation of diesel fuel in the tubes and can be seen in Table 4.5. Degradation was found in all the uncontaminated soils varying in depth.

4.5.1.3 Somerset and Agricultural Soils

The contaminated soil near the town of Somerset had a significantly greater numbers of HC degraders than its uncontaminated soil. A thousand-fold difference in numbers between contaminated and uncontaminated sites can be observed in Table 4.5. The surface soil from St.Claude had slightly fewer microorganisms than the other agricultural

soils (Table 4.6), which was not found to be significantly less than the numbers from the other agricultural soils (α=0.05).

The subsurface samples (90-100 cm) for the soils from Glenlea and Brandon yielded no diesel degraders (Table 4.6). The only subsurface diesel degraders were found in the soil sample from St.Claude, which was the only coarse textured soil among the three soils studied. Confirmation of diesel degradation was done using 1/100-soil dilutions and the decrease in TEH levels in inoculated versus sterile tubes can be seen in Table 4.7 for soils from Somerset and Table 4.8 for agricultural soils.

Table 4.3 MPN averages of diesel degraders in contaminated and adjacent uncontaminated soils from Somerset (± standard deviation).

Site and Depth (cm)	Uncontaminated MPN	Contaminated MPN	t-test (α=0.05)
Somerset 0-10	130±46	97000±60000	S*

* significantly different

Table 4.4 MPN averages of diesel degraders in agricultural surface and subsurface soils (± standard deviation).

Site	Depth (cm)	MPN
Glenlea	0-10	67±46a
	90-100	0
Brandon	0-10	76±47a
	90-100	0
St.Claude	0-10	28±6a
	90-100	18±2

a = not significantly different (α=0.05)

Table 4.5 Confirmation of diesel fuel degradation from decreased levels of TEH (μg/g) in test tubes from MPN experiment for contaminated and adjacent uncontaminated soils from Somerset (± standard deviation).

Site	Depth (cm)	TEH in test tubes (sterile)	TEH in test tubes (inoculated)
Somerset uncontaminated	0-10	5100±100	4500±70
Somerset contaminated	0-10		4200±100

Table 4.6 Confirmation of diesel fuel degradation from decreased levels of TEH (μg/g) in test tubes from MPN experiment for agricultural soils, contaminated and adjacent uncontaminated soils from Somerset (± standard deviation).

Site	Depth (cm)	TEH in test tubes (sterile)	TEH in test tubes (incoluated)
Glenlea	0-10	5100±100	4600±100
Brandon	0-10		4700±100
St.Claude	0-10		4600±50
St.Claude	90-100		4800
Somerset uncontaminated	0-10		4500±70
Somerset contaminated	0-10		4200±100

4.5.2 Most Probable Number of Phenanthrene Degraders

The following estimates of degrader populations were obtained using phenanthrene as the sole source of carbon and energy for microbial growth in the MPN procedure.

4.5.2.1 Thicket Portage and Pikwitonei

Significantly greater numbers of phenanthrene degraders were found in the contaminated soil samples versus their respective uncontaminated at all depths, for both Thicket Portage and Pikwitonei (Table 4.9).

A decrease in microbial numbers with increasing depth can be observed from results of soils from Thicket Portage. A decrease in microbial numbers with depth was not

consistent with the results for soils from Pikwitonei. All uncontaminated soils for these two sites displayed the presence of organisms able to degrade phenanthrene. Confirmation of phenanthrene degradation is summarized in Table 4.10 for Thicket Portage and Pikwitonei samples.

Table 4.7 Average most probable numbers (MPNs) of phenanthrene degraders in contaminated versus uncontaminated soils according to depth (± standard deviation).

Site	Depth (m)	Uncontaminated MPN	Contaminated MPN	t-test (α=0.05)
Thicket Portage	0.2-1.0	150±80	31000±20000	S[a]
	1.5-2.0	20±10	660±390	S
Pikwitonei	0.75-1.0	380±130	12200±8500	S
	1.25-1.75	60±45	3900±1900	S
	1.75-2.25	40±20	350±125	S
	2.0-2.5	360±160	6400±3600	S
	2.5-3.0	105±100	1100±625	S
Brandon (sand)	1.5-2.15	4900±2000	3300± 770(sand 1)	NS[b]
	2.6-3.1	20±10	400±150 (sand 1)	S
			14000±8200 (sand 2)	S
	3.1-3.7	3500±1800	1300± 140(sand 1)	S
Brandon (loam)	1.5-2.0	750±225	945000±450000 (loam 1)	S
	2.0-2.5	320±25	22000±5500 (loam 2)	S
	2.75-3.5	750±350	50±30 (loam 2)	S
			24000±10000 (sand 2)	S
	3.5-4.0	300±200	230000±1000 (sand 2)	S
	4.0-4.5	10±4	17000±6400 (loam 1)	S
			18000±4400 (sand 2)	S

a = significantly different, b = not significantly different

4.5.2.2 Brandon

Soils were compared according to texture, as previously mentioned in section 4.5.1.2. Most of the contaminated soils from the Brandon site yielded significantly higher numbers than their uncontaminated soils (α=0.05; Table 4.9). Microbial numbers between contaminated sand 1 and control sand 1 resulted in no significant difference. As

was the case with the diesel-MPN, controls sand 1 at 3.1-3.7 m and loam 1 at 2.65-3.5 m had significantly higher numbers than their respective contaminated soils.

Table 4.8 Confirmation of phenanthrene degradation from decreased levels (μg/g) in test tubes from MPN experiment for uncontaminated soils from Thicket Portage, Pikwitonei, and Brandon (± standard deviation).

Site	Depth (m)	Phenanthrene in test tubes (sterile)	Phenanthrene in test tubes (inoculated)
Thicket Portage	0.2-1.0	260±5	190±2
	1.5-2.0		240±5
Pikwitonei	0.75-1.0	250±6	200±10
	1.25-1.75		230±2
	1.75-2.25		220±3
	2.0-2.5		240±5
	2.5-3.0		240±2
Brandon (sand)	1.5-2.15	270±7	240± 7 (sand 1)
	2.6-3.1		250±2 (sand 2)
	3.1-3.7		250±6 (sand 1)
Brandon (loam)	1.5-2.0	270±7	200±6 (loam 1)
	2.0-2.5		230±15 (loam 1)
	2.75-3.5		240±5 (loam 1)
	3.5-4.0		250±5 (loam 2)

Also similar to the diesel-MPN experiments, high numbers of phenanthrene degraders were found at considerable depths (3.5-4.5 m) in the contaminated samples. All uncontaminated samples from this site yielded numbers of organisms capable of phenanthrene degradation. Uncontaminated soils were again used to confirm the degradation of phenanthrene in the test tubes. Degradation was found in all the uncontaminated soils (Table 4.10).

4.5.2.3 Agricultural Soils

Phenanthrene degraders were present in both surface and subsurface samples of all agricultural soils (Table 4.12). No significant differences were found either among the surface samples or among the subsurface samples. The number of phenanthrene degraders in previously contaminated soil near Somerset and its uncontaminated soil were not significant different (Table 4.11). The only significant difference that was found was when comparing surface and subsurface samples. Surface samples numbers were approximately 40-fold more numerous than their respective subsurface samples. Confirmation of phenanthrene degradation can be observed in Table 4.13 for the soils from Somerset and Table 4.14 for the agricultural surface and subsurface soils.

Table 4.9 MPN averages of phenanthrene degraders in contaminated and adjacent uncontaminated soils from Somerset (± standard deviation).

Site and Depth (cm)	Uncontaminated MPN	Contaminated MPN	t-test (α=0.05)
Somerset 0-10	1400±700	1800±560	NS*

* not significantly different

Table 4.10 MPN averages of phenanthrene degraders in agricultural soils, contaminated and adjacent uncontaminated soils from Somerset (± standard deviation).

Site	Depth (cm)	MPN
Glenlea	0-10	1500±800a
	90-100	41±7b
Brandon	0-10	960±290a
	90-100	30±13b
St.Claude	0-10	1200±700a
	90-100	17±7b

a = not significantly different, b = not significantly different

Table 4.11 Confirmation of phenanthrene degradation from decreased levels (μg/g) in test tubes from MPN experiment for contaminated and adjacent uncontaminated soils from Somerset (± standard deviation).

Site	Depth (cm)	Phenanthrene (μg/g) (sterile)	Phenanthrene (μg/g) (inoculated)
Somerset uncontaminated	0-10	250±7	210±7
Somerset contaminated	0-10		200±10

Table 4.12 Confirmation of phenanthrene degradation from decreased levels of (μg/g) in test tubes from MPN experiment for agricultural soils (± standard deviation).

Site	Depth (cm)	Phenanthrene (μg/g) (sterile)	Phenanthrene (μg/g) (+inoculated)
Glenlea	0-10	250±7	220±7
	90-100		240±7
Brandon	0-10		230±10
	90-100		240±5
St.Claude	0-10		210±7
	90-100		230±7

4.5.3 MPN-TEH time assay

Both uncontaminated and previously contaminated soils from Somerset were contaminated with the addition of 5000 μg of diesel fuel per gram of dry soil. The change in MPN of diesel degraders over the six-week incubation period in the uncontaminated and contaminated soils can be seen in Figure 4.1. After a small initial increase in diesel degraders, the uncontaminated soil had numbers similar to the contaminated soil (α=0.05) at weeks 2 and 3. Numbers from the uncontaminated soil decreased significantly compared to the contaminated soil for the remainder of the experiment. The increase in the number of diesel degraders coincided with a decrease in TEH concentrations extracted from the soil, an indicator of diesel fuel degradation (Figure 4.2). Surprisingly, the uncontaminated soil degraded more diesel fuel than the

contaminated soil. The extraction efficiency for this experiment ranged from 94% to 97% with an average recovery of 95%. Extraction of diesel fuel from the test tubes did not reveal any changes from week to week, but did confirm that degradation was occurring. The summary of these results can be found in Table 4.15.

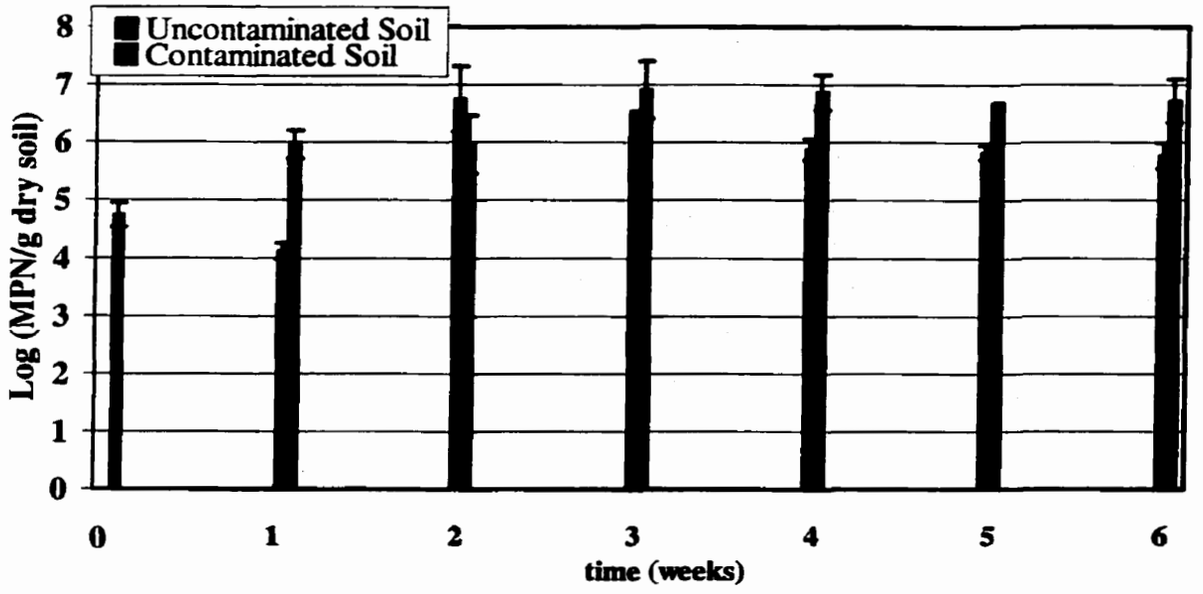


Figure 4.1 Comparison of MPN of diesel degraders in contaminated vs. uncontaminated soils from Somerset. Error bars represent the standard deviation of three replicates.

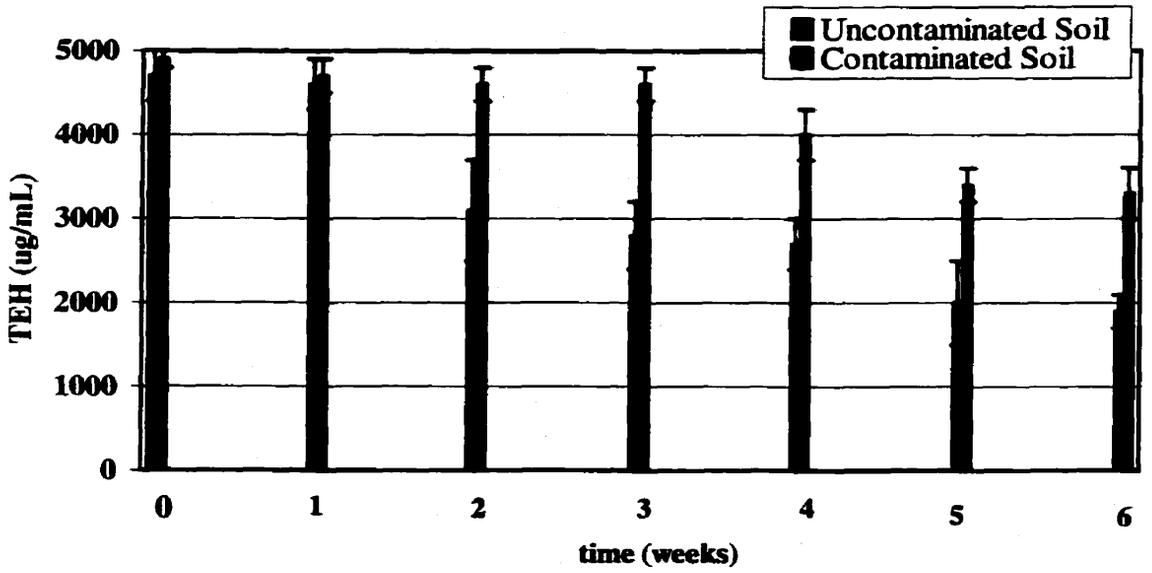


Figure 4.2 Decrease in TEH levels over six week period in contaminated and uncontaminated soils from Somerset. Error bars represent the standard error from three replicates.

Table 4.13 Average values from extraction of diesel fuel from MPN experiment over six-week period.

	TEH (µg/ml)						
	Week 0	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
Control (no soil)	5200+/-200	5000+/-100	5000+/-200	4900+/-300	4900+/-200	5100+/-100	5000+/-200
Uncontaminated Soil	5200+/-200	4600+/-200	4400+/-100	4700+/-100	4600+/-50	4500+/-100	4300+/-200
Contaminated Soil	4600+/-200	4700+/-300	4500+/-200	4600+/-50	4500+/-100	4600+/-100	4400+/-200

4.6 Discussion and Conclusions

4.6.1 MPN of diesel and phenanthrene degraders

4.6.1.1 Contaminated vs. Uncontaminated Soils

The enumeration of MPN using diesel fuel or phenanthrene as sole carbon sources indicated that uncontaminated soils, at all depths sampled, yielded microbial populations with the potential to degrade these hydrocarbons. This confirms a previous finding that uncontaminated soils contain small populations of hydrocarbon degrading microorganisms (Carmichael and Pfaender, 1996). The only exceptions were the agricultural subsurface soils (90-100 cm) from Glenlea and Brandon. As the microbial population decreases with increasing depth, the microbial population of these subsurface samples was most likely minimal, resulting in too few organisms capable of HC degradation.

As the microorganisms that degrade aromatic hydrocarbons (phenanthrene) are distinct from those that attacks aliphatic hydrocarbons (diesel) (Foght *et al.*, 1990; Atlas, 1991; Wrenn and Venosa, 1996), these uncontaminated soils contained microbial populations that could degrade both aromatic and aliphatic hydrocarbons. The analysis of the test tubes with 'positive' growth (ie. colour change) using gas chromatography confirmed the breakdown of the diesel fuel or phenanthrene. The use of control tubes containing neither of the hydrocarbons also confirmed that the organisms were degrading the hydrocarbons and not soil organic matter extracted during the preparation of the microbial suspensions.

A significant difference between contaminated and uncontaminated soils from Somerset was found using diesel fuel as the growth substrate but not when phenanthrene was used. The favoured selection of aliphatic degraders in the contaminated soil may be due to factors such as the characteristics of the substrate and nature of the contaminant. The degradative processes occurring in this soil may have been more adept in utilizing simpler substrates (aliphatic) than complex substrates (PAHs).

The MPN time-assay experiment showed that a large population of hydrocarbon degraders, which was steadily maintained throughout the experiment, dominated the contaminated soil from Somerset. As this soil had previous hydrocarbon exposure, no drastic change in the HC-degrading population was observed. The population was initially very numerous and was steadily maintained throughout the experiment. Few HC-degraders were found in the uncontaminated soil at the start of the experiment. With time, the population of degraders increased in the uncontaminated soil, having similar numbers to the contaminated soil within two weeks following diesel fuel addition. The increase in HC-degraders is also consistent with the decrease in levels of TEH throughout the incubation. The uncontaminated soil degraded more diesel fuel than the contaminated soil. The activity of the microbial community in the uncontaminated soil increased at a higher rate than in the contaminated soil, possibly due to the dramatic increase in HC-degraders, which required more substrate. This may also reflect other impediments to the population of HC degraders in the contaminated soil, such as toxicity from addition of diesel fuel. The population of HC-degraders in the contaminated soil was more constant, which resulted in a more stable microbial activity or possibly that the

microbial population was constrained by factors other than substrate availability resulting in a lower concentration of diesel degraded in the six-week period.

The increased numbers of diesel and phenanthrene degraders in the diesel-contaminated soils of Thicket Portage and Pikwitonei reflect the adaptation of these microbial communities to the presence of diesel fuel. This adaptation exhibits selective enrichment of competent organisms (Atlas, 1991). A high number of diesel-degrading microorganisms in diesel-contaminated soils can be explained by the long-lasting contamination of the soil (Margesin and Schinner, 1997). Both soils from Thicket Portage and Pikwitonei had a contamination event lasting approximately 30 years, which resulted in a change in the composition of the microbial community. Both contaminated and uncontaminated soils displayed the potential to also degrade aromatic hydrocarbons for these two sites. The presence of the diesel fuel supported the growth of the aliphatic degraders to a greater extent than for the aromatic degraders. Polyaromatics can make up a significant component of diesel fuel varying from 10-30% (Block *et al.*, 1991). This likely reflects the relative recalcitrance of aliphatic versus aromatic compounds. The decrease in numbers with increasing depth for these two sites was expected as microbial populations decrease with increasing depth, again reflecting the relative availability of substrates to support growth.

For the most part, soils from the Brandon coal tar contaminated site yielded higher numbers than their respective uncontaminated soils for both MPN using diesel and phenanthrene. The contaminated soil samples that were not significantly different from their uncontaminated soils (contaminated sand 1) were found to have the lowest concentrations of PAHs. This could indicate that the concentration of PAH was less than

that required for the induction of enzymatic pathways capable of degrading hydrocarbons. PAH-contaminated sites may contain a considerable number of PAH-degrading microorganisms, but a minimum concentration of PAH must be present in the soil in order to select and stimulate the growth of the PAH-degrading microflora (Kästner *et al.*, 1994). Kästner *et al.* (1994) did not give these minimum concentrations, but the two contaminated soils that were examined in their study had levels of 3700 μg PAH/g soil and 2000 μg/g soil, with both soils having high numbers of HC degraders (MPN>10^5). Carmichael and Pfaender (1997) suggested that pre-exposure of soils to concentrations of PAHs greater than background (in this case being >0.08 μg/g soil) appeared to be needed to develop a substantial population of PAH-degrading microorganisms.

The temperature for the MPN experiments was 20°C, which is above the ambient soil temperature for all of the sites. This was done to ensure rapid growth of HC-degrading population from the soils, if any was to be found. Sims and Overcash (1983) reviewed a study of the effect of temperature change on rates of degradation. It was found that degradation of phenanthrene increased by approximately 20% for each 5°C temperature over a range of 15°C to 37°C, with maximum degradation of 90% occurring at 37°C (at concentration of 1000 μg/L). The determination of strategies for implementing intrinsic remediation techniques would require that the laboratory conditions closely relate to the actual conditions found at these sites. This would accurately test the HC-degrading ability of the natural microbial population, providing a better assessment of the intrinsic remediation potential. Results from a study using soils from a crude oil spill near the Alaska-British Columbia border, conducted MPN experiments ranging from 5 to 8°C.

Wilson (1999) found that an indigenous cold-adapted bacterial population was present and capable of bioremediation of the HC-contamination in the soil. This population was adequate to decrease the levels of the contaminants to acceptable levels. Cold-adapted HC-degrading populations would most likely be found in the soils used for this study.

This study confirms the ubiquitous distribution of both aliphatic and aromatic hydrocarbon degraders in native microbial populations found in uncontaminated and agricultural soils throughout the soil profile. Contaminant preexposure generally resulted in increased numbers of HC degraders, reflecting the development and adaptation of the appropriate microbial communities, through either growth or enzyme induction (Carmichael and Pfaender, 1990; Atlas 1991; Wrenn and Venosa, 1996). Previous findings that microorganisms that degrade aromatic HCs are distinct from those that attack aliphatic HC (Foght *et al.*, 1990; Atlas, 1991; Wrenn and Venosa, 1996) confirmed the importance of finding both groups of HC degraders in native microbial populations. Even when a microbial population with the capability to degrade both aliphatic and aromatic HC are present in a soil, important information regarding the structure of the community can be obtained by separately enumerating the alkane- and aromatic-degraders (Wrenn and Venosa, 1997). In this case, diesel fuel provided a reasonable source for aliphatic the HC-degrading community. The use of a single HC, such as hexadecane, would have provided a better indication of aliphatic degradation. Aliphatic and aromatic HC appear to be degraded by two distinct subsets of HC-degrading microbial populations and thus the ability to distinguish between alkane- and PAH-degrading microorganisms is an important feature of these MPN procedures (Wrenn and Venosa, 1997). The long lasting contamination of the previously contaminated examined

in this study, having high numbers of HC degraders, likely reflects environmental restrictions on the activity of these HC degraders, such as low substrate availability due to sorption. This would indicate that the system is not necessarily limited by the composition of the microbial community. This work concludes that the MPN procedure provides a means for simultaneously determining the populations of aliphatic- and aromatic-degraders. Variations of this method, such as using microtiter plates that would eliminate the need for test tubes, could be simple enough for the field such as and should provide a useful tool for characterizing petroleum-contaminated environments or for monitoring the progress of bioremediation (Wrenn and Venosa, 1997).

Although this method does provide important information of the presence of aliphatic and aromatic degraders in uncontaminated soils, the number of degraders required for successful intrinsic remediation to occur is not known. Although this method does provide information of the numbers of HC degraders, it does not give any indication of the activity of microorganisms in the soils. From this study, the usefulness of enumeration as a management tool for implementing intrinsic remediation cannot fully be determined. A more detailed study identifying the numbers of HC degraders required for successful remediation could be done using the MPN procedure and would provide a more accurate description of the usefulness of enumeration as a management tool for implementing intrinsic remediation.

CHAPTER 5

SOIL METABOLIC DIVERSITY AS A POTENTIAL MANAGEMENT TOOL FOR INTRINSIC REMEDIATION

5.1 Abstract

The functional diversity of microbial communities in hydrocarbon contaminated soils was examined using Gram-negative Biolog™ plates. The functional diversity of contaminated soils was compared to adjacent uncontaminated soils according to depth. Agricultural soils varying in texture and climate, taken from three locations in Manitoba, were also evaluated to observe the microbial diversity of autochthonous microbial communities in surface and subsurface samples. The results indicated no significant differences on the microbial diversity in contaminated versus uncontaminated soils. Most results revealed no significant negative effect on the substrate utilization patterns of microbial communities in contaminated soils. In some cases, especially at greater depths, microbial communities in contaminated soils had demonstrated enhanced substrate utilization capabilities, indicating an adaptive response of the populations to the presence of the contaminants or the selection of microorganisms with broad substrate utilization capabilities. The contamination event did not seem to have an affect on the microbial diversities of soil microorganisms. As a potential remediation tool, metabolic diversity provided useful information indicating that the contamination event did not disrupt the

diversity of the site, seen from the lack of difference between contaminated and uncontaminated soils indicate

5.2 Introduction

Assessments of strategies and outcomes of bioremediation would benefit from comprehensive knowledge of the microbial diversity of the autochthonous communities of natural ecosystems and their degradative potential (Wünsche *et al.*, 1995; Becker *et al.*, 1995). Environmental stress or disturbances upset the ecological balance of population interactions, generally resulting in decreased microbial diversity (Atlas *et al.*, 1991) and a shift in the dominant population. As the dominant population can persist within the disturbed communities, they become highly specialized in adapting to the selection pressure of the "new" environment. This shift in population results in a change in the structure or function of the microbial communities, which might be identified by changes in substrate utilization patterns. The dominant populations within perturbed communities have enhanced physiological tolerances and substrate utilization capabilities (Atlas *et al.*, 1991). Gram-negative Biolog plates (Biolog Inc., 3938 Trust Way, Hayward, CA 94545, USA) are useful in producing sole-carbon-source-utilization patterns of microbial samples to estimate community structure. Biolog plates are an advantageous technique because it is rapid and inexpensive, providing data to assess similarities and differences between microbial communities (Garland & Mills, 1991; Zak *et al.*, 1994).

5.3 Objective of Study

The objective of this study was to determine the usefulness of metabolic diversity in contaminated and adjacent uncontaminated soils as a potential management tool for implementing strategies for intrinsic remediation. In conjunction, three agricultural soils varying in texture were also used in this study to determine the substrate utilization patterns of the indigenous microflora and potential for remediation efforts.

5.4 Materials and Methods

5.4.1 Soils

All soils, contaminated, adjacent uncontaminated, and agricultural, have been described in Chapter 3.

5.4.2 Biolog test for metabolic diversity

Gram-negative Biolog plates containing 95 sole carbon sources were purchased from Biolog Inc. (Hayward, CA). Each plate has 96 wells, 95 having different carbon sources and 1 control with no carbon source. A dried mixture of peptone, nutrients, salts, and redox tetrazolium dye are included with the carbon source to ensure growth of the organisms. The dye provides an indication for metabolism. After inoculation, wells remained colourless until metabolic activity reduced the tetrazolium dye to a purple colour indicating a positive test (Zak *et al.*, 1994). Physiological saline (0.85%, 8.5 g NaCl in 1000 mL distilled water) and Bushnell-Haas broth were prepared and dispensed into 100 mL milk dilution bottles. Bottle 1 contained 90 mL of 0.85% saline while bottle 2 contained 99 mL of Bushnell-Haas broth. Bushnell-Haas broth was used for this experiment as it was also used with the MPN experiments. The broth would allow the

growth of similar microbial populations to those grown in the MPN experiments, as it contains a variety of salts. All bottles were then autoclaved. Once the cycle was complete, all bottles were cooled to room temperature. Ten grams of soil (oven dry weight) was added to Bottle 1 and shaken for 5 minutes. A 1 mL subsample of this solution was then transferred to Bottle 2 (10^{-3} dilution) and shaken. This final dilution was then poured into sterile plastic reagent reservoirs (Eppendorf, Inc.) while inside a sterile hood (LABCONCO® Purifier™ Clean Bench). Soil solutions were dispensed into the 96 well plates in 100μL volumes using a multichannel pipette (Eppendorf 8-channel Repeater™ Pipette) and incubated at 25°C for 7 days. This was the same incubation time that was followed for the MPN experiment. The differentiation and appropriate grouping of natural microbial communities according to the number of utilized substrates seem to be possible using incubation periods of 48 hours or more (Wünsche *et al.*, 1995). At this time, the Biolog patterns most probably reflect the metabolic diversity of the quantitatively dominating components of the microbial communities (Wünsche *et al.*, 1995). A substantial increase in the number of utilized substrates after 48 hours might indicate metabolic activities of taxa which only occur in low quantities or which can grow very slowly under the given conditions (Wünsche *et al.*, 1995). Quadruplicate plates were inoculated from each soil sample.

A change in the tetrazolium dye colour was assigned a value ranging from 1 to 4, with one indicating a slight colour change and four indicating an intense change. This was done using the author's judgement. The change in colour was always compared to that in the control well to ensure that the control remained colourless. The degree to which a particular substrate is utilized can be quantified by observing a colour change caused by

the incorporation of tetrazolium dye into a respiring bacterial community (Zak *et al.*, 1994). Substrate richness represent the percentage of substrates metabolized over the seven-day incubation and is an indicator of the functional diversity or metabolic capability of the microbial community.

5.4.3 Data Analysis

Statistical analysis was performed using SAS (Statistical Analysis System, SAS Institute, Cary, NC, USA) version 8.0. Principle Component Analysis (PCA) and Cluster Analysis were used to analyze the data for the contaminated sites of Thicket Portage, Pikwitonei, and Brandon. PCA required the reduction in the number of variables, as the procedure cannot be performed on a data set containing more variables than observations. The Biolog data were grouped into six categories according to Zak *et al.* (1994). The categories were as follows: (1) carbohydrate, 30 substrates; (2) carboxylic acids, 24 substrates; (3) polymers, 5 substrates; (4) amines/amides, 6 substrates; (5) amino acids, 20 substrates; (6) miscellaneous, 10 substrates. A list of substrates from the Gram-negative Biolog plates and their category placement can be found in Table 4.2. A total of four PCA's were done to evaluate the data between contaminated and adjacent uncontaminated soils. PCA's were divided as follows: (1) Thicket Portage; (2) Pikwitonei; (3) Brandon; (4) agricultural soils, which included contaminated and uncontaminated soils from Somerset. For each of these locations, the Biolog data sheets for each soil sample were individually analyzed. From the four replicates for each soil sample from a location, if each individual substrate was utilized three or more times, a score of one was given for that substrate in one of the six categories. If less than three was utilized, no score was given. This was done for all six substrate categories. The sums of each category were divided by total substrates for that category. The percentage

of substrate utilized for each category was used for PCA to directly compare the utilization patterns between the soil samples for a specific location.

For cluster analysis, in order to directly compare pairs of substrate utilization patterns, simple matching coefficients S_m were calculated:

$$S_m = N_p/N_o$$

N_p = number of positive matches; N_o = number of total substrates (here $N_o = 95$)

For each soil sample from a location, the quadruplicate replicates were compared and coded by a value of one if three or greater of an individual substrate was utilized. If less than three, no score was given.

The goal was to uncover structural differences between samples for a specific site, using a hierarchical cluster analysis. A total of four cluster analyses were done, divided similarly as with the PCA.

Table 5.1 Carbon substrates included in the Gram-negative Biolog plates categorized by substrate categories.

Carbohydrates	Carboxylic Acids	Amino Acids	Miscellaneous
adonitol	acetic acid	D,L-carnitine	2,3-butanediol
α-D-glucose	α-hydroxybutyric acid	D-alanine	bromosuccinic acid
α-D-lactose	α-ketobutyric acid	D-serine	D,L,-α-glycerolphosphate
α-methyl-D-glucoside	α-ketoglutaric acid	γ-aminobutyric acid	glucose-1-phosphate
cellobiose	α-ketovalerica acid	glycyl-L-aspartic acid	glucose-6-phosphate
D-arabitol	β-hydroxybutyric acid	glycyl-L-glutamic acid	glycerol
D-fructose	*cis*-aconitic aicd	hydroxy-L-proline	inosine
D-galactose	citric acid	L-alanine	thymidine
D-mannitol	D,L-lactic acid	L-alanyl-glycine	uridine
D-mannose	D-galactonic acid lactone	L-asparagine	urocanic acid
D-meliobiose	D-galacturonic acid	L-aspartic acid	
D-raffinose	D-gluconic aicd	L-glutamic aicd	
D-psicose	D-glucosaminic acid	L-histidine	
D-sorbitol	D-glucuronic acid	L-leucine	**Amines/Amides**
D-trehalose	D-saccharic acid	L-ornithine	2-amino-ethanol
gentiobiose	formic acid	L-phenylalanine	alaninamide
i-erythritol	γ-hydroxy-butyric acid	L-proline	glucuronamide
L-arabinose	itaconic acid	L-pyroglutamic acid	phenyl-ethylamine
L-fucose	malonic acid	L-serine	putrescine
L-rhamnose	*p*-hydroxy-phenylacetic acid	L-threonine	succinamic acid
lactulose	propionic acid		
m-inositol	quinic acid		
maltose	sebacic acid		
methyl pyruvate	succinic acid	**Polymers**	
mono-methyl succinate		α-cyclodextrin	
N-acetyl-D-galactosamine		β-cyclodextrin	
N-acetyl-D-glucosamine		dextrin	
sucrose		glycogen	
tyranose		tween 40	
xylitol		tween 80	

5.5 Results

5.5.1 Metabolic Diversity of Soils

5.5.1.1 Thicket Portage, Pikwitonei, and Brandon

The percentage of substrate utilized (metabolic diversity) was significantly greater in the contaminated sample than in the uncontaminated sample for only the soil from 0.2-1.0 m depth from Thicket Portage (Table 5.3). Contaminated soils from 0.75-1.0 and 2.5-3.0 meters from Pikwitonei had greater metabolic diversity than their respective uncontaminated soils (Table 5.3). The metabolic diversity for the contaminated samples

at depths of 1.25-175m and 2.0-2.5m were not significantly different than their respective uncontaminated soils, while the contaminated sample at the 1.75-2.25m depth lower metabolic diversity than the uncontaminated.

The differences in metabolic diversity between contaminated and uncontaminated soil samples from Brandon varied considerably and are summarized in Table 5.3. The contaminated sand 2 samples, having high concentrations of PAHs, were found to have the highest metabolic diversity (Table 5.3). The contaminated loams also had high levels of PAHs but only in one case (contaminated loam 1 at 4.0-4.5m) was the metabolic diversity significantly greater than its respective uncontaminated soil.

Table 5.2 Percent substrate utilization of contaminated vs. adjacent uncontaminated soils from Thicket Portage, Pikwitonei, and Brandon, according to depth.

Site	Depth (m)	Percent substrate utilization		t-test (α=0.05)
		Uncontaminated soils	Contaminated soils	
Thicket Portage	0.2-1.0	10.4±2.6	23.4±2.7	S[a]
	1.5-2.0	35.9±3.1	32±3.9	NS[b]
Pikwitonei	0.75-1.0	8.3±2.5	41.1±3.4	S
	1.25-1.75	43.8±5.2	41.4±2.6	NS
	1.75-2.25	21.1±2.6	7.3±1.2	S
	2.0-2.5	32±0.9	28.6±4.6	NS
	2.5-3.0	11.5±0.9	29.2±0.7	S
Brandon (sand)	1.5-2.0	56.5±1.6	64.3± 2.2(sand 1)	S
	2.6-3.1	39.8±3.5	52.9±1.5 (sand 1)	NS
			77.1±1.9 (sand 2)	S
	3.1-3.7	57.3±4.5	52.9±3.1(sand 1)	NS
Brandon (loam)	1.5-2.0	59.4±2.8	57.3±1.5 (loam 1)	NS
	2.0-2.5	57.3±1.5	57.8±3.5 (loam 2)	NS
	2.75-3.5	56±2.6	42.7±3.1 (loam 2)	NS
			80.5±3.5 (sand 2)	S
	3.5-4.0	67.2±1.3	79.2±0.9 (sand 2)	S
	4.0-4.5	2.2±1.4	55.7±6.9 (loam 1)	S
			73.2±1.0 (sand 2)	S

a = significantly different, b = not significantly different

Generally, a decrease in microbial diversity is observed with increase in depth from the lack of substrate and nutrients (Smith *et al.*, 1993). For the most part, this was not the case in this study as some subsurface were more active than surface soils in utilizing greater number of substrates, probably due to the presence of hydrocarbons (substrate) to the subsurface.

5.5.1.2 Agricultural Soils

In the soils sampled from agricultural sites, surface soils were much more active in degrading substrates than the subsurface. A high percentage of substrates utilized for the agricultural surface soils were expected, as the microbial communities would have an abundance of nutrients available to them, promoting microbial growth and diversity Table 5.4). The metabolic diversity of the contaminated soil from Somerset was not significantly different than its uncontaminated soil (α=0.05) (Table 5.5).

Table 5.3 Percent substrate utilization in surface and subsurface agricultural soils.

Site	Depth (cm)	%Substrate utilization
Glenlea	0-10	86.2±1.8
	90-100	61.5±2.4
Brandon	0-10	86.7±1.6
	90-100	58.6±2.9
St.Claude	0-10	71.9±3.0
	90-100	64.3±1.0

Table 5.4 Percent substrate utilization in contaminated and uncontaminated soils from Somerset.

Site	Depth (cm)	%Substrate utilization	t-test (α=0.05)
Somerset contaminated	0-10	73.4±2.8	NS*
Somerset uncontaminated	0-10	78.9±1.0	

* not significantly different

5.5.2 Principle Component Analysis

A principle component analysis (PCA) was performed to identify the minimum number of common elements or principle components, defined as eigenvalues. The information will describe the variation of the first two or three principle components. The number of components to best summarize the data was chosen with an accountability of total variance of $\geq$85%. Substrates were grouped in functional classes for this analysis (Table 5.1) to reduce the number of variables. The first principle component (Prin1) identified the overall substrate utilization patterns of the various soils samples, and in the following examples, Prin1 was represented on the x-axis (Fig. 5.1). A decrease in the overall utilization patterns is represented moving from right to left on the PCA graphs. The second component (y-axis) represented the substrate categories with the highest positive and highest negative loadings (percentages), defined as eigenvectors. On the PCA graphs from the y-axis (Prin2 or Prin3) viewpoint, points at the bottom of the graph represented data with a low positive to negative loading ratios while those at the top had high positive to negative ratios. The substrate categories with these positive and negative loadings as well as the number of components required are summarized in Table 5.6 for each of the locations.

Table 5.5 Summary of results obtained from PCA for all locations used in study.

Location	# components required for data summary (≥85%)	Accountability of total variance	Component with high (+) loading (1^{st})	Component with high (-) loading (1^{st})	Component with high (+) loading (2^{nd})	Component with high (-) loading (2^{nd})
Thicket Portage	2	94%	amines/ amides	carbohydrates	-*	-
Pikwitonei	3	89%	amines/ amides	miscellaneous	polymers	carbohydrates
Brandon	2	85%	polymers	amines/ amides	-	-
Ag soils	2	89%	amines/ amides	carbohydrates	-	-

*not required

5.5.2.1 Thicket Portage, Pikwitonei, and Brandon

PCA of samples from Thicket Portage (Fig. 5.1) revealed differences in substrate utilization patterns between contaminated and uncontaminated soils. The contaminated samples were closely grouped reflecting similar substrate consumption, while the uncontaminated samples were distanced in regards to the second component (Prin2). Analysis of data from Pikwitonei for components Prin1 and Prin2 did not show differences in substrate consumption patterns between uncontaminated and contaminated samples (Fig. 5.2), as data points for both were clustered together. The same was observed with components Prin1 and Prin3 (Fig. 5.3).

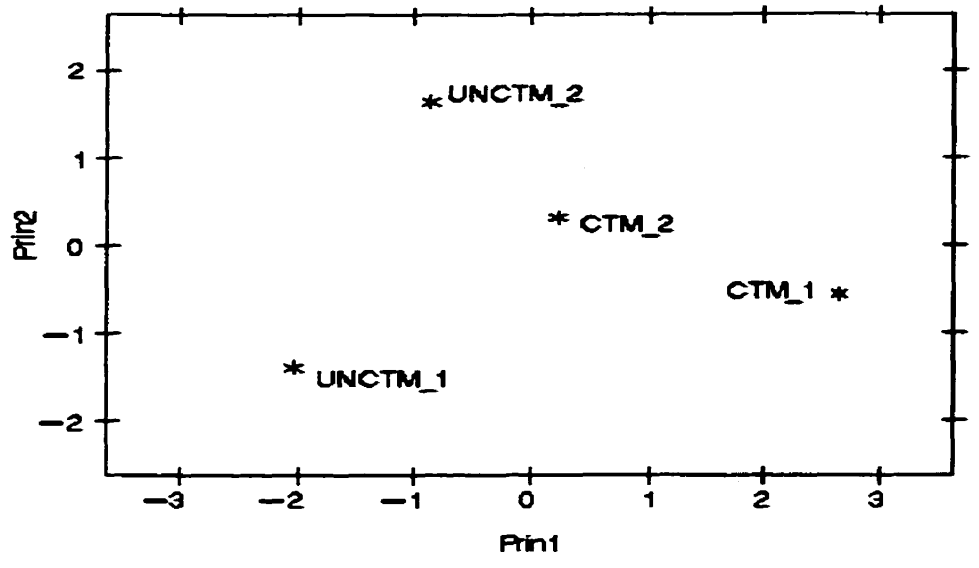


Figure 5.1 Relationship between microbial communities in diesel contaminated (CTM) soils and adjacent uncontaminated (UNCTM) soils from Thicket Portage as depicted by PCA (1=0.5-1.0 m, 2=1.5-2.0 m).

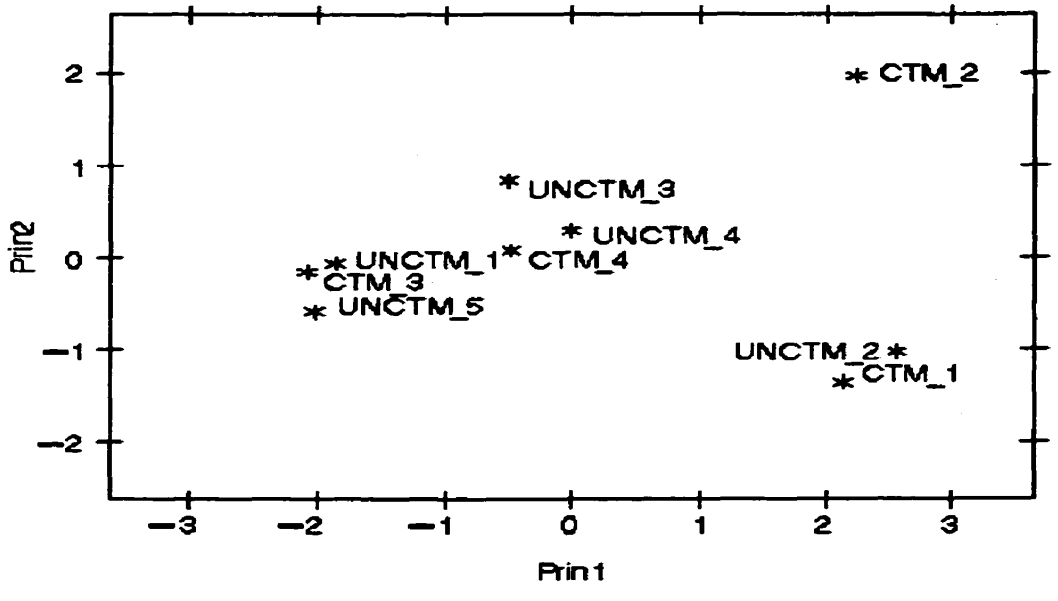


Figure 5.2 Relationship between microbial communities in diesel contaminated (CTM) soils and adjacent uncontaminated (UNCTM) soils from Pikwitonei depicted by principle components 1 and 2 (1=0.75-1.0 m, 2=1.25-1.75 m, 3=1.75-2.25 m, 4=2.0-2.5 m, 5=2.5-3.0 m).

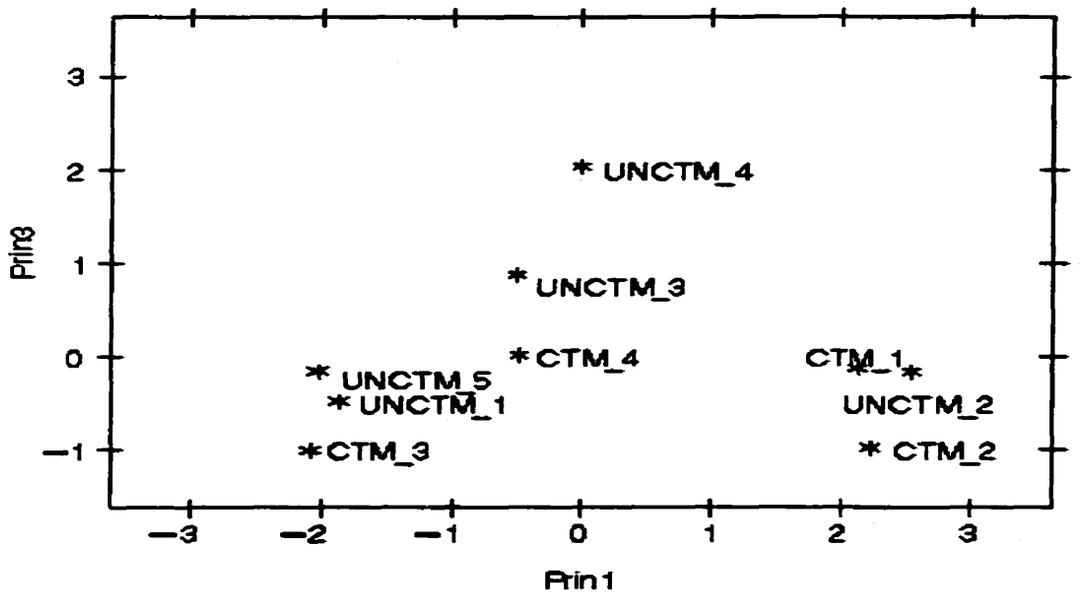


Figure 5.3 Relationship between microbial communities in diesel contaminated (CTM) soils and adjacent uncontaminated (UNCTM) soils from Pikwitonei depicted by principle-components 1 and 3 (1=0.75-1.0 m, 2=1.25-1.75 m, 3=1.75-2.25 m, 4=2.0-2.5 m, 5=2.5-3.0 m).

Observation of PCA of the soils from Brandon industrial site did not reveal distinct differences between contaminated and uncontaminated samples (Fig. 5.4). A list of the soil samples found in the Figure 4.8 with their corresponding depths can be found in Table 5.1. All contaminated soil samples were grouped together for easy differentiation from the uncontaminated soils. Contaminated samples from the same borehole (contaminated sand 2a, contaminated loam (sand) 3b, contaminated loam (sand) 4, and contaminated loam (sand) 5b) were found grouped together in the upper right hand corner of the graph. Most of these samples had very high levels of PAHs extracted from them.

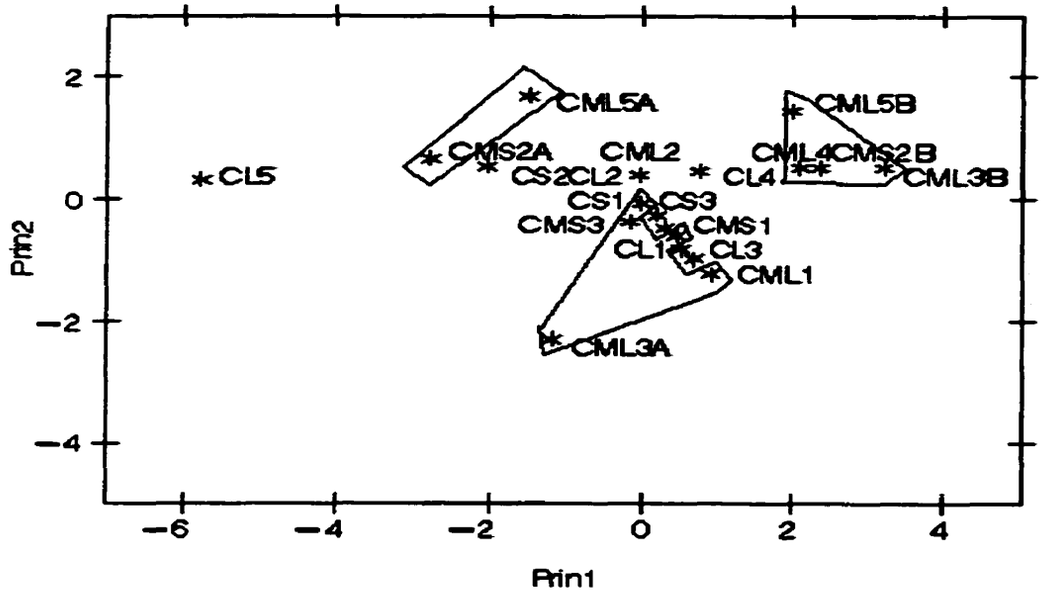


Figure 5.4 Relationship between microbial communities of PAH contaminated (CM) soils (in boxes) and adjacent uncontaminated (CL) soils from Brandon as depicted by PCA.

(CS1=uncontaminated sand 1, CMS1=contaminated sand 1, CS2=contaminated sand 2, CMS2A=contaminated sand 2A, CMS2B=contaminated sand 2B, CS3=control sand 3, CMS3=contaminated sand 3, CL1=control loam 1, CML1=contaminated loam 1, CL2=control loam 2, CML2=contaminated loam 2, CL3=control loam 3, CML3A=contaminated loam 3A, CML3B=contaminated sand 3B, CL4=control loam 4, CML4=contaminated sand 4, CL5=control loam 5, CML5A=contaminated loam 5A, CML5B=contaminated sand 5B).

5.5.2.2. Agricultural soils and contaminated soil from Somerset

PCA of the agricultural soils grouped the subsurface soils on the left and surface soils on the right of the graph (Figure 5.5). This separation was indicative of the overall substrate utilization patterns of the subsurface soils, being significantly lower than the surface soils. The contaminated soil from Somerset was grouped with surface samples from Glenlea and Brandon with reference to the second component (Prin2).

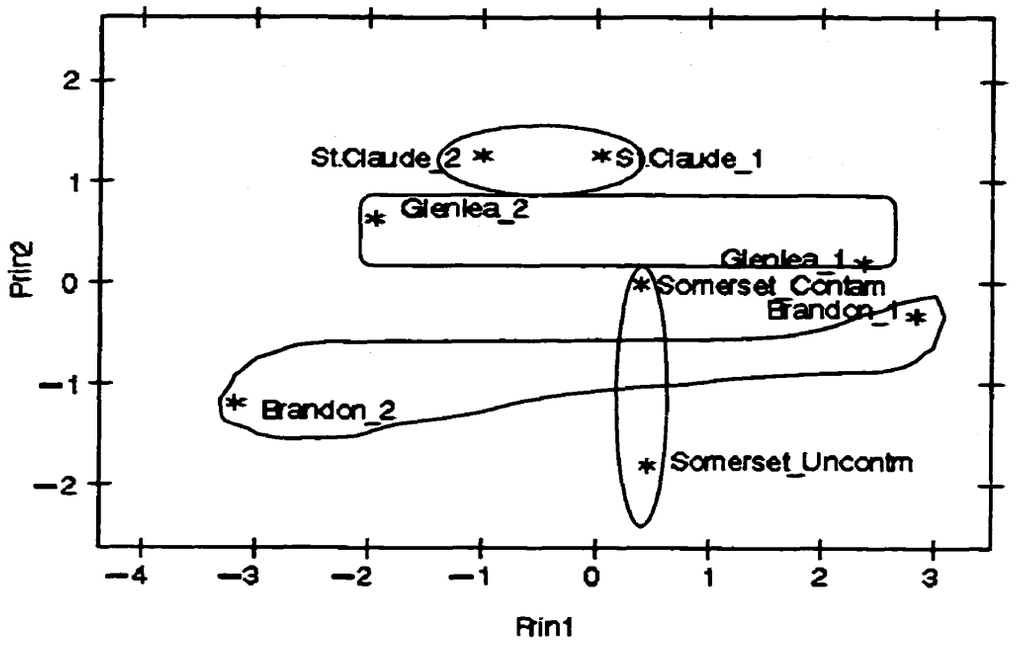


Figure 5.5 Relationship between microbial communities in agricultural soils and contaminated soil from Somerset as depicted from PCA (1=0-10 cm, 2=90-100 cm).

5.5.3 Cluster Analysis

The relationship between the substrate utilization patterns of the soil samples for each location was evaluated using cluster analysis. Dendograms resulting from a hierarchical clustering for each location (Fig. 5.6 to 5.9) indicated the substrate utilization patterns between the soils forming separate clusters. The greater the average distances between the clusters, the greater the difference between the soils.

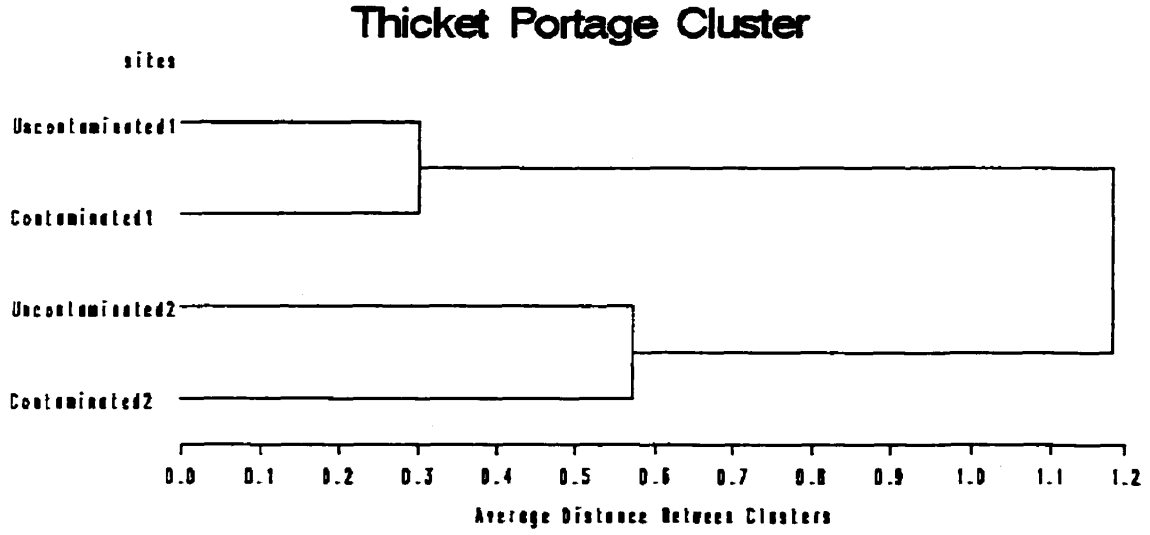


Figure 5.6 Relationship between microbial communities in diesel contaminated and adjacent uncontaminated soils from Thicket Portage resulting from cluster analysis (1=0.5-1.0 m, 2=1.5-2.0 m).

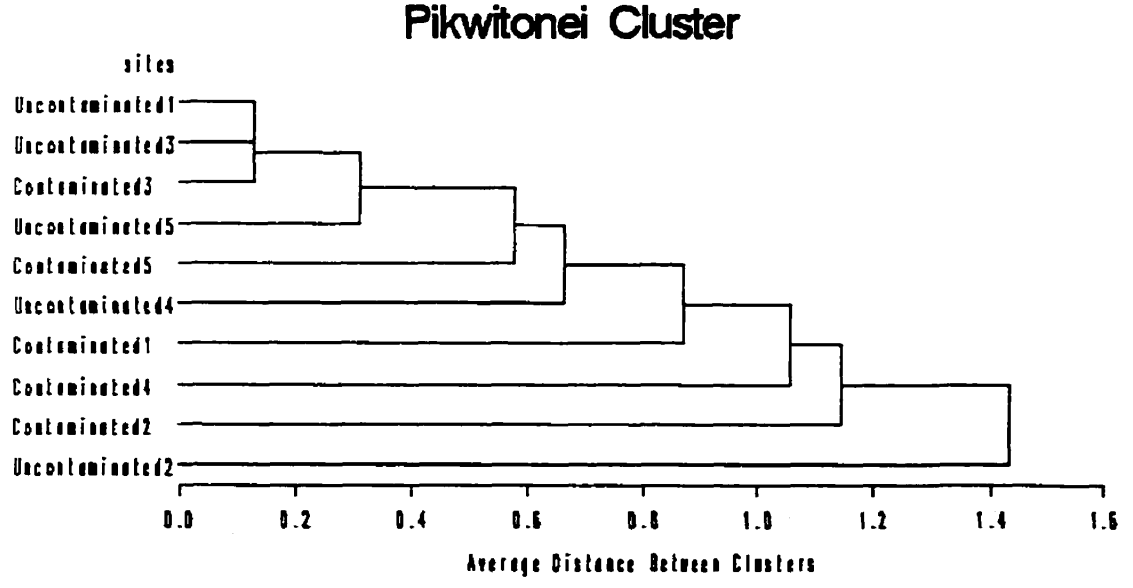


Figure 5.7 Relationship between microbial communities in diesel contaminated and adjacent uncontaminated soils from Pikwitonei resulting from cluster analysis (1=0.75-1.0 m, 2=1.25-1.75 m, 3=1.75-2.25 m, 4=2.0-2.5 m, 5=2.5-3.0 m).

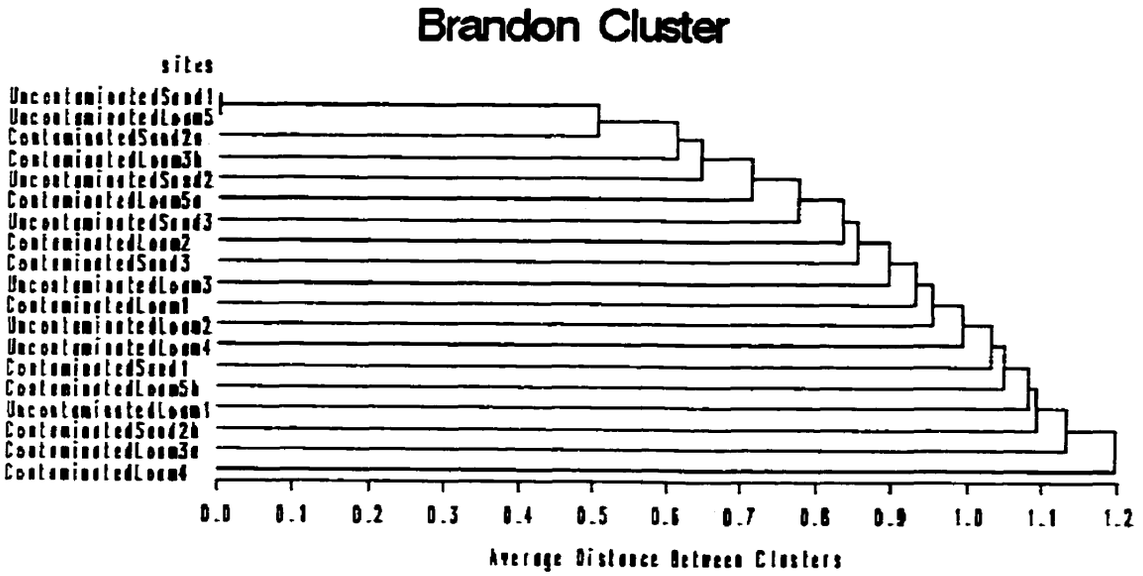


Figure 5.8 Relationship between microbial communities in PAH contaminated and adjacent uncontaminated soils from Brandon resulting from cluster analysis (sand1 = 1.5-2.0 m, sand2 = 2.6-3.1 m, sand3 = 3.1-3.7 m, loam1 = 1.5-2.0 m, loam2 = 2.0-2.5 m, loam3 = 2.75-3.25 m, loam4 = 3.5-4.0 m, loam 5 = 4.0-4.5 m).

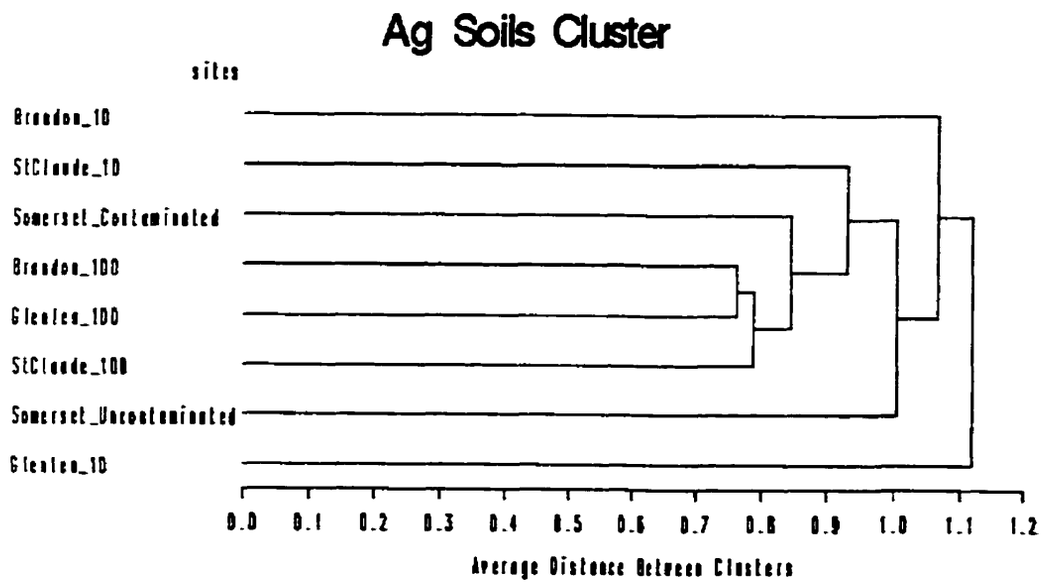


Figure 5.9 Relationship between microbial communities in agricultural soils and contaminated soil from Somerset resulting from cluster analysis (10=0-10 cm, 100=90-100 cm).

5.6 Discussion and Conclusions

5.6.1 Metabolic Diversity

For the most part, the metabolic diversity was not significantly impacted by the contamination event. Measure of microbial diversity did not discriminate the effects of hydrocarbon contamination on the microbial communities of soils. The only trend visible for all the contaminated sites, with the exception of one sample from Pikwitonei, was that the contamination event did not have a negative effect on the overall substrate utilization patterns of the microorganisms. Most studies that demonstrated significant differences in microbial diversities between contaminated and uncontaminated soils were examined a few hours after contamination (Derry *et al.*, 1998). All the soils used for this study had lengthy contamination periods (30 years for Thicket Portage and Pikwitonei, 90 years for Brandon, and 6 years for Somerset). The lack of significance in metabolic diversity between some contaminated and uncontaminated soils may reflect processes of microbial adaptation to hydrocarbon contamination. Disturbances, which are a form of environmental variability such as hydrocarbon contamination, may be a basis for selecting populations with increased versatility (Atlas *et al.*, 1991).

The substrate utilization of all soils, contaminated and uncontaminated, from Thicket Portage and Pikwitonei were less than 50%. In some cases, substrate utilization barely surpassed 10%. If this trend were only observed with the contaminated samples, one might conclude that the presence of hydrocarbon contaminants altered the metabolic diversity of the microbial communities. This was not the case. Some other factor, either present in the soil or some other form of disturbance such as spill of a more toxic contaminant, might have caused the low percentages of substrate utilization.

A typical observation usually seen using Biolog plates is a decrease in metabolic diversity with increasing soil depth (Maurice, 1998). The majority of microbial activity will occur at the surface due to the presence of organic matter and other nutrients, which are lacking in the subsurface (Smith *et al.*, 1993). For both Thicket Portage and Pikwitonei, this trend was not observed. Therefore, again some other factor, possibly a toxic compound present in these soils, affected the metabolic diversity of these soil microbial communities.

For the contaminated soils from Brandon, the heavily contaminated samples of sand 2 showed substantial substrate utilization percentage at all depths in relation to their uncontaminated soils. The increased metabolic diversity in these samples strongly suggests that the microbial population were highly versatile due to the presence of the various PAHs, allowing for greater substrate utilization capabilities. These soils were contaminated for a several decades, allowing the establishment of well-adapted population of hydrocarbon degraders. At such great depths (4.0-4.5 m), the metabolic diversity would usually be low but the contamination obviously altered the enzymatic capabilities of the microbial communities. The contamination of soils can be expected to induce a considerable change in the microbial habitats (Wünsche *et al.*, 1995). The loam 1 sample at the 4.0-4.5m depths also had a significant percent of substrates utilized for such a great depth. Although this soil was not high in PAHs levels, the presence of the contaminants seems to have affected the microbial diversity. A comparison in metabolic diversity between sand and loam samples with similar levels of PAHs was observed at 2.75-3.5m with the sand sample having approximately 40% greater substrate utilization. This could be due to the fact that coarser soils have quicker water infiltration allowing

more oxygen, contaminant and microorganisms to flow through the profile. As oxygen is required for the metabolism of PAHs, its presence might be more limited in the finer texture loam soil preventing the development of a more diverse microbial community.

PCA and cluster analysis describing results of sole carbon utilization patterns, failed to discriminate between contaminated and uncontaminated sites. As a tool for implementing strategies for intrinsic remediation, metabolic diversity provided important information from the lack of difference observed between contaminated and uncontaminated soils. The lack of difference indicates that the contamination event did not disrupt the microbial diversity of the contaminated sites or is no longer disrupting microbial diversity because of the age of the contamination. The lack of change in substrate utilization patterns between contaminated and uncontaminated soils might reflect a return to a steady-state level governed by factors other than substrate supply. The stability of the population in the contaminated soils may also indicate that the contamination event favoured the selection of microorganisms having broad substrate utilization capabilities. When assessing the suitability of contaminated soils for intrinsic remediation strategies, the effect of the contamination event on the microbial diversity is always a concern, especially issues of contaminant toxicity (Wünsche *et al.*, 1995). The results from this study indicate that the contamination event does not disrupt the microbial population.

CHAPTER 6

THE EXAMINATION OF MICROBIAL RESPIRATION OF LABELLED SUBSTRATES IN CONTAMINATED AND UNCONTAMINATED SOILS AS A POTENTIAL MANAGEMENT TOOL FOR IMPLEMETING INTRINSIC REMEDIATION

6.1 Abstract

The fate of radiolabeled (^{14}C) phenanthrene was examined in four contaminated soils from Manitoba, two diesel fuel, one coal tar, and one crude oil. Agricultural soils varying in texture were sampled from three locations in Manitoba to determine the potential of their microbial populations to degrade phenanthrene. Agricultural soils were collected from 0-10 cm and 90-100 cm depths in order to compare surface and subsurface hydrocarbon degrading populations. Phenanthrene degradation was compared between contaminated and adjacent uncontaminated soils according to depth, to determine the influence of preexposure on the rate of degradation. Microbial activity was also determined by measuring evolution of carbon dioxide in order to compare the activity in contaminated versus uncontaminated soils. Soils from the two diesel fuel contaminated sites degraded higher amounts of phenanthrene than their respective uncontaminated soils at all depths, with ^{14}C recoveries ranging from 10 to 50%. A decrease in degradation was also observed with an increase in depth for these sites. Two uncontaminated sand soils and one loam soil produced greater amount of degradation than their respective contaminated samples for the coal tar contaminated site. All contaminated soils from the

coal tar site, except for one, were able to degrade phenanthrene, with recoveries ranging from 31 to 49%. The absence of a lag phase and a linear rate of degradation, were the only differences upon comparison between coal tar contaminated soil and adjacent uncontaminated soil, having similar recoveries of approximately 43%. ^{14}C-phenanthrene degradation from subsurface soils indicated the presence of microorganisms capable of hydrocarbon degradation in aerobic environments. Indigenous soils microorganisms were capable of phenanthrene degradation, as all surface agricultural soils degraded the PAH, following a similar degradation pattern beginning with a slight lag phase and percent recoveries. Two of the three subsurface agricultural soils were able to degrade phenanthrene, prior to a 20-day lag phase. Determination of microbial activity from CO_2 evolution between contaminated and uncontaminated soils did not demonstrate significant differences. In only two cases, the contaminated soils had higher activity levels than their respective uncontaminated soils. Microbial activity did not seem to be stimulated by the addition of phenanthrene in both contaminated and uncontaminated soils.

6.2 Introduction

In most cases, higher rates of hydrocarbon degradation have been shown to occur in previously contaminated soils than in uncontaminated soils (McGill *et al.*, 1981; Wilson and Jones, 1994; Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997) from the adaptation of selected microbial community capable of hydrocarbon degradation. An adaptation period often occurs when contamination occurs in previously uncontaminated soils, before the onset of significant metabolism. The adaptation period is thought to allow for the development of the appropriate microbial communities through growth or enzyme induction (Aelion *et al.*, 1989; Carmichael and Pfaender, 1997). Higher rates of

microbial activity from measurement of CO_2 have also been found in contaminated soils when compared to uncontaminated (Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997). It was suggested that the selection of tolerant species with increased metabolic versatility due to the contamination event, probably maintained high levels of activity. Miethe *et al.* (1994) found that the microbial activity in pristine soils increased after the addition of diesel fuel confirming the ubiquitous distribution of hydrocarbon degrading microorganisms in soils. The relationship between previous contamination and the adaptive response of the soil microbial community is important as it might influence hydrocarbon degradation. Knowledge of the microbial activity of microorganisms with the potential to degrade hydrocarbon contaminants in agricultural soils would provide important information when assessing strategies for intrinsic remediation.

6.3 Objective of Study

The objective of this study was to determine if previous exposure to hydrocarbons has resulted in a microbial community with an increased ability to degrade phenanthrene. To achieve this objective a study was undertaken to examine the kinetics of phenanthrene degradation for selected diesel fuel, coal tar, and crude oil contaminated soils in comparison to adjacent uncontaminated soils by depth. Phenanthrene degradation was also determined for four agricultural soils varying in texture. Microbial activity from total respiration rates was determined and compared between contaminated and uncontaminated soils to determine its effectiveness as an indicator of a soil's potential to degrade polycyclic aromatic hydrocarbons, such as phenanthrene.

6.4 Materials and Methods

6.4.1 Soils

All soils used in this experiment were discussed in Chapter 3.

6.4.2 Preparation of soils for degradation study

For all soils samples used in this experiment, four replications of 10 g of soil (oven dry basis) were weighed into 30 mL beakers. All samples were wet to field capacity and incubated at room temperature for approximately three months. Soils were wet to field capacity every week during the incubation time.

6.4.3 Microcosm apparatus

The apparatus used to contain the 10 g of soil consisted of a 500 mL glass jar with an airtight fitting lid. In addition to the beaker of soil, the microcosms contained a CO_2 trap with 15 mL of 0.1 M NaOH in a 20 mL scintillation vial, 3 mL of acidified water (pH 3) to help maintain humidity, and 0.5 g Ambersorb® 563 to trap volatile components. Ambersorb® 563 is a very porous charcoal based substance which traps any volatile hydrocarbon.

6.4.4 ^{14}C-phenanthrene

^{14}C phenanthrene was purchased from Sigma Chemical Co. (St. Louis, MO) as phenanthrene-9-^{14}C (12.4 mCi/mmol, purity >98%) (Figure 6.0). The ^{14}C-label is positioned in the most accessible site for microbial degradation to begin. The ^{14}C would be released soon after the microorganisms have initiated degradation. Stock solution was first made up in hexane such that each 1 mL addition to the soils would contain 5000 μg/g diesel fuel and 500 μg/g phenanthrene. Hexane was a carrier solvent used to distribute the phenanthrene in the soil sample. The unlabeled and ^{14}C-labeled phenanthrene was added to the stock solution so that the level of activity added to each sample did not

exceed 0.5 μCi. The level of phenanthrene added was ten times the acceptable level for an industrial site according to the Canadian Council of Ministers of the Environment (CCME) Soil Quality Guidelines, March 1997. The level of diesel fuel added to the soil would also require remedial according to Manitoba Conservation Guidelines (Manitoba Environment, 1993).

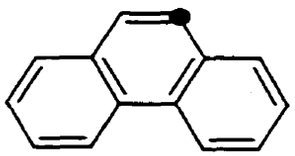


Figure 6.0 Structure of phenanthrene and location of labeled ^{14}C (black dot).

After the addition of the stock solution (1 mL), the beakers of soil remained in the fumehood for approximately one hour to allow the volatilization of the carrier solvent. The beakers were then weighed, wet to field capacity, and placed into the microcosm apparatus.

6.4.5 Respiration monitoring and $^{14}CO_2$ evolution

The NaOH traps were removed and replaced every day for four days, every two days (four times), then every four days (four times), and finally once a week until the end of the experiment. A 1 mL aliquot of the removed NaOH traps was placed in a 7 mL scintillation vial and combined with 5 mL of ScintiSafe 30% liquid scintillation cocktail (Fisher Scientific, Fairlawn, NJ). This mixture was allowed to equilibrate in the dark for a minimum of 24 hours before counting to prevent any erroneous readings from the ion interaction with the scintillation fluid. A Beckman LS 7500 scintillation counter was used to determine the disintegrations per minute (DPM). Final DPMs were corrected for

background and blanks then related to the original radioactivity added to each microcosm to give the percent degradation of phenanthrene in diesel.

The measurement of the total CO_2 respired utilized a modified version of the persulfate oxidation method used for total organic carbon analysis (Technicon method #455-76W/A). Acidifying the sample and passing carbon dioxide across a gas diffusion membrane determined dissolved carbon dioxide. The carbon dioxide was then collected in a colour reagent containing $3.1x10^{-5}$ mol of phenolphthalein per litre of solution. Colour change was determined at 550 nm wavelength. The results were given in mg C/L of trap that were then converted to mg C/trap basis and eventually expressed per gram of soil in the microcosm. Samples at every second time point were analyzed during this experiment. The specific activity (moles $^{14}CO_2$/mole total C per trap) was calculated at five intervals (1 to 6 days, 10 to 24 days, 35 to 63 days, 77 to 105 days, and 119 to 147 days), to determine possible fluctuations in the proportion of $^{14}CO_2$ evolved during the incubation period.

6.4.6 Kinetic Analysis of $^{14}CO_2$ evolution

Determination of the degradation rate of phenanthrene was done using a linear or first order rate model. Curve fitting was performed using the statistical software JMP IN and SAS version 8.0 (Statistical Analysis Software Institute Inc., Cary, NC). Each of the soil replications used in the degradation experiment had a linear and first order curve fit to its data by use of the following equations:

$$A_t = K_L t + b_L \qquad \textbf{Equation 6.1}$$

$$A_t = A_F(1-e^{-K_F t}) \qquad \textbf{Equation 6.2}$$

Equation 6.1 describes the linear curve where A_t = percent degradation at time t (days), K_L = the linear degradation rate constant (%/day), b_L = the y intercept. Equation 6.2

describes the first order curve where A_F = percent of added ^{14}C that has evolved as $^{14}CO_2$ at time infinity, K_F = the first-order degradation rate constant ($days^{-1}$). All curve fitting procedures were performed using cumulative percent degradation data at time t. Once all curve fitting was completed, a test was done to determine which of the two equations produced the best fit for the data, from observing significant reduction in the residual sum of squares (RSS) with increasing complexity of the model. The difference between the RSS of the two models and dividing the difference by the residual mean square (RMS) of the more complex model resulted in the F-statistic. The calculated F value can be compared to the tabulated value in the F table in order to determine if the increase in model complexity resulted in a better fit. The degrees of freedom at $P \geq 0.05$ level can be derived by using the value 1 and the number of data points minus the number of parameters from the model (Robinson, 1985).

The half-life for phenanthrene degradation was calculated for each soil sample from the K_F values determined from the best fitting curves. The calculation is based on the percent of the chemical degraded with the assumption that the entire compound is available and has been degraded.

$$t_{1/2} = \ln 2 / K_F \qquad \textbf{Equation 6.3}$$

6.4.7 Volatilization

A Packard Oxidizer Model 306 was used to combust the Ambersorb sample to determine the amount of ^{14}C-labeled compound that had volatilized during the experiment. At the end of the experiment, the vials containing the Ambersorb® 563 were removed from the microcosms and sealed. A 0.2 g sample of the Ambersorb was weighed and placed in a Combusto-Cone sample holder with an equal amount of

cellulose. The cellulose was added to help moderate the burn of the Ambersorb. A small amount of Combustaid (0.3 mL) was added to the Ambersorb-cellulose mixture, to assist in the ignition of the sample. A three-minute combustion time was required to completely burn the Ambersorb samples. As the burning Ambersorb was converted to CO_2, a strong base (Carbo-Sorb®E) was used to trap all the CO_2. Once the burn was complete, 5 mL of the Carbo-Sorb®E was mixed with 15 mL of ScintiSafe 30% liquid scintillation cocktail. The samples were allowed to equilibrate in the dark for a minimum of 24 hours before counting to prevent any erroneous readings from the ion interaction with the scintillation fluid. A Beckman LS 7500 scintillation counter was used to determine the disintegrations per minute (DPM). Final DPMs were corrected for background and blanks, and then related to the original radioactivity added to each microcosm to give the percent volatilization of ^{14}C-phenanthrene.

6.5 Results

6.5.1 Degradation Study

Curve fitting analysis of the phenanthrene degradation data indicated that two separate trends occurred (Table 6.1). The soils that experienced a degradation plateau fit the first-order model while the other soils were best suited to the linear model. The rate of phenanthrene degradation did not level off for the soils with the linear fit during the entire duration of the experiment. The range of half-life values for phenanthrene degradation is quite broad, ranging from 15.5 days to 54.1 days.

6.5.1.1 Contaminated Soils

Most of the contaminated soils from Thicket Portage (TP), Pikwitonei (Pik), and Brandon were able to degrade phenanthrene (Figures 6.1, 6.2, and 6.3, 6.4, respectively). The only contaminated soil that was not able to degrade phenanthrene was the contaminated loam 2 (2.75-3.5m). Most of the uncontaminated soils from all the sites were also able to degrade phenanthrene. For both soils from TP and Pik, higher rates of degradation occurred with the contaminated samples compared to their respective uncontaminated soils. All the soils from these two sites were best fit with the linear model, indicating that phenanthrene degradation had yet to stabilize. A decrease in the rates of phenanthrene mineralized for the contaminated soils from TP and Pik were observed with an increase in soil depth. Soils from greater depth (control 2 at 2.5-3.0m) for the uncontaminated soils from Pik had greater degradation than the control 1 soil at 1.75-2.25 meters. Both uncontaminated soils from TP had little degradation. Soils from Brandon were divided by texture, sand and loam (Figure 6.3 and 6.4, respectively) for better visualization.

Table 6.1 Curve fitting analysis of the results for phenanthrene degradation and half life values. Values shown represent the mean of four replicates.

Sites	Best Fit Model	Rate Constant (K_L %/day) or (K_F day^{-1})	b or A_F (%^{14}C)	Degradation half life (days)
TP[1] Control 1 (0.5-1.0m)	linear*	0.0100	-0.18	
TP Contaminated 1	linear	0.3712	-7.18	
TP Control 2 (1.5-2.0m)	linear	0.0090	-0.18	
TP Contaminated 2	linear	0.0723	-1.93	
Pik[2] Control 1 (0.75-1.0m)	linear	0.2013	-4.04	
Pik Contaminated 1	linear	0.0576	-2.09	
Pik Control 1 (1.75-225 m)	linear	0.1275	-1.77	
Pik Contaminated 1	linear	0.0061	-0.27	
Pik Control 2 (2.5-3.0m)	linear	0.0907	-2.26	
Pik Contaminated 2	linear	0.0376	-0.768	
BD[3] Control Sand 1 (1.5-2.0m)	1st order	0.0447	64.10	15.5
BD Contaminated Sand 1	linear	0.2051	3.33	
BD Control Sand 2 (2.6-3.1m)	1st order	0.0128	64.42	54.1
BD Contaminated Sand 1	linear	0.3776	-8.30	
BD Contaminated Sand 2	linear	0.2228	0.08	
BD Control Loam 1 (1.5-2.0m)	linear	0.0196	-0.63	
BD Contaminated Loam 1	1st order	0.0425	36.82	16.3
BD Control Loam 1 (2.75-3.5m)	linear	0.0748	-2.9	
BD Contaminated Loam 2	linear	0.0010	0.0	
BD Contaminated Sand 2	1st order	0.0095	64.37	73.1
BD Control Loam 2 (4.1-4.5m)	1st order	0.0246	56.62	28.2
BD Contaminated Sand 3	linear	0.2709	5.24	
Somerset Control	1st order	0.0253	43.39	27.4
Somerset Contaminated	1st order	0.0146	50.75	47.5
Glenlea 0-10 cm	1st order	0.0253	46.10	26.3
St.Claude 0-10 cm	1st order	0.0342	47.98	20.3
Brandon 0-10 cm	1st order	0.0325	51.15	21.3
Glenlea 90-100 cm	linear	0.1775	3.87	
St.Claude 90-100 cm	linear	0.0606	-2.473	
Brandon 90-100 cm	linear	0.0100	-0.39	

[1] TP = Thicket Portage, [2] Pik = Pikwitonei, [3] BD = Brandon
* R^2<0.95 were rejected for the linear fit model

For the sandy soil, both uncontaminated soils had higher rates of phenanthrene degradation than their respective contaminated soils at all depths. A slight lag phase was visible for all of the sandy soils. Due to the lack of contaminated loam samples, two contaminated sandy soils were used at two depths, 3.15-3.65 m and 4.0-4.5 m, and were

compared to the loam soils (described in Chapter 3). The highest rate of degradation was observed with control 1 loam sample (1.5-2.0m) for the loam soils, while the uncontaminated soil from 4.0-4.5 meters had the lowest degradation rate among the uncontaminated samples. Soils sampled from deeper depths had lower rates of phenanthrene degradation for the uncontaminated soils from both textures. All contaminated loam soils, except for contaminated loam 2 (2.75-3.5m), displayed moderate phenanthrene degradation. An effect from soil depth was not as obvious with the contaminated soils from Brandon for both soil textures. The loam samples did not have an initial lag phase, which was seen with the coarse textured soils.

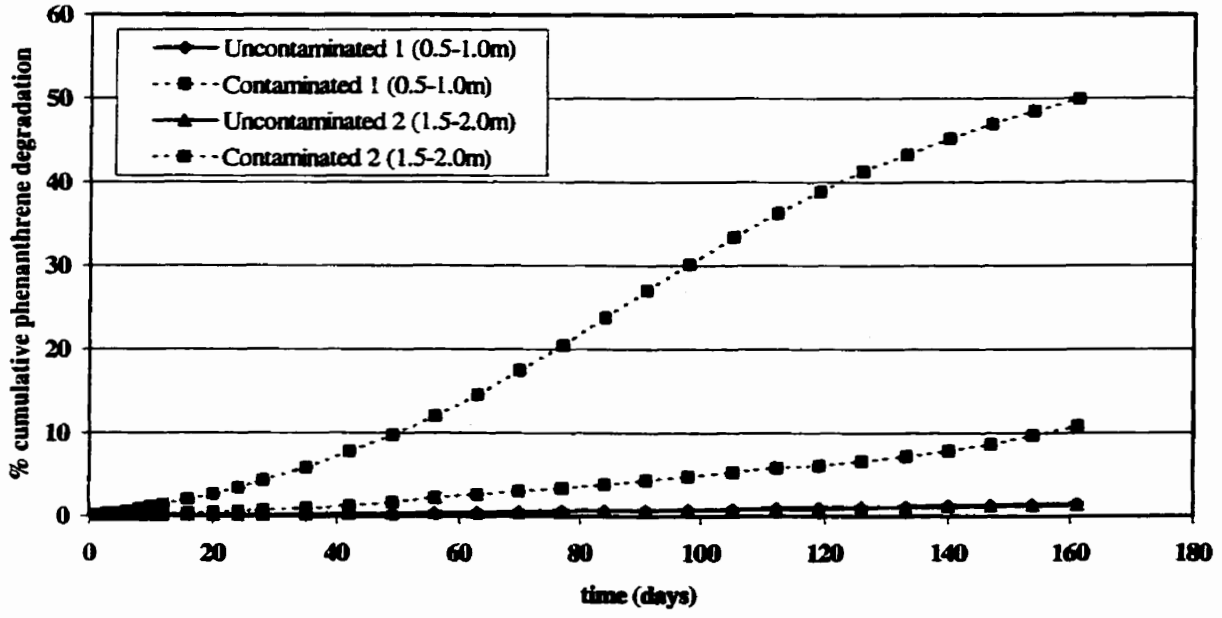


Figure 6.1 Cumulative phenanthrene degradation in contaminated and uncontaminated soils from Thicket Portage. Error bars (hidden by data points) represent the standard error of four replicates.

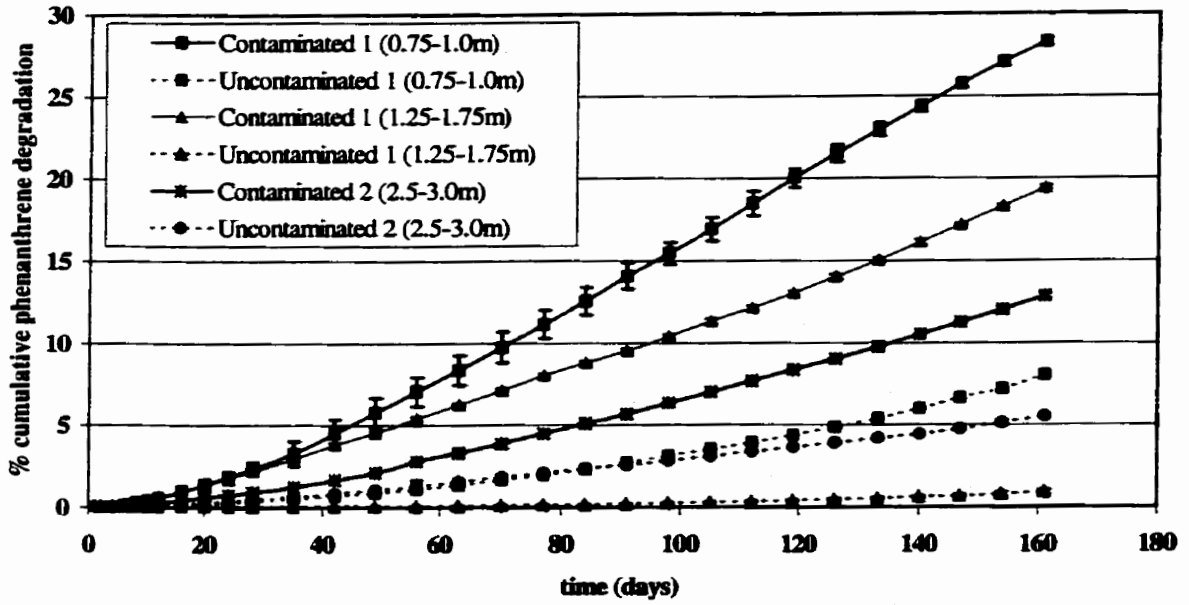


Figure 6.2 Cumulative phenanthrene degradation in contaminated and uncontaminated soils from Pikwitonei. Error bars represent the standard error of four replicates.

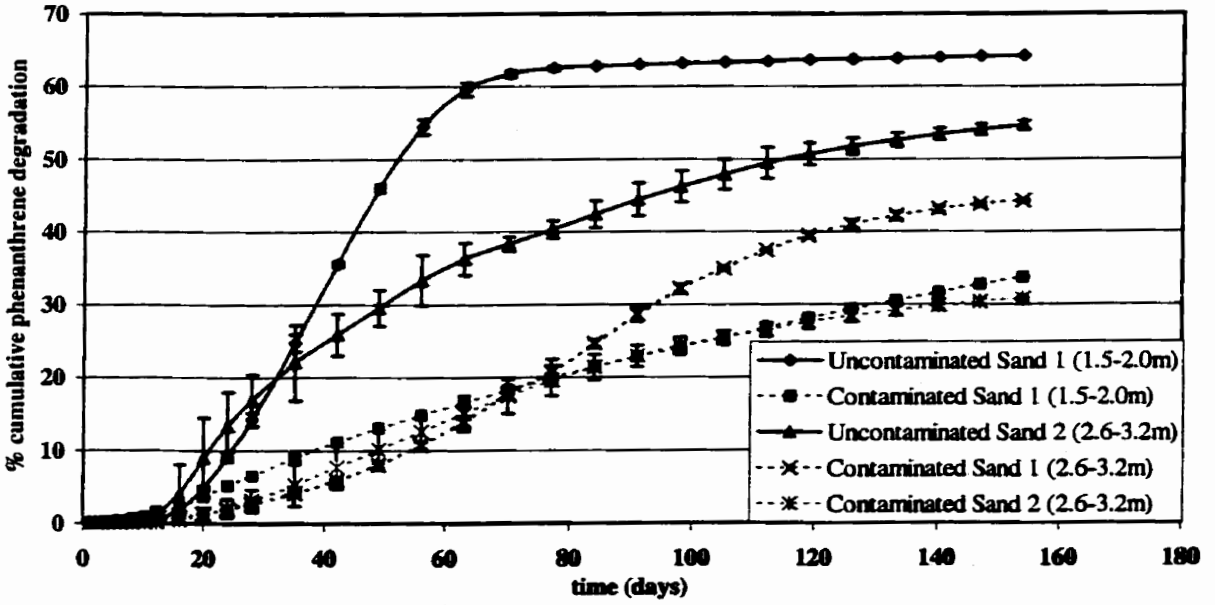


Figure 6.3 Cumulative phenanthrene degradation in contaminated and uncontaminated sand soils from Brandon. Error bars represent the standard error of four replicates.

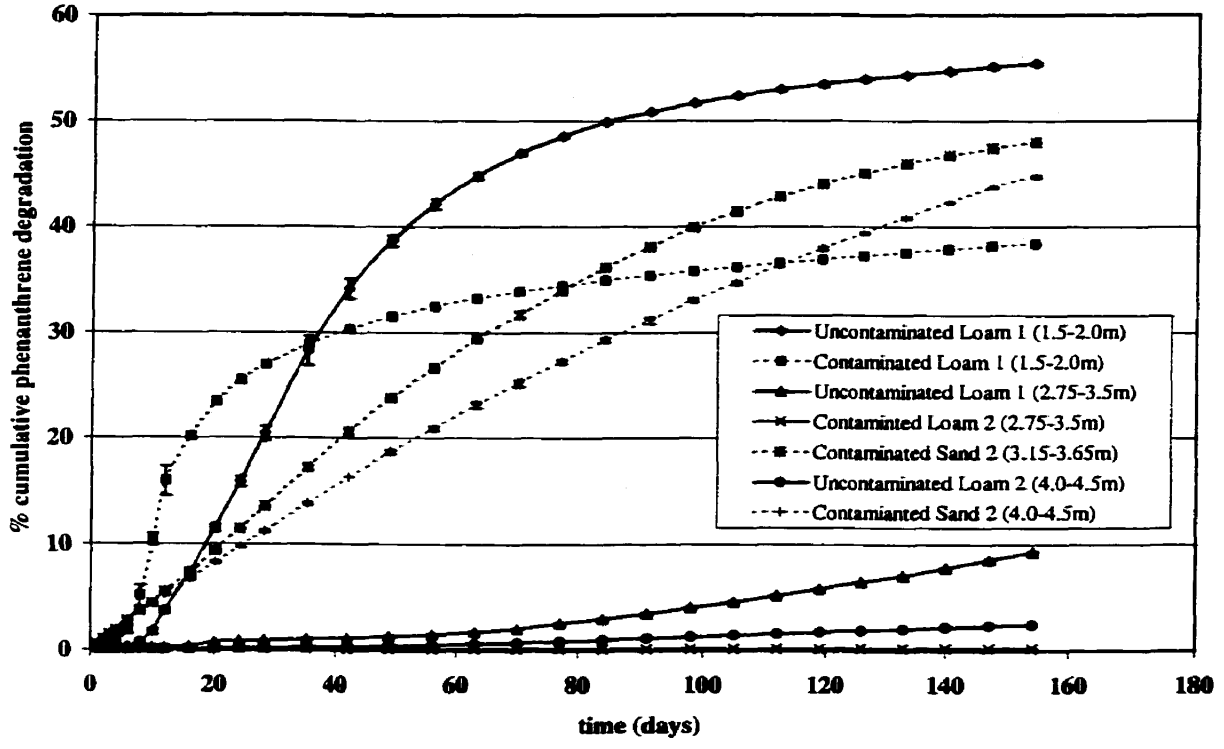


Figure 6.4 Cumulative phenanthrene degradation in contaminated and uncontaminated loam soils from Brandon. Error bars represent the standard error of four replicates.

6.1.1.2 Agricultural soils

All surface agricultural soils were able to degrade phenanthrene and were fitted by the first-order model (Figure 6.5). Soils from Brandon and St.Claude had very similar degradation patterns, while soil from Glenlea had slightly less phenanthrene degradation. All surface soils except for the contaminated soil form Somerset had a slight lag phase before degradation began. The uncontaminated soil from Somerset had higher phenanthrene degradation than the contaminated sample up until approximately 120 days of incubation. The subsurface soils had lower rates of phenanthrene degradation compared to the surface samples (Figure 6.6). The subsurface soil from Glenlea had the

highest rate phenanthrene degradation among the subsurface samples, followed by the soil from Brandon. Very little degradation occurred with the St.Claude subsurface soil. A lag phase of approximately 20 days was seen with the subsurface soil from Glenlea, and about a 40 days lag phase with the subsurface soil from Brandon. All subsurface soils were best fitted with the linear model, indicating that phenanthrene degradation had yet to stabilize.

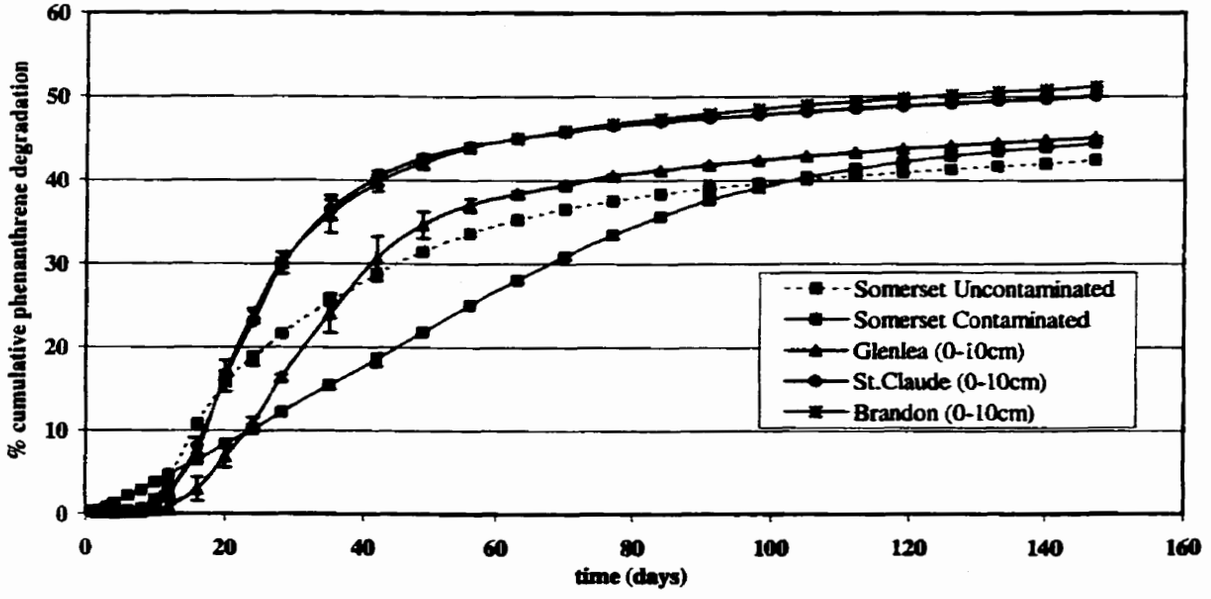


Figure 6.5 Cumulative phenanthrene degradation in surface agricultural soils and contaminated soil from Somerset. Error bars represent the standard error of four replicates.

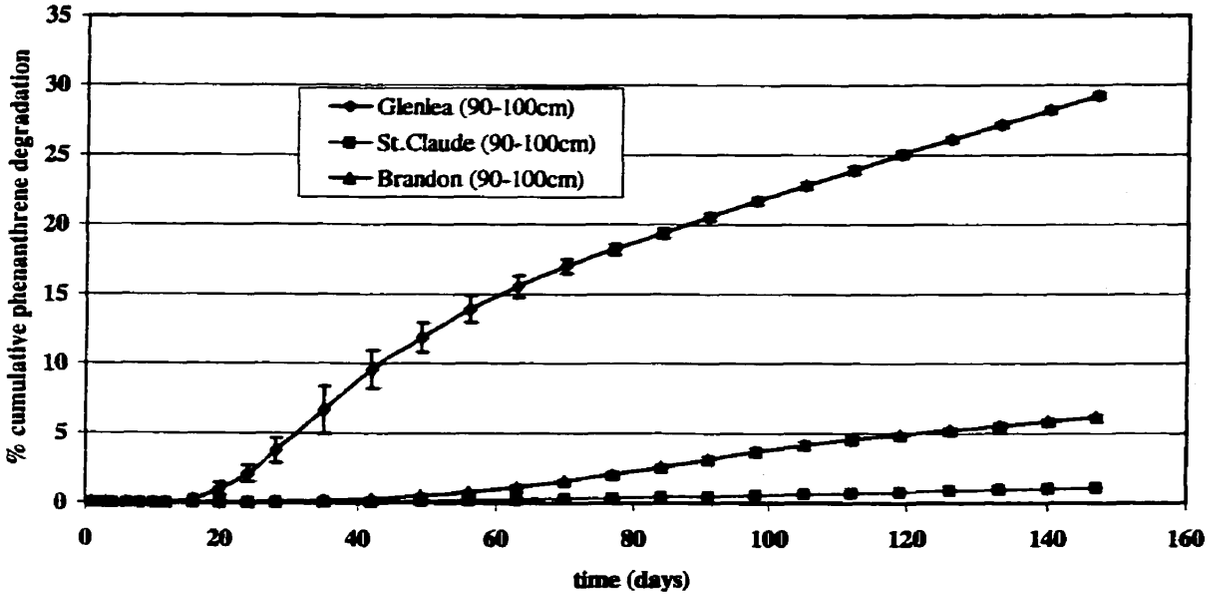


Figure 6.6 Cumulative phenanthrene degradation in agricultural subsurface soils. Error bars represent the standard error from four replicates.

6.5.2 Respiration Activity

Total CO_2 respired for contaminated soils from TP were higher than their respective uncontaminated soils, with the surface sample (0.5-1.0m) being higher than the subsurface sample (1.5-2.0m) (Figure 6.7). Both TP uncontaminated soils had similar evolution of CO_2. Little difference in respiration activity was observed between contaminated and uncontaminated soils from Pik (Figure 6.8) and coarse textured soil from Brandon (Figure 6.9). Highest rate of respiration for the loam soils from Brandon was observed with contaminated loam 1 from 1.5-2.0 meters. Similar levels of respiration were seen with the remaining loam samples. Both uncontaminated and contaminated soils from Somerset had similar levels of respiration activity (Figure 6.11), with the uncontaminated soil having slightly higher amounts of CO_2.

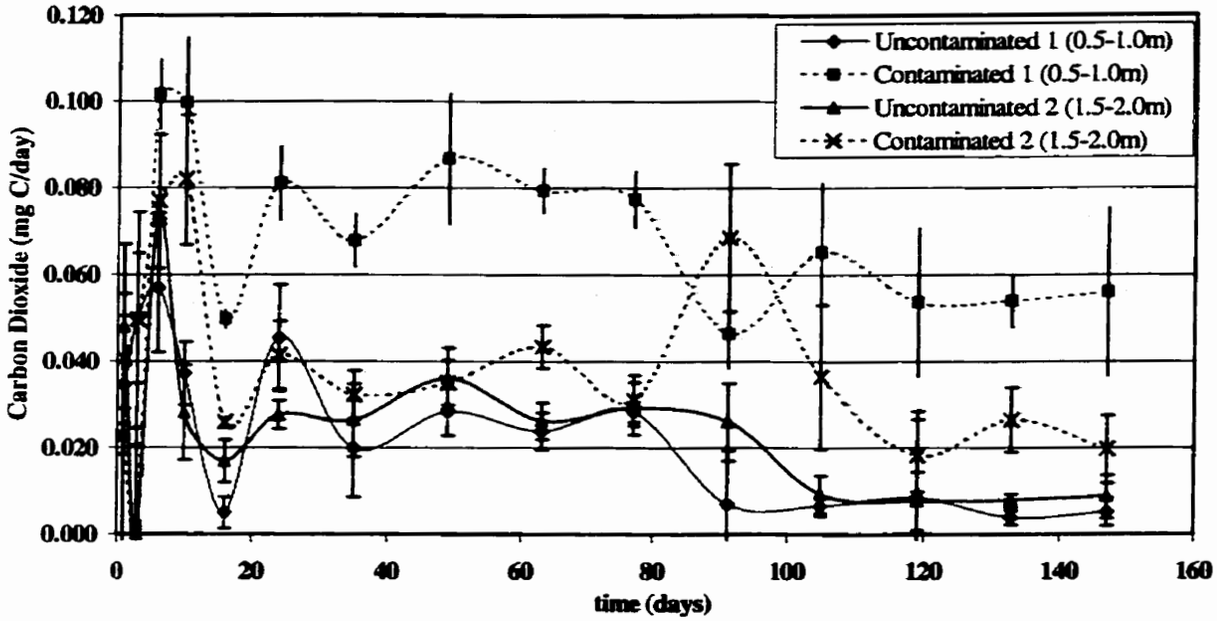


Figure 6.7 Respiration activities of contaminated and uncontaminated soils from Thicket Portage. Points represent the mean of four replicates. Error bars represent the standard error of four replicates.

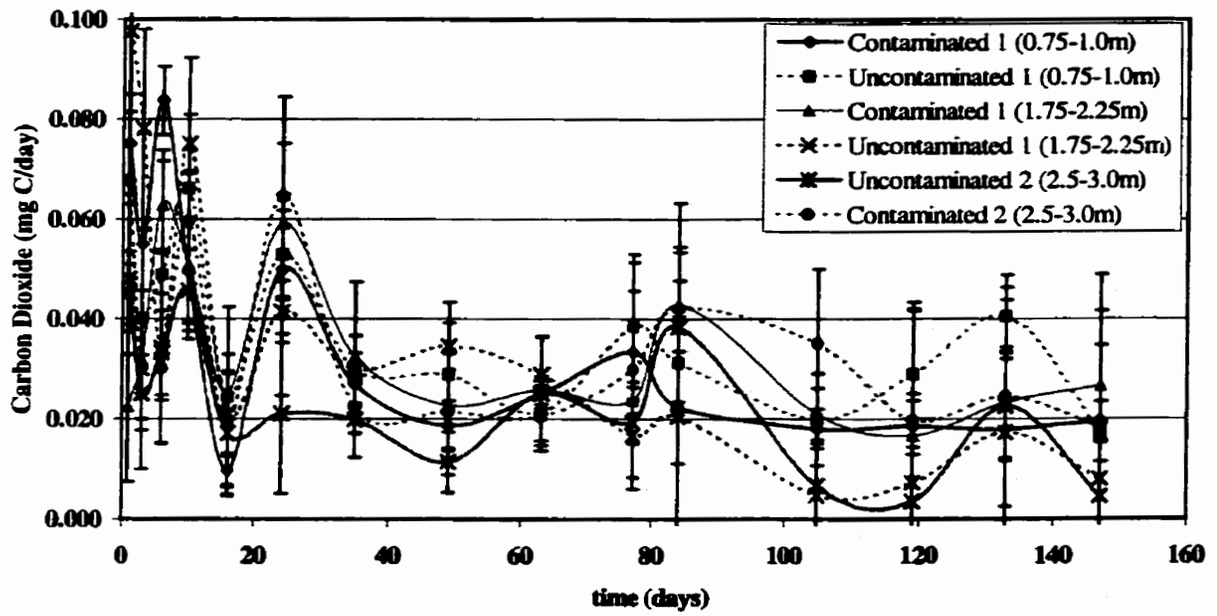


Figure 6.8 Respiration activities of contaminated and uncontaminated soils from Pikwitonei. Points represent the mean of four replicates. Error bars represent the standard error of four replicates.

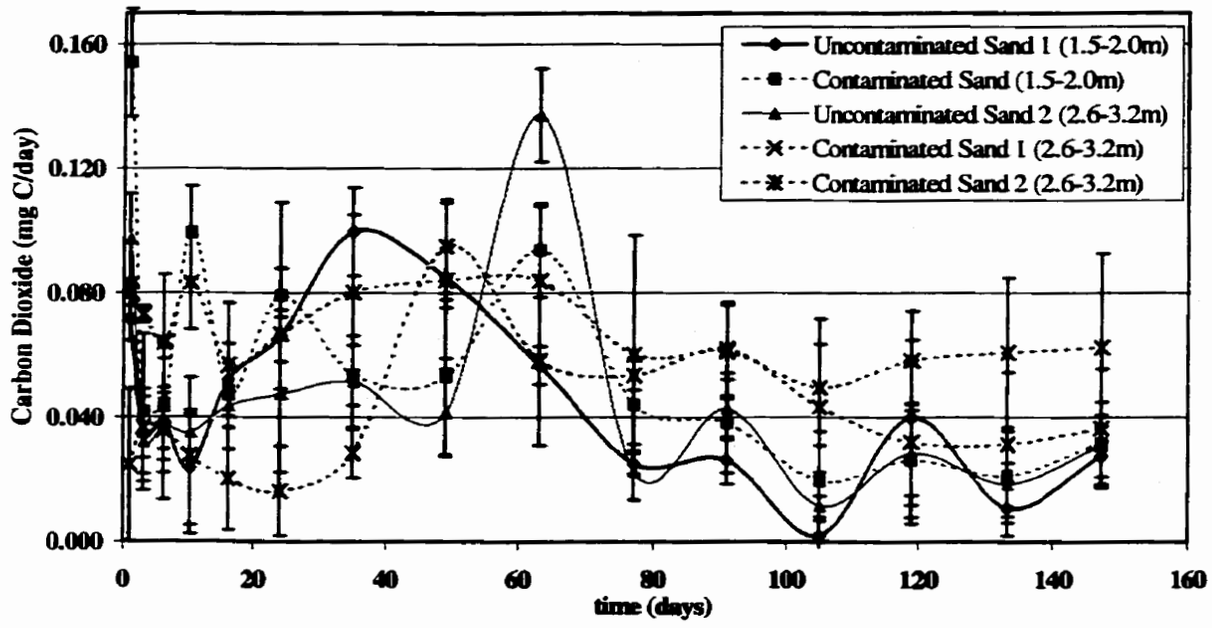


Figure 6.9 Respiration activities of contaminated and uncontaminated sand soils from Brandon. Points represent the mean of four replicates. Error bars represent the standard error of four replicates.

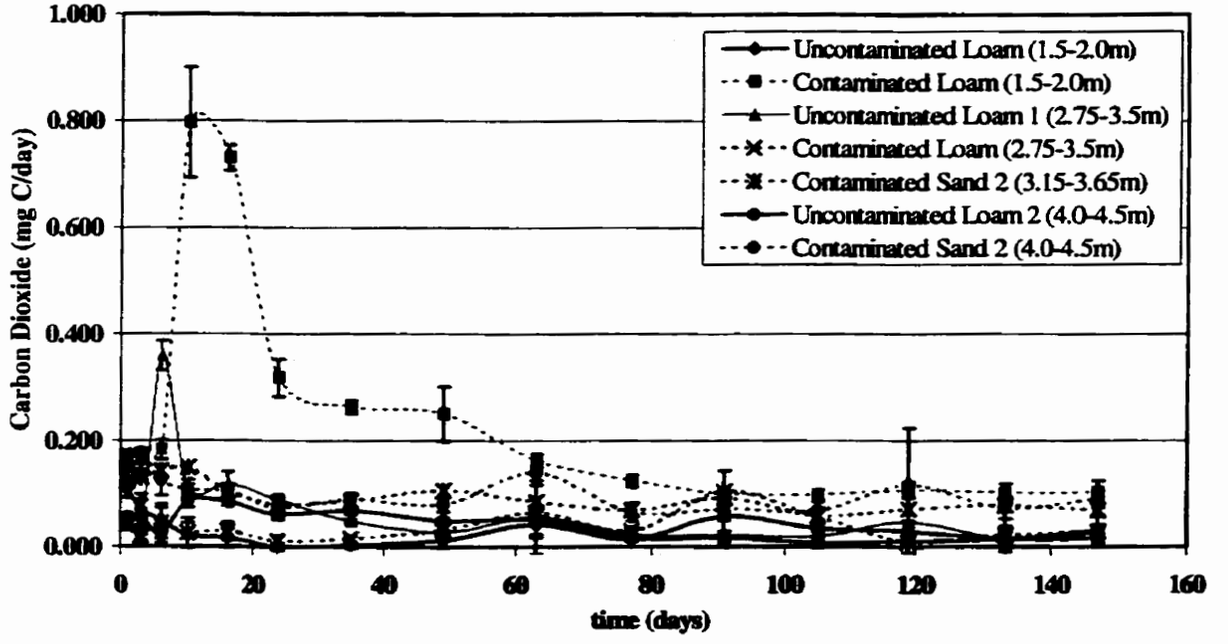


Figure 6.10 Respiration activities of contaminated and uncontaminated loam soils from Brandon. Points represent the mean of four replicates. Error bars represent the standard error of four replicates.

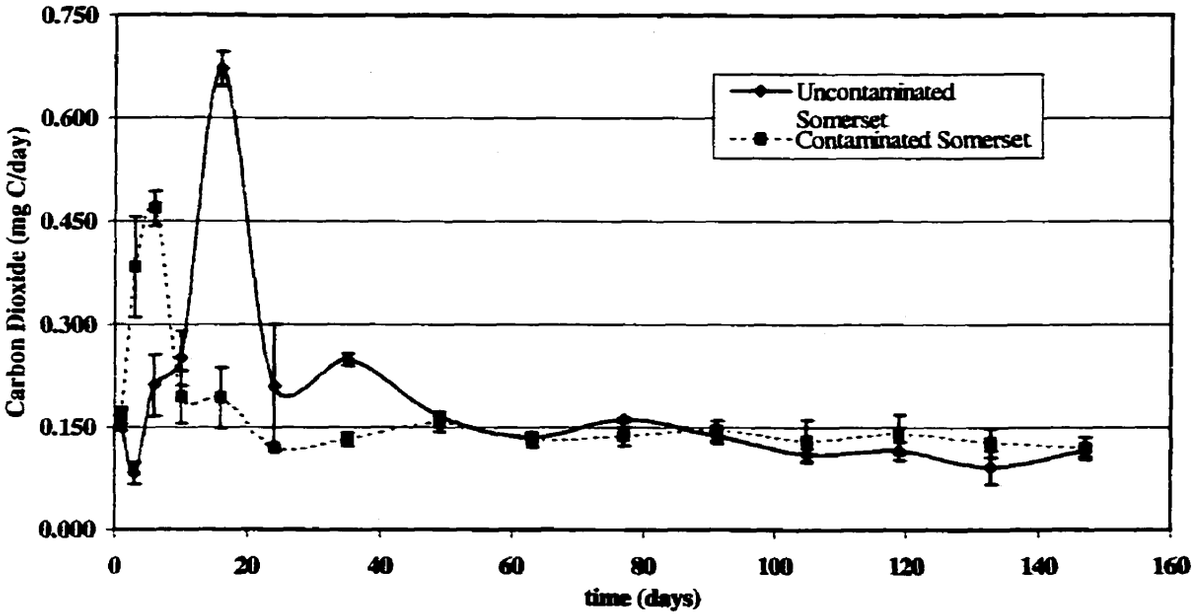


Figure 6.11 Respiration activities of contaminated and uncontaminated soils from Somerset. Points represent the mean of four replicates. Error bars represent the standard error of four replicates.

6.5.3 Specific Activity

The specific activity was calculated to determine the percentage of $^{14}CO_2$ produced compared to total CO_2 production throughout the incubation period (Table 6.2). Increases in specific activity reflect increased utilization of ^{14}C-phenanthrene by the microorganisms. Some soils revealed an overall increase in specific activity throughout the incubation period (denoted by *), with highest values found toward the end of the experiment. Values for both contaminated and uncontaminated soils from Somerset remained consistent, reflecting a constant balance of $^{14}CO_2/CO_2$ production.

Table 6.2 Comparison of specific activities between uncontaminated and contaminated soils at five intervals throughout the incubation period.

Sites	Specific Activity (%)				
	1-6 days	10-24 days	35-63 days	77-105 days	119-147 days
TP[1] Uncontaminated 1 (0.5-1.0m)	3.55e-4	1.64e-4	3.73e-4	3.89e-4	1.20e-3*
TP Contaminated 1	5.61e-3	1.06e-3	2.12e-3	4.20e-3	7.89e-3
TP Uncontaminated 2 (1.5-2.0m)	2.34e-4	1.25e-4	2.74e-4	3.42e-4	1.29e-3*
TP Contaminated 2	7.55e-4	3.17e-4	7.38e-4	1.75e-3	3.64e-3
Pik[2] Uncontaminated 1 (0.75-1.0m)	9.00e-4	1.08e-3	2.67e-3	5.79e-3	1.13e-2*
Pik Contaminated 1	4.13e-4	2.73e-4	4.79e-4	8.89e-3	1.51e-3*
Pik Uncontaminated 1 (1.75-225 m)	2.73e-3	1.15e-3	1.92e-3	5.79e-3	8.22e-3
Pik Contaminated 1	2.69e-4	1.42e-4	1.85e-4	1.37e-4	4.48e-3*
Pik Uncontaminated 2 (2.5-3.0m)	6.11e-4	5.52e-4	1.28e-3	3.50e-3	2.39e-2*
Pik Contaminated 2 (2.5-3.25m)	3.25e-4	3.25e-4	7.26e-4	1.31e-3	2.07e-3
BD[3] Uncontaminated Sand 1 (1.5-2.0m)	4.20e-4	4.10e-3	7.06e-3	6.82e-2	2.01e-2*
BD Contaminated Sand 1	9.12e-4	1.54e-3	4.77e-3	7.67e-3	1.06e-2*
BD Uncontaminated Sand 2 (2.6-3.1m)	7.16e-4	5.76e-3	1.40e-2	2.69e-2	1.64e-2*
BD Contaminated Sand 1	1.14e-3	1.87e-3	3.77e-3	5.33e-3	1.58e-2*
BD Contaminated Sand 2	4.37e-4	5.82e-4	2.10e-3	6.43e-3	6.96e-3*
BD Uncontaminated Loam 1 (1.5-2.0m)	1.89e-3	6.61e-3	1.03e-2	5.65e-2	4.23e-2*
BD Contaminated Loam 1	5.00e-3	4.27e-3	2.18e-3	2.27e-3	1.60e-3*
BD Uncontaminated Loam 1 (2.75-3.5m)	1.58e-3	6.15e-4	4.50e-4	9.29e-4	9.58e-4
BD Contaminated Loam 2	1.76e-4	9.11e-5	2.92e-4	3.84e-4	2.05e-4
BD Contaminated Sand 2	4.81e-3	2.39e-3	4.16e-3	8.16e-3	6.43e-2*
BD Uncontaminated Loam 2 (4.1-4.5m)	2.87e-4	2.33e-4	1.50e-3	5.94e-4	7.05e-4
BD Contaminated Sand 3	3.86e-3	2.74e-3	3.07e-3	6.39e-3	3.36e-2*
Somerset Uncontaminated	1.08e-3	4.53e-3	2.48e-3	3.76e-3	2.72e-3
Somerset Contaminated	4.84e-3	2.00e-3	2.68e-3	4.08e-3	3.91e-3

[1] TP = Thicket Portage, [2] Pik = Pikwitonei, [3] BD = Brandon, * = denotes an increase

6.5.4 Volatilization

Combustion of the Ambersorb® indicated that volatilization of ^{14}C-phenanthrene had taken place for all soils during the incubation period (Table 6.3). All the samples had low levels of radioactivity from the Ambersorb trap, none exceeding 1%.

Table 6.3 Percent of radioactivity added as 14C-labeled phenanthrene recovered from volatilization. Means and standard deviations are from four replicates.

Site (depth in meters)	% Volatilization
TP[1] Uncontaminated 1 (0.5-1.0m)	0.99±0.18
TP Contaminated 1	0.14±0.06
TP Uncontaminated 2 (1.5-2.0m)	0.78±0.10
TP Contaminated 2	0.28±0.05
Pik[2] Uncontaminated 1 (0.75-1.0m)	0.33±0.13
Pik Contaminated 1	0.50±0.12
Pik Uncontaminated 1 (1.75-2.25m)	0.42±0.04
Pik Contaminated 1	0.60±0.12
Pik Uncontaminated 2 (2.5-3.0m)	0.44±0.11
Pik Contaminated 2	0.41±0.06
BD[3] Uncontaminated Sand 1 (1.5-2.0m)	0.21±0.06
BD Contaminated Sand 1	0.35±0.04
BD Uncontaminated Sand 2 (2.6-3.1m)	0.27±0.14
BD Contaminated Sand 1	0.33±0.06
BD Contaminated Sand 2	0.46±0.18
BD Uncontaminated Loam 1 (1.5-2.0m)	0.43±0.17
BD Contaminated Loam 1	0.03±0.03
BD Uncontaminated Loam 1 (2.75-3.5m)	0.75±0.09
BD Contaminated Loam 2	0.16±0.03
BD Contaminated Sand 2	0.06±0.02
BD Uncontaminated Loam 2 (4.1-4.5m)	0.07±0.01
BD Contaminated Sand 2	0.06±0.02
Somerset Uncontaminated	0.04±0.01
Somerset Contaminated	0.12±0.03
Glenlea 0-10 cm	0.06±0.01
St.Claude 0-10 cm	0.04±0.00
Brandon 0-10 cm	0.03±0.01
Glenlea 90-100 cm	0.22±0.02
St.Claude 90-100 cm	0.29±0.04
Brandon 90-100 cm	0.27±0.06

[1] TP = Thicket Portage, [2] Pik = Pikwitonei, [3] BD = Brandon

6.6 Discussion and Conclusions

6.6.1 Degradation of phenanthrene

Hexane was used to evenly distribute the diesel fuel and phenanthrene mixture onto the soils. Although the addition of hexane may have caused the death of some microorganisms from initial exposure, which has been shown to be toxic (Riser-Roberts, 1992), it was assumed that the majority of the hexane was volatilized. The beakers containing the soils were placed in a fumehood for one hour after the addition of hexane mixture, allowing for most of the hexane to be volatilized.

6.6.1.1 Contaminated vs. uncontaminated soils

The results from the degradation study of soils from TP and Pik suggest that the rate of phenanthrene degradation was related to previous hydrocarbon contamination of the soil and the difference in soil depth. Contaminated soils from both TP and Pik had higher levels of degradation than their respective uncontaminated soils at all depths. A decrease in degradation was seen with an increase in soil depth for these sites, suggesting decreased rates of microbial activity with increased depth. The relation between soil depth and phenanthrene degradation was more obvious than between the level of contamination to the soils and the amount of phenanthrene degraded. Concentrations of total extractable hydrocarbon (TEH) for the contaminated soils from TP and Pik can be found in Table 6.4. Levels of phenanthrene degradation had yet to stabilize in most of the soils from TP and Pik, indicating that degradation was not limited by phenanthrene concentration.

Table 6.4 Concentrations of total extractable hydrocarbon (TEH) in contaminated soils from Thicket Portage and Pikwitonei.

Site	Depth (meters)	TEH (μg/g)
Thicket Portage	0.2-1.0	6500
	1.5-2.0	5200
Pikwitonei	0.75-1.0	4400
	1.75-2.25	5700
	2.5-3.0	700

(detection limit 5μg/g)

Highest degradation of phenanthrene for both sand and loam soils from Brandon were observed with the uncontaminated samples from 1.5-2.0 meters. Most researchers have found the opposite, usually finding soils from contaminated sites to have higher rates of PAH degradation when compared to uncontaminated soils (McGill *et al.*, 1981; Wilson and Jones, 1993; Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997). High degradation of phenanthrene in these uncontaminated soils might be indicative of previous hydrocarbon exposure. Although all the uncontaminated soils had levels of contamination below the limit of detection (Chap. 3), it is possible that these soils experienced some contaminant exposure during the lengthy contamination event.

Soils of both textures that were able to degrade the phenanthrene had similar rates of ^{14}C-PAH recovery, ranging from 30% to 65%. Both contaminated sand soils used to compare against the loam soils (Figure 6.4) had greater percent phenanthrene degradation than the contaminated loam soils. Carmichael and Pfaender (1997) discovered a relationship between the fraction of silt and clay in contaminated soils and the percent of ^{14}C-PAH mineralized, which included ^{14}C-phenanthrene. They found that soils dominated by larger particles (~90% sand) usually had greater extents of degradation and higher total ^{14}C-PAH recoveries. They also compared a sand (90% sand) and loam (40%

silt) soil from a same site and found greater degradation in the sand. They attributed this difference to the increased interaction of ^{14}C-PAH with organic coatings on silt and clay sized particles, or the particle themselves, as opposed to the sand. Silt and clays have larger surface areas than sand so they have a greater fraction of organic matter on their surfaces and therefore larger partition coefficients than sand. As the range of depths for the samples from Brandon was between 1.5-4.5 m, it seems unlikely that amount the of organic matter at these depths would have influenced phenanthrene degradation as organic matter content decreases with increase depth. The interaction would be probably be caused by only the silt and clay particles.

No effect of previous levels of contamination on phenanthrene degradation was observed. Contaminated loam 2 from 2.75-3.5 meters had similar levels of PAH to the other soils studied in Figure 6.4, including the contaminated sands from 3.15-3.65 and 4.0-4.5 meters, but degraded little phenanthrene throughout the incubation period. The microbial community at that specific location may have been disturbed by the presence of some other toxic product utilized at this industrial site.

Patterns of phenanthrene degradation differed among the various soils. The presence of a plateau period towards the end of the incubation period for some soils is likely due the result of sorption of phenanthrene to the soil and subsequent slow rates of desorption for the sorbed ^{14}C-phenanthrene. Carmichael and Pfaender (1997) found similar results of a mineralization plateau from their work using a variety of ^{14}C-PAHs, even after an additional two month incubation period. Volkering *et al.* (1992) concluded from their work that the rate by which pollutant desorb, thereby becoming available for microbial degradation, might play a crucial part in the success of bioremediation programs.

The possibility of hydrocarbon degradation occurring at significant depths at the actual sites would be difficult, as the process requires oxygen. An increase in soil depth results in an increase in bulk density, resulting in a decrease in air porosity (Paul and Clark, 1996). Under anaerobic conditions, unsubstituted PAHs are extremely resistant to microbial degradation (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). Anaerobic degradation of aliphatic hydrocarbons has been identified but the process is extremely slow. The results obtained from the contaminated sites indicated that in the presence of oxygen, microorganisms from subsurface soils could degrade hydrocarbons. Remedial procedures such as bioventing or the addition of oxygen releasing compounds such as hydrogen peroxide would provide the oxygen necessary for more successful microbial hydrocarbon degradation.

6.6.1.2 Agricultural soils

All agricultural surface soils were able to degrade phenanthrene with similar rates of recovery, ranging from 42 to 51%. Little difference was observed between contaminated and uncontaminated soils from Somerset, the only differences being the lack of a lag phase and a linear degradation rate for the contaminated soil. Adaptation to the presence of phenanthrene did not seem to be needed for the microbial community in the contaminated soil from Somerset, allowing degradation to begin quicker. The presence of a plateau period towards the end of the incubation period for all surface soils may be due to sorption of the phenanthrene (Manilal and Alexander, 1991; Maurice, 1998).

A lag phase was also observed for the subsurface soils from Glenlea and Brandon, indicating an initial adaptation stage. Low rates of degradation in the subsurface soils were probably attributed to the presence of hydrocarbon degraders having decreased

microbial respiration, resulting in a longer adaptation period. Microbial communities from surface and subsurface fertile agricultural soils have the potential to degrade considerable amounts of phenanthrene.

As previously mentioned, examination of the degradation of labelled compounds does provide a good indication of the potential of uncontaminated soils for biodegradation, but the presence of a degradation plateau does seem to indicate that substrate availability due to sorption becomes an important issue to address.

6.6.2 Respiration and Specific activity

For the most part, respiration data indicated insignificant differences in microbial activity during phenanthrene degradation between contaminated and uncontaminated soils at all depths. Two contaminated soils, one from TP at 0.5-1.0 meters and one from Brandon (contaminated loam 1 from 1.5-2.0 meters), had higher levels of activity than their respective uncontaminated soils. Geiselbrecht *et al.* (1996) and Carmichael and Pfaender (1997) had found higher rates of CO_2 production in contaminated versus uncontaminated soils. They claimed that tolerant microbial species with greater metabolic versatility probably maintained activity. This might explain the two contaminated soils previously mentioned. In most cases, high rates of phenanthrene degradation from $^{14}CO_2$ analysis did not correspond with high respiration activity from CO_2 measurements. Results from Grosser *et al.* (1991) showed that one soil having the lowest microbial activity of all soils studied had the highest degradation potential with the PAHs examined. This suggests that additional factors, such as bioavailability of substrate and selection for degrading populations may be involved in the breakdown of PAHs. The majority of the previously contaminated soils from this study did not exhibit higher rates of CO_2. The abundant supply of carbon in these soils should have resulted in higher rates

of respiration from the microorganisms. The issue of substrate availability arises again. The issue of contaminant toxicity can also be questioned. Results from the enumeration study (Chapter 4) showed that numbers of HC degraders from these contaminated soils were abundant, ruling out the possibility of toxicity.

All contaminated soils displayed the potential to degrade phenanthrene. The length of the contamination event should have provided the microorganisms with ample time to degrade the contaminants. If levels of contaminant have been persistent for many years in a soil system, several factors prevented successful biodegradation to occur resulting in its sorption, therefore decreasing its bioavailability. The rate of contaminant sorption may be greater than the rate of degradation. Therefore, in some contaminated soils, the availability of PAHs due to sorption may be a controlling factor for intrinsic degradation. This suggest that efforts to increase the availability of the PAH may be necessary if the metabolic capability shown to exist by this study and others is to be used in intrinsic bioremediation strategies. Efforts such as the use of surfactants to prevent contaminant sorption to occur or to cause desorption should be examined.

The low percentages for specific activity seem to indicate that phenanthrene degradation accounted for a small portion of the total microbial activity, which was not surprising as phenanthrene is a complex structure for microorganisms to degrade and was added at a low concentration. Increases in specific activity toward the end of the incubation period for some the uncontaminated soil could indicate stages of adaptation by the microorganisms from increased ^{14}C-phenanthrene utilization. All of the sand textured soils from Brandon had increases in specific activity towards the end of the incubation period.

The usefulness of microbial respiration as a management tool for intrinsic remediation does provide important information of the potential of soils for degradation. Measures of PAH degradation based on the use of freshly added compounds should be used with caution for assessing the fate of aged on site contaminants. Availability of the compound over time due to sorption becomes an important issue to address, limiting the possibility of complete degradation.

6.6.3 Volatilization

Little loss of ^{14}C-phenanthrene was detected by burning of the Ambersorb using the oxidizer. Recovery of Ambersorb standards spiked with ^{14}C-phenanthrene was approximately 85%. Some volatilization of phenanthrene would be expected due its moderate vapour pressure of 90.7 mPa at 25°C (Government of Canada, 1994). All soils from all depths had similar losses of phenanthrene from volatilization. This suggests that the use of Ambersorb for trapping volatilized phenanthrene provided reasonable amounts.

CHAPTER 7

GENERAL DISCUSSION

The objective of the study was to determine the usefulness of microbial enumeration, diversity, and activity as potential tools for implementing strategies for intrinsic remediation. Chapter 4 surveyed differences in numbers of alkane and phenanthrene-degrading microbes, using a most-probable-number technique, in contaminated versus adjacent uncontaminated soils and agricultural soils at a variety of depths. Chapter 5 compared substrate utilization patterns between contaminated and adjacent uncontaminated soils for possible differences caused by the contamination event. Chapter 6 investigated the effect of previous hydrocarbon exposure on the degradation of phenanthrene in diesel fuel and respiration activity from comparisons between contaminated and uncontaminated soils and examined the degradative potential of agricultural surface and subsurface soils.

7.1 Enumeration of alkane and phenanthrene degrading microorganisms

Soils consist of heterogeneous microorganisms that include naturally occurring population with the ability to degrade petroleum products and low molecular weight PAHs such as phenanthrene (Atlas, 1978; Bossert and Bartha, 1984; Dragun, 1988, Kästner *et al.*, 1994; Atlas and Cerniglia, 1995). Hydrocarbon (HC) degrading bacteria are ubiquitously distributed, their proportions in heterotrophic bacterial soil communities

ranging from 0.13% to 50% (Wünsche *et al.*, 1995). In this study, both aliphatic (diesel fuel) and aromatic (phenanthrene) utilizing bacteria were found in all uncontaminated soils, including agricultural soils at both surface and subsurface levels. As microorganisms that degrade aromatic HCs are frequently distinct from those that attack aliphatic HCs (Foght *et al.*, 1990; Atlas, 1991; Wrenn and Venosa, 1996), the isolation of both groups in the examined soils was very important because both groups are not always found in soils. Kästner *et al.* (1994) demonstrated no zone forming units in freshly sampled uncontaminated soil using a method of soil extraction and plating on minimal medium plates with phenanthrene as the sole source of carbon. In the majority of cases, soils previously contaminated had higher numbers of aliphatic and aromatic degrading populations, which was in accordance with numerous findings found in the literature (Atlas, 1981; Kästner *et al.*, 1994; Duncan *et al.*, 1998; Williams *et al.*, 1998; Nyman, 1999). The addition of petroleum products to soils selectively favours and enriches the microbial population that is able to adapt and utilize the new substrate (Bossert and Bartha, 1984).

7.2 Relationship between microbial enumeration, diversity, and respiration

In the majority of cases, enumeration of hydrocarbon degrading microorganisms from contaminated and uncontaminated soils revealed that contaminated soils yielded significantly greater numbers of HC-degraders. As a tool for implementing strategies for intrinsic remediation, the enumeration experiment showed that the organisms required for HC degradation were present in the soils. The increase in numbers of HC degraders in contaminated versus uncontaminated soils at a variety of depths indicated that selection

and adaptation of microorganisms capable of HC degradation had occurred during the contamination event. The presence of aliphatic and aromatic degraders in agricultural surface and subsurface soils demonstrated that various groups of HC degraders are found in native microbial populations. The use of enumeration as a management tool was not fully examined in this study, as the number of HC degraders required for successful intrinsic remediation to occur was not determined. Further study in examining the number of degraders required for remediation would provide a more accurate description of the usefulness of enumeration. Enumeration results do not provide any information on the microbial activity in the soils. Researchers have found that high numbers does not always indicate high levels of activity in contaminated soils (Grosser *et al.*, 1991; Miethe *et al.*, 1994; Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997). The combination of results from both enumeration and activity studies provides a more detailed description of the HC degrading population. The number of HC degraders was shown, by the degradation study, not be a limiting factor in bioremediation. During the microcosm incubation period for some soils, carbon became limiting to the soil microbial community resulting in a degradation plateau. Enumeration could be used as a contaminant toxicity indicator, as high numbers would indicate that toxicity did not inhibit growth and reproduction of the soil microbial community.

Results from the metabolic diversity experiment did not provide a clear indication of the effects of contamination on the microbial community. Contaminated soils that had higher percent substrate utilization than their respective uncontaminated soils, might be from the selection and adaptation of microorganisms having increased metabolic capabilities from the contamination event. In terms of the adaptability of microorganisms at contaminated sites, metabolic diversity could provide information of increased

substrate versatility from possible shifts in population dynamics. This phenomenon was observed in a study done by Wünsche *et al.* (1995) where a pristine soil was contaminated with oil resulted in a shift in substrate utilization patterns and bacterial genera favouring *Pseudomonads*, which was found to dominate a previously long-term contaminated soil. The lack of change in substrate utilization patterns might also reflect a return to a steady-state level governed by factors other than substrate supply. The addition of petroleum products to soils has been shown to decrease the diversities of the microbial communities by upsetting the ecological balance of population interactions within the community (Atlas *et al.*, 1991). Changes in microbial diversity have been observed through shifts in substrate utilization patterns (Wünsche *et al.*, 1995). Derry *et al.* (1998) examined soils contaminated for more than three years with creosote and found no significant differences in diversity between uncontaminated and contaminated soils, possibly reflecting processes of microbial adaptation to creosote. Atlas *et al.* (1991) found that microbes within disturbed communities demonstrated enhanced physiological tolerances and substrate utilization capabilities. Soils from Thicket Portage, Pikwitonei, and Brandon had significant exposure time to the contamination, all exceeding a period of twenty-five years. Most results from these three soils revealed no significant negative effect from previous HC exposure, with the majority of the cases showing the contaminated soils having greater percent substrate utilization than the uncontaminated soils (Table 7.1). The usefulness of metabolic diversity was seen from the lack of difference between uncontaminated and contaminated soils, indicating that 30 years after the contamination event, there was no impact of the microbial community. The stability of the population in the contaminated soil was due to the selection of microbes having broad substrate utilization capabilities. As was the case with enumeration study, metabolic diversity

might also be useful in assessing the toxicity effect on the soil microbial community. Soils having high number of HC degraders and high substrate utilization capabilities would indicate that contaminant toxicity did not affect the microbial community.

In most cases, higher rates of HC degradation have been shown to occur in previously contaminated soils compared to pristine soils by tolerant species having increase metabolic versatility (McGill *et al.*, 1981; Wilson and Jones, 1993; Geiselbrecht *et al.*, 1996; Carmichael and Pfaender, 1997). Since contaminated soils have adapted microbial communities with higher proportions of HC-degraders, they can quickly respond to the presence of HC pollutants. This was the case for all but one soil from Thicket Portage and Pikwitonei. In six of the ten soil samples examined, the effect of previous contamination exposure resulted in greater number of HC-degraders having increased metabolic capabilities and higher rates of phenanthrene degradation when compared to adjacent uncontaminated soils (Table 7.1). This was also the case for the contaminated sand soils at depth of 3.1-3.5 and 4.1-4.5 meters. Although yielding greater numbers of HC-degraders, the contaminated soil from Somerset had similar percent substrate utilization and $^{14}CO_2$ recovery than its uncontaminated soil. The major difference between the soils from Somerset was the lack of lag phase in the degradation study for the contaminated soil, indicating the lack of an acclimation period for degradation to proceed. Both uncontaminated sand soils and uncontaminated loam from 1.5-2.0 meters from Brandon mineralized higher amount of phenanthrene than their respective contaminated soils. Although contaminant levels were below detection limit, this could indicate that these uncontaminated soils did have previous contaminant exposure throughout the lengthy contamination period of this site.

Table 7.1 Comparison of most probable number (MPN) of diesel degraders, % substrate utilization, and $\%^{14}CO_2$ recovery from microbial enumeration, diversity, and respiration, respectively.

Site and depth (m)	Results		
	MPN (diesel)	% Utilized substrate	$\%^{14}CO_2$ recovery
TP[1] uncontaminated 1 (0.5-1.0)	*420*	*10.4*	*1.48*
TP contaminated	51000	35.9	49.89
TP uncontaminated (1.5-2.0)	*150*	*23.4*	*1.38*
TP contaminated	6000	32.0	10.76
Pik[2] uncontaminated 1 (0.75-1.0)	*90*	*8.3*	*8.04*
Pik contaminated	57000	41.1	28.27
Pik uncontaminated 1 (1.25-1.75)	*61*	*21.1*	*0.89*
Pik contaminated	6600	7.3	19.45
Pik uncontaminated 2 (2.5-3.0)	*9*	*11.5*	*5.53*
Pik contaminated	340	29.2	12.86
BD[3] uncontaminated sand 1 (1.5-2.0)	*4300*	*56.5*	*64.20*
BD contaminated sand 1	3300	64.3	33.69
BD uncontaminated sand 2 (2.6-3.2)	*65*	*39.8*	*54.68*
BD contaminated sand 1	230	52.9	44.26
BD contaminated sand 2	227000	77.1	30.68
BD uncontaminated loam (1.5-2.0)	*540*	*59.4*	*55.40*
BD contaminated loam 1	21400	57.3	38.38
BD uncontaminated loam (2.75-3.5)	*2800*	*56.0*	*9.26*
BD contaminated loam 2	150	42.7	0.47
BD contaminated sand 2 (3.1-3.5)	60000	80.5	47.98
BD uncontaminated loam 2 (4.1-4-5)	*3*	*22.0*	*2.43*
BD contaminated sand 2	9200	73.2	44.74
Somerset uncontaminated	130	78.9	42.37
Somerset contaminated	97000	73.4	44.42

[1]TP=Thicket Portage, [2] Pik=Pikwitonei, [3]BD=Brandon

Therefore in summary, these soils contain high numbers HC degrading microorganisms, having increased metabolic versatility, and enhanced microbial activity, however the contaminants were still present in the soils after decades. An important factor causing reduced biodegradation of HC compounds is sorption on soil particles, primarily clays, and soil organic matter. The hydrophobic nature of diesel fuel and PAHs allows adsorption onto hydrophobic areas of soil surfaces. Carmichael and Pfaender

(1997) found similar results of a degradation plateau from their work using a variety of ^{14}C-PAHs even after an additional two month incubation period. Soils that did not experience a plateau in this study (soils from TP and Pik) could simply be due to the continued availability of the phenanthrene for degradation. Continued incubation might have resulted in a degradation plateau caused by increased sorption of the phenanthrene with time or by decrease in microbial activity.

Carmichael and Pfaender (1997) also found a relation between the fraction of silt and clay in contaminated soils and the percent of ^{14}C-PAHs mineralized, which included phenanthrene. They found that soils dominated by larger particles (90% sand) usually had greater extents of degradation and higher total recoveries. They also compared a sand (90% sand) and loam (40% silt) soils taken from the same site, having similar amounts of organic carbon, and found greater PAH degradation in the sand. Silt and clays have greater surface areas than sand, allowing for larger partition coefficients. This trend was also visible in this degradation experiment, as sand soils had overall greater percent recoveries than the loam textured soils. Measure of PAH degradation based on the use of freshly added compounds may be useful as a management tool for assessing the potential of microbial communities, but should be used with caution for assessing the fate of aged on site contaminants.

Although the presence of HC degraders were found in all surface agricultural soils, the development of a plateau at approximately the same duration of the incubation and percent ^{14}C-phenanthrene recoveries no greater than 51%, would seem to indicate a problem with substrate availability. The carbon supply became limited to the soil microbial community during the incubation period. The increase in MPN of diesel and phenanthrene degraders as well as the lack of a lag phase in the degradation study for the

contaminated soil from Somerset compared to its uncontaminated soil does seem to indicate that selection and adaptation occurred during the contamination event. An adaptation period was required to select these organisms and allow for their adaptation and growth in the contaminated environment. Therefore, as this adaptation period for microbial adaptation would proceed, a greater fraction of the contaminants could become sorbed and no longer available for degradation. In terms of site management, the success of intrinsic remediation using naturally occurring microorganisms would be dependent on the prevention of contaminant sorption within the soil matrix at or near the initial time of a spill, which would allow continued availability to the microorganisms as they become adapted to the contaminated environment. The microorganisms required to breakdown the contaminants are found in the natural environment and their numbers most often increase because of processes of adaptation. The numbers of naturally occurring HC degraders does not seem to be the limiting factor in successful bioremediation strategies, but the availability of contaminants as substrate over time would be the important issue to address.

7.3 The effect of previous hydrocarbon exposure in relation to soil depth

Soil depth can be a controlling factor in the degradation of HC contaminants in the environment (Rainwater *et al.*, 1993). The number of organisms is usually expected to decrease with increasing soil depth, due to many factors such as decreases in substrates and lack of nutrients and oxygen, resulting in decreases in microbial enumeration, diversity, and respiration. Generally, this trend was observed in the examined soils. Findings of significant numbers of HC degraders having substantial metabolic versatility

yielding high ^{14}C-phenanthrene recovery at depths exceeding 2.5 meters was very important. Examples of these are soils from Pik contaminated 2 at 2.5-3.0 meters and especially Brandon contaminated sand 2 from 2.6-4.5 meters (Table 7.1). PAH contamination of the contaminated sand 2 soils from Brandon was significant, with phenanthrene concentrations of 1900 μg/g at 4.1-4.5 meters. Even at these great depths, adaptation of microbial communities with the potential to degrade hydrocarbons having increased metabolic versatility was occurring. PAHs have been shown to be extremely resistant to microbial degradation under anaerobic condition (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). The main requirement for HC degradation at these depths would be a supply of oxygen, either through bioventing or the introduction of oxygen releasing compounds, such as hydrogen peroxide.

7.4 Current state of the contaminated sites

To date no remediation has taken place at Brandon or Thicket Portage. The Brandon contaminated site was taken over by Morrow Consultants and no Remedial Action Plan has yet to be developed. Thicket Portage is not scheduled for remediation until next year, while remediation was started last summer in Pikwitonei. The contaminated soil from Pikwitonei will be taken to a landfarm for remediation. The landfarm has been built and the work will continue this summer. Remediation strategies for the contamination site near Somerset began after the spill occurred and are continuing.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The objective of this study was to determine the usefulness of microbial enumeration, diversity, and respiration as potential management tool for implementing strategies for intrinsic remediation. The combined information from all three studies proved to be a much better tool that the individual results. Relations between the three studies provided important information on the effects of the contamination event on the soil microbial community. Enumeration proved to be an important tool as it was concluded that native microbial populations consisted of both aliphatic and aromatic degrading microbes throughout the soil profile, whose numbers increased from the adaptation of previous hydrocarbon exposure. Results from the enumeration study did not provide information of the numbers of degraders required for successful remediation strategies. The relation of enumeration and respiration values obtained from this study, it can be concluded that the presence of native HC degraders and their increase in numbers during the contamination event was not a limiting factor in degradation. The presence of a degradation plateau towards the end of the incubation period indicated that carbon became limiting. The introduction of HC degrading laboratory strains would not be required for degradation to proceed in these soils. The microbial community from these contaminated soils often had increased metabolic versatilities and were capable of higher

recoveries of ^{14}C-phenanthrene. Even at depths ranging from two and half to four meters, the presence of HC degraders was observed.

In most cases, examination of percent substrate utilization using Gram-negative BIOLOG plates showed that previous hydrocarbon contamination generally had no negative effect on the microbial populations in these environments. In some cases, greater substrate utilization was seen in the contaminated soils. Analysis of substrate utilization patterns through principle component analysis and cluster analysis did not prove to be a useful indicator of the effects of previous hydrocarbon exposure for these soils, as inconsistent results were obtained from both contaminated and uncontaminated soils. These results concluded the usefulness of metabolic diversity in indicating that the contamination event did not disrupt the microbial diversity of the sites. The contamination event selected microorganisms with broad substrate utilization capabilities, resulting in a stable population. The increased metabolic versatility observed in some contaminated soils does seem to indicate that selection and adaptation had occurred due to the contamination event. The high number of degraders having broad substrate utilization capabilities indicated that contaminant toxicity did not affect the soil microbial population in the contaminated soils. Both enumeration and diversity could be used as toxicity indicators for contaminated soils.

Measurements of $^{14}CO_2$ from the degradation of ^{14}C-phenanthrene were a much more consistent indicator of which soils had the greatest phenanthrene degrading potential, than respiration activity from analysis of total CO_2. The majority of the contaminated soils yielded higher recoveries of phenanthrene than their respective uncontaminated soils. The two uncontaminated soils from Brandon that had high rates of degradation and broad substrate utilization capabilities indicated that these soils might have been exposed to the

contaminants. Although contaminant concentrations for these uncontaminated sites were below detection limit, the possibility of contaminant exposure cannot be excluded. Only three contaminated soils had higher microbial activity than their uncontaminated soils, indicating that in most cases, the presence of diesel fuel did not stimulate microbial activity. The microcosm apparatus differs from conditions found at the contaminated sites. The environment created by the microcosm is a closed system that would be more representative of a bioreactor, where soil conditions and inputs can be strictly regulated and controlled.

Comparison of results obtained from the three experiments revealed that in six of the ten contaminated soils studied, the contaminated soils produced greater numbers of hydrocarbon degraders that had increased metabolic capabilities with higher recovery of ^{14}C-phenanthrene. In these cases, previous hydrocarbon exposure resulted in the selection of microbes able to utilize numerous substrates, including hydrocarbons, as sources of carbon and energy, resulting in quicker response to the addition of ^{14}C-labelled phenanthrene yielding higher rates of recovery. The contamination present in some of the soils used in this study was very old, allowing microbial communities ample time to degrade the contaminants. However, high concentrations of diesel fuel and PAHs remained in the soils, even low molecular weight PAHs such as naphthalene, which have been shown to be readily degradable. Therefore in some contaminated soils, the availability of the contaminants was a controlling factor for intrinsic remediation of contaminants. The increase in time required for microbial adaptation to occur causes greater portion of the pollutants to become sorbed within the soil matrix. This suggest that efforts to impact the availability of hydrophobic contaminants may be necessary if the metabolic potential shown to exist by this study and other is to be used in intrinsic

remediation strategies. Measures of PAH degradation based on the use of freshly added compounds may be useful in assessing the potential of the microbial community as in this study, but should be used with caution for assessing the fate of aged on site contaminants.

Further study is required in determining the effects of hydrocarbon exposure on the substrate utilization patterns of microorganisms in soils. Research has found inconsistent results from the use of BIOLOG plates due to the extreme diversity of microorganisms in soils and the many factors that affect their distribution and the many ways of interpreting the results. Standardized methods of analysing and interpreting results from the plates would be extremely helpful, which would then remove an important variable when comparing results. Analysing a soil near the time of contamination and consistent observations throughout a period of time would provide valuable information of shifts in substrate utilization patterns, which has been observed by some researchers such as Wünsche *et al.* (1995). The BIOLOG method does provide important information on the functional diversity and interactions of microbial populations in soil. Interpretations of these results need to be refined for optimization of this information.

This study found that hydrocarbon degraders are ubiquitously distributed in a variety of native soil environments. The next step would be to optimize their distribution and growth in the presence of a contamination event. Contaminant availability, nutrient status, organic matter, temperature, oxygen and moisture content are all important factors in effective bioremediation strategies. Since no soil environments are similar, determination of the optimal conditions for each of these factors would be important.

The determination of intermediates from microbial degradation might provide important information on the potential of a soil to degrade hydrocarbons. Although some of the contaminated soils degraded less phenanthrene when compared to uncontaminated

soils, it is possible that the highly adapted communities in the contaminated soils might be forming intermediates of phenanthrene instead of complete breakdown to CO_2. If this was the case, the communities in the contaminated soils might actually be degrading more phenanthrene than those in the uncontaminated soils.

The use of a more complex PAH such as benzo[a]pyrene would provide information of the microbial communities to degrade more recalcitrant molecules. The structure of phenanthrene is found within that of benzo[a]pyrene, so the induction of enzymatic pathways for both PAHs may be similar, as was suggested by Rogoff (1962). The major problems with complex PAHs are that they are highly insoluble and adsorb tightly to soil particles. Research showing degradation of benzo[a]pyrene is limited. Any finding of microbial communities with the potential to degrade benzo[a]pyrene would be important.

CHAPTER 9

CONTRIBUTION OF KNOWLEDGE

The study identified that the usefulness of microbial enumeration, diversity, and respiration was a better remediation tool when the results from the three separate experiments were combined. These three microbial parameters provided important information of the effect of the contamination event on the soil microbial population. Enumeration provided information on the presence of native HC degraders in soils, which increased in numbers in the presence of HC contaminants by processes of adaptation. Further study in identifying number of HC degraders required for successful remediation would provide a better indication of the usefulness of enumeration as a management tool. Enumeration in relation to metabolic diversity indicated that contaminant toxicity did not affect the microbial population as high numbers of degraders having broad substrate utilization capabilities were found. Important information was observed from the lack of difference in the metabolic diversity between contaminated and uncontaminated soils, indicating that the contamination event may have disrupted the microbial community but the effect did not persist. The contamination event selectively favoured HC degraders with generalized substrate consumption. Microbial respiration provided details of the degradative potential of the HC degrading population. Relation between enumeration and respiration indicated that high number of degraders does not always indicate high rates of

degradation. These two experiments also revealed that the supply of carbon was the limiting factor in degradation and not the number of degraders, indicating a problem with continued substrate availability. The use of freshly added substrates may be useful in assessing the potential of the microbial community, but should be used with caution when assessing the fate of aged on site contaminants.

This work has demonstrated that through the study of microbial enumeration, diversity, and respiration, native to soils in Manitoba have the microorganisms with the potential to degrade hydrocarbon contaminants. Strategies such as bioaugmentation, with the introduction of 'super bugs' would not be necessary. Although degradation of phenanthrene in some soils was limited, certain factors such as aeration, nutrient addition, irrigation, temperature, and especially availability would enhance degradation. The long-term persistence of diesel fuel in soils from Thicket Portage and Pikwitonei and PAHs in soils from Brandon strongly suggests that availability was an important controlling factor for intrinsic remediation. The presence of plateau phase in the degradation experiments toward the end of the incubation period confirms this finding. The use of surfactants would be necessary in situations where the contaminants are not bioavailable. The effectiveness of the surfactants would seem to be dependent on when they were applied. If applied quickly after a HC spill, the surfactants would prevent sorption to occur, allowing the microbial communities an adaptation period, and once adapted, then begin the degradation of a greater portion of available substrate.

Even at depths of 2.5 to 4 meters, microorganisms were found with the potential to degrade HC contaminants. Introduction of oxygen through bioventing or oxygen releasing compounds would be required to activate and maintain degradation, as oxygen would be limiting as these depths. Microorganisms required to remove hydrocarbon

contaminants are omnipresent in the natural environment. Studies to increase their presence and degradative abilities during contamination events would result in effective strategies for successful intrinsic remediation.

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APPENDIX

Contaminated ?

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

THICKET PORTAGE
Decommissioned Diesel Site

TP-010
1 OF 1
Prj.# 200531

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0	AS/AG	1	OH	(0.00-0.20) Top soil, brown, trace rootlets.
	AS/AG	2	OH	(0.20-0.50) Clay, organic, dark brown, trace rootlets, soft insitu, above PL.
	AS/AG	3	CH	(0.50-1.00) Clay, dark brown, medium insitu, above PL, slight diesel odor.
1	AS/AG	4		(1.00-1.50) Clay, dark brown, trace silt pockets, medium insitu, above PL. Strong diesel odor to 2.00m.
	AS/AG	5		(1.50-2.00) Clay, dark brown, trace silt pockets, medium insitu, above PL.
	AS/AG	6		(2.00-3.00) Clay, dark brown, trace silt pockets, trace oxidation, medium insitu, above PL. No diesel odor below 2.00m.
2	AS/AG	7		
	AS/AG	8		
	AS/AG	9		
	AS/AG	10		
	AS/AG	11		
3	AS/AG	12		
	AS/AG	13		(3.00-4.50) Clay, light brown, varved, silt layers 5-10mm thick, clay layers 5-13mm thick, trace oxidation, medium to soft insitu, above PL.
	AS/AG	14		
	AS/AG	15		
	AS/AG	16		
4	AS/AG	17		
	AS/AG	18		
	AS/AG	19	CI	(4.50-4.75) Clay, light brown, silt varves, trace oxidation, silt reacts to shaking, silt layers 5-14mm thick, clay layers 4-16mm thick, above PL.
	AS/AG	20		(4.75-5.00) Clay as above, trace fine grained sand.
5	AS/AG	21	SM	(5.00-5.25) Sand, brown, fine to coarse grained, with silt, saturated.
				(5.25) End of Hole due to auger refusal. Well installed.

Moisture: PL 20% | M.C. 40% 60% 80% | LL

Total Extractable Hydrocarbons (ppm) ⊕: 100 200 300 400

Microtip PID Reading ○: 100 200 300 400

Oil and Grease (ppm) ■: 20 40 60 80

DEPTH (m)	Total Extractable Hydrocarbons (ppm) ⊕	Microtip PID Reading ○	Oil and Grease (ppm) ■
0		31.4	
		178.0	
	>>4000	308.0	
		278.0	
1		425.0	
	>>11000	487.0	
		370.0	
		303.0	
2	>>1800	278.0	
		20.0	
		25.7	
		16.8	
3		15.2	
		14.9	
		16.2	
4		8.0	
		8.3	
		12.4	
5		11.8	
		8.0	
		3.7	

WATER TABLE ▽
Depth @ 0.20 (m)
on 98/07/18

NORTH: 207.800
EAST: 201.900
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 99.07 (m)

EQUIPMENT: RM-30
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling

COMPLETION: 98/07/15
INSPECTOR: P. Pantel
DEPTH: 5.25 (m)

STATUS:
FINAL: DES 98/04/08 0/8
Overlays: HCX600 HCPID ATT10 OG100 LINE USCS TITLE
Printed: 98/06/19 11:10 TP98A GEOTE

Uncontaminated 1

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

THICKET PORTAGE
Decommissioned Diesel Site

TP-033
1 OF 1
Prj.# 200531

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL — M.C. — LL; 20% 40% 60% 80%

Oil and Grease (ppm) ■: 20 40 60 80

Total Extractable Hydrocarbons (ppm) ⊕: 100 200 300 400

Microtip PID Reading ○: 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION	Total Extractable Hydrocarbons (ppm) ⊕	Microtip PID Reading ○
		1 AS/AG	OH	(0.00-0.25) Top soil, dark brown, roots.		16.8
		2 AS/AG	CH	(0.25-1.00) Clay, brown, trace rootlets, above PL. No diesel odor detected in hole.		14.3
1		3 AS/AG		(1.00-1.50) Clay, brown, above PL.		10.1
		4 AS/AG		(1.50-2.00) Clay as above, trace silt pockets.		6.0
		5 AS/AG				4.4
2		6 AS/AG		(2.00-2.50) Clay as above, medium insitu.		6.6
		7 AS/AG				8.5
		8 AS/AG		(2.50-3.00) Clay, brown, trace silt seams, medium insitu, above PL.		8.9
3		9 AS/AG				4.4
		10 AS/AG		(3.00-3.50) Clay, brown, slightly varved, trace oxidation, medium insitu, above PL.		4.3
		11 AS/AG				4.0
		12 AS/AG	CI	(3.50-4.75) Clay, light brown, varved, soft insitu, above PL.		6.4
		13 AS/AG				6.6
4		14 AS/AG				1.5
		15 AS/AG				1.6
		16 AS/AG				10.0
		17 AS/AG				2.5
5		18 AS/AG	ML	(4.75-4.90) Clay, light brown/grey, varved, trace fine sand in silt varves, trace oxidation, above PL.		2.4
				(4.90-5.25) Silt grey, trace sand, trace gravel.		
				(5.25) End of Hole due to auger refusal.		

DEPTH (m)

WATER TABLE
No water level recorded.

NORTH: 240.200
EAST: 197.200
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 98.50 (m)

EQUIPMENT: RM-30
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling

COMPLETION: 98/07/17
INSPECTOR: P. Pantel
DEPTH: 5.25 (m)

STATUS: FINAL: DES 98/11/18 O/B
Overlays: HCX500 HCPID ATT10 OG100 LINE USCS TITLE
Printed: 99/05/19 11:13 TP98A GEOTE

Contaminated !

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

PIKWITONEI
Decommissioned Diesel Site

PI-012
1 OF 1
Prj.# 200533

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
		1 AS/AG	CH	(0.00-0.20) Clay filled.
		2 AS/AG		(0.20-1.50) Clay, dark brown, blocky, diesel odor, above PL.
		3 AS/AG		
1		4 AS/AG		
		5 AS/AG		
		6 AS/AG		
		7 AS/AG		(1.50-2.50) Clay, brown, some silt, varved, diesel odor, above PL.
2		8 AS/AG		
		9 AS/AG		
		10 AS/AG		
		11 AS/AG	CI	(2.50-3.50) Clay, brown, with silt varves.
3		12 AS/AG		
		13 AS/AG		
		14 AS/AG		
		15 AS/AG		(3.50-4.20) Clay, light brown, with silt varves, diesel odor, above PL.
4		16 AS/AG		
		17 AS/AG		
		18 AS/AG	CL	(4.20-4.45) Clay, light brown, some sand seams, wet, diesel odor.

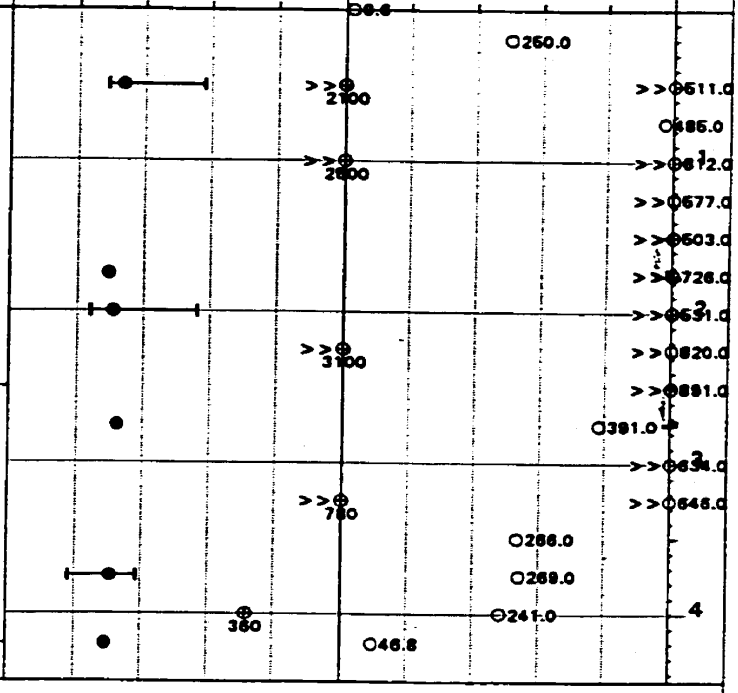
(4.45) End of Hole due to auger refusal on bedrock.

Moisture: PL 20% | M.C. 40% 60% | LL 80%

Total Extractable Hydrocarbons (ppm) ⊕: 100 200 300 400

Microtip PID Reading ○: 100 200 300 400

Oil and Grease (ppm) ■: 20 40 60 80



WATER TABLE No water encountered during drilling.	NORTH: 217.600 EAST: 230.000 TREND: ------ PLUNGE: -90.0 (deg) ELEV G/S: 97.89 (m)	EQUIPMENT: RM-30 METHOD: Hollow Stem Auger DRILLER: Paddock Drilling	COMPLETION: 98/07/07 INSPECTOR: F. Demchenko DEPTH: 4.45 (m)
		STATUS: FINAL: DES 99/02/18 O/B Overlays: HCX600 HCPID ATT10 OG100 LINE USCS TITLE	Printed: 99/06/19 11:27 PI98A GEOTE

Uncontaminated /

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

PIKWITONEI
Decommissioned Diesel Site

PI-016
1 OF 1
Prj.# 200533

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
		1 AS/AG	CH	(0.00-0.50) Clay fill, with gravel, rootlets.
		2 AS/AG		(0.50-1.25) Clay, brown, slightly blocky, above PL.
1		3 AS/AG		
		4 AS/AG		
		5 AS/AG		
		6 AS/AG		(1.25-3.00) Clay, brown, some silt, varved, slightly blocky, above PL.
		7 AS/AG		
2		8 AS/AG		
		9 AS/AG		
		10 AS/AG		
		11 AS/AG		
		12 AS/AG		
3		13 AS/AG	CI	(3.00-4.25) Clay, brown, some silt varves, slightly blocky, trace oxidation, above PL.
		14 AS/AG		
		15 AS/AG		
		16 AS/AG		
4		17 AS/AG		
		18 AS/AG		(4.25-4.70) Clay, light brown, trace sand, varved, damp, stiff insitu, above PL, no diesel odor.
		19 AS/AG		
		20 AS/AG	ML	(4.70-5.00) Silt, some sand, some clay, trace fine gravel, trace oxidation, varved, damp, no diesel odor.
5		21 AS/AG	SM	(5.00-5.20) Sand, some silt, some fine to coarse gravel, trace oxidation, wet.
				(5.20) End of Hole due to auger refusal on bedrock.

Moisture: PL 20% | M.C. 40% 60% | LL 80%

Total Extractable Hydrocarbons (ppm) ⊕: 100 200 300 400

Oil and Grease (ppm) ⊠: 20 40 60 80

Microtip PID Reading ○: 100 200 300 400

DEPTH (m)	Microtip PID Reading ○
0	10.0
1	12.2, 10.9, 9.7, 12.3
2	10.7, 10.1, 8.8, 9.3, 8.1, 12.5, 10.9
3	11.4, 8.6, 12.4, 13.4
4	9.8, 8.1, 11.2, 14.0
5	16.0

WATER TABLE No water encountered during drilling.	NORTH: 227.300 EAST: 220.000 TREND: ------ PLUNGE: -90.0 (deg) ELEV G/S: 98.51 (m)	EQUIPMENT: RM-30 METHOD: Hollow Stem Auger DRILLER: Paddock Drilling	COMPLETION: 98/07/07 INSPECTOR: F. Demchenko DEPTH: 5.20 (m)

STATUS: FINAL: DES 98/03/18 O/B
Overlays: HCX600 HCPID ATT10 OG100 LINE USCS TITLE
Printed: 98/06/18 11:28 PI98A GEOTE

Contaminated 2

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

PIKWITONEI
Decommissioned Diesel Site

PI-008
1 OF 1
Prj.# 200533

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

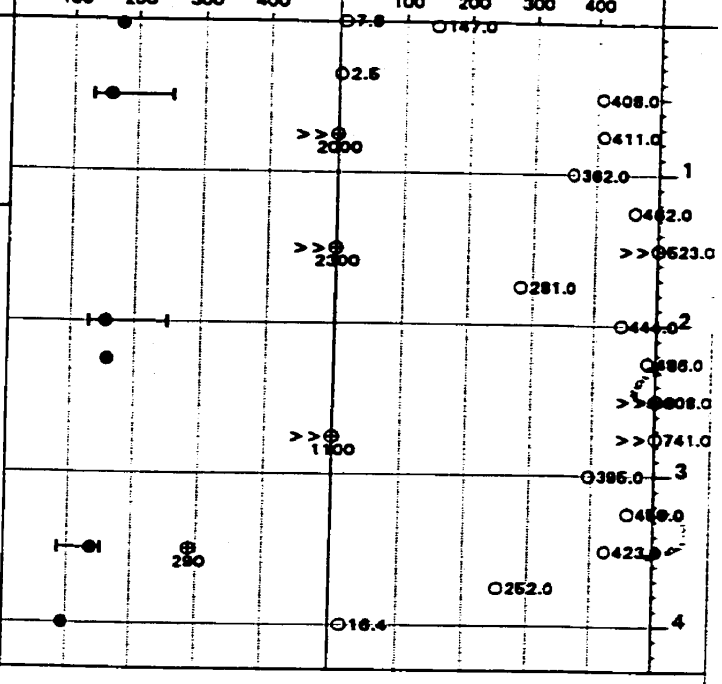
DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0		1 AS	CH	(0.00-1.00) Clay, brown, trace sand, diesel odor, above PL.
		2 AS/AG		
		3 AS/AG		
		4 AS/AG		
		5 AS/AG		
1		6 AS/AG		(1.00-1.25) Clay, brown, some silt, blocky.
		7 AS/AG	CI	(1.25-2.50) Clay, brown, some silt varves, medium insitu, diesel odor, above PL.
		8 AS/AG		
		9 AS/AG		
2		10 AS/AG		
		11 AS/AG		
		12 AS/AG		(2.50-3.00) Clay as above, with silt.
		13 AS/AG		
3		14 AS/AG		(3.00-3.75) Clay, brown, some silt varves, trace sand, medium insitu, above PL.
		15 AS/AG		
		16 AS/AG		
		17 AS/AG	CL	(3.75-4.00) Clay, light brown, some sand, varved, wet.
4		18 AS/AG	ML	(4.00-4.30) Silt, light brown, with sand, saturated.
				(4.30) End of Hole due to auger refusal on bedrock.

Moisture: PL / M.C. / LL — 20% 40% 60% 80%

Oil and Grease (ppm) — 20 40 60 80

Total Extractable Hydrocarbons (ppm) ⊕ — 100 200 300 400

Microtip PID Reading ○ — 100 200 300 400



WATER TABLE No water encountered during drilling.	NORTH: 212.600 EAST: 223.400 TREND: ------ PLUNGE: -90.0 (deg) ELEV G/S: 98.11 (m)	EQUIPMENT: RM-30 METHOD: Hollow Stem Auger DRILLER: Paddock Drilling	COMPLETION: 98/07/07 INSPECTOR: F. Demchenko DEPTH: 4.30 (m)
STATUS:	FINAL: DES 99/02/16 O/S	Overlays: HG2500 HGPID ATT10 OG100 LINE USCS STYLE	Printed: 99/06/19 11:27 PI08A GEOTE

Uncontaminated 2

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

PIKWITONEI
Decommissioned Diesel Site

PI-010
1 OF 1
Prj.# 200533

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL 20% 40% M.C. 60% 80% LL

Oil and Grease (ppm) ■: 20 40 60 80

Total Extractable Hydrocarbons (ppm) ⊕: 100 200 300 400

Microtip PID Reading ○: 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION	Total Extractable Hydrocarbons (ppm) ⊕	Microtip PID Reading ○	DEPTH (m)
		1 AS	PT	(0.00-0.10) Peat.		3.5	
		2 AS/AG	CH	(0.10-1.25) Clay, dark brown, trace rootlets, blocky, above PL.		0.7	
		3 AS/AG				3.8	
		4 AS/AG				2.4	
1		5 AS/AG				4.7	
		6 AS/AG				0.2	1
		7 AS/AG	CI	(1.25-2.50) Clay, brown, some silt varves, above PL.		1.1	
		8 AS/AG				4.4	
2		9 AS/AG				0.0	
		10 AS/AG				0.1	2
		11 AS/AG				0.0	
		12 AS/AG		(2.50-4.00) Clay as above, with silt varves.		0.0	
▽ 3		13 AS/AG				0.0	
		14 AS/AG				0.0	3
		15 AS/AG				0.0	
		16 AS/AG				1.7	
		17 AS/AG				1.0	
4		18 AS/AG	CL	(4.00-4.50) Clay, light brown, and silt varves.		0.0	4
		19 AS/AG			⊕ 170	93.1	
		20 AS/AG	ML	(4.50-4.75) Silt, light brown, some clay, varved, above PL.		3.1	
		21 AS/AG		(4.75-5.00) Silt, light brown, some clay, trace sand, wet.		0.0	
5				(5.00) End of Hole due to auger refusal on bedrock.			5
				Well installed.			

WATER TABLE ▽
Depth @ 2.80 (m)
on 98/07/10

NORTH: 204.700
EAST: 230.000
TREND: ----
PLUNGE: -90.0 (deg)
ELEV G/S: 98.09 (m)

EQUIPMENT: RM-30
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling

COMPLETION: 98/07/07
INSPECTOR: F. Demchenko
DEPTH: 5.00 (m)

STATUS: FINAL: DES 98/02/18 O/B
Overlays: HCX600 HCPID ATT10 OG100 LINE USCS TITLE
Printed: 98/05/19 11:54 PIBBA GEOTE

Contaminated Sand ?

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

BRANDON GASSIFICATION PLANT

BG-023
1 OF 1
Prj.# 201001

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL 20% 40% M.C. 60% 80% LL
Standard Penetration (N): 20 40 60 80
Vane Shear (kPa): 25 50 75 100
Unconfined Compression / Penetrometer (kPa): 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0		1 DS/DG	OL	(0.00-0.20) Top soil, black.
			COAL	(0.20-0.25) Coke.
			SP	(0.25-0.63) Sand, black.
		2 DS/DG	ML	(0.63-0.86) Silt, dark brown/ black, some black substance (difficult to drill), trace sand, trace gravel.
1		3 DS/DG	N/R	SSA to 1.05m.
		EG		(1.34) Difficult drilling. Drill with SSA to 1.60m; grinding ends. Drill HSA with plug to 1.65m.
		4 DS/DG	CL	
2		5 DS/DG	GP	(1.65-1.85) Clay, grey, some silt, medium stiff insitu, above PL.
		6 DS/DG		(1.85-4.20) Gravel, with sand.
	▽1	7 DS/DG		
3		8 DS/DG		
		DG		
4	▽2 ▽3	9 DS/DG		(4.20-4.67) Gravel, some sand, odor.
		10 DS/DG		(4.67-5.19) Gravel, tan brown, some coarse grained sand, saturated, odor.
5		11 DS/DG		(5.19-5.71) Gravel, saturated, odor.
		DG 12 DS/DG	ML	(5.71-5.86) Sand, coarse grained, and gravel.
6		13 DS		
		14 DG		
		DS		(6.66) End of Hole. No bedrock encountered. Installed well.

WATER TABLE
1) 2.65 (m) 98/05/04
2) 4.15 (m) 97/11/06
3) 4.26 (m) 98/01/28

NORTH: 37.300
EAST: 31.400
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 100.18 (m)

EQUIPMENT: Canterra
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling Ltd

COMPLETION: 97/10/29
INSPECTOR: F. Demchenko
DEPTH: 6.66 (m)

STATUS:
FINAL: DES 98/10/22 O/B
Overlays: SPT UNC1 ATT10 VAN10 LINE USCS TITLE
Printed: 99/05/19 11:61 BG97A GEOTE

Contaminated Sand 2

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

BRANDON GASSIFICATION PLANT

BG-025
1 OF 1
Prj.# 201001

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL 20% 40% M.C. 60% 80% LL
Standard Penetration (N): 20 40 60 80
Vane Shear (kPa): 25 50 75 100
Unconfined Compression △ Penetrometer ▲ (kPa): 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0		1 DS	PT	(0.00-0.23) Peat, brown.
		2 DS/DG	CL	(0.23-0.65) Clay, brown, with gravel, with cinders.
1		3 DS/DG	SP-SM	(0.65-1.65) Black cinders, with sand, some gravel.
		4 DG		
		5 DS/DG		
		6 DS/DG		
2		DG		(1.65) Black unidentified substance.
		DG		(2.15-2.65) Tar, black, soft liquid form, some sand, some gravel, strong odor.
3		6 DS/DG		(2.65-3.15) Sand, brown, with gravel, some silt, damp.
		7 DS/DG	SP	(3.15-3.65) Sand, coarse, some gravel, and black tar, soft fluid form.
4		8 DS/DG	GP	(3.65-5.15) Gravel, with coarse grained sand, with non-fluid tar.
	▽	DG		
		9 DS/DG		
5		10 DS/DG	SP-SM	(5.15-6.15) Sand, brown, coarse grained, some fine gravel, damp, some tar odor.
6		DG		
		DG		(6.15-6.63) Sand, medium grey, some silt, damp, odor.
7		11 DS/DG		(6.63-8.15) Sand as above, saturated, odor.
		12 DS/DG		
8		13 DS/DG		
		14 DS/DG		(8.15-9.86) Sand, medium brownish grey, fine grained, saturated.
9		AG		
				(9.26) End of Hole. Installed well.

WATER TABLE ▽
1) Gone-Bldg Demolished (m) 98/09/04
2) 4.28 (m) 97/11/05

NORTH: 20.500
EAST: 40.000
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 100.25 (m)

EQUIPMENT: Canterra
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling Ltd

COMPLETION: 97/10/29
INSPECTOR: F. Demchenko
DEPTH: 9.26 (m)

STATUS: FINAL: DES 98/10/22 O/B
Overlays: SPT UNC1 ATT10 VAN10 LINE USCS TITLE
Printed: 98/06/19 11:48 BG97A GEOTE

Un Contaminated Sand

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GEOTECHNICAL
OVERBURDEN LOG

BRANDON GASSIFICATION PLANT

BG-010
1 OF 1
Prj.# 201001

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL 20% | M.C. 40% 60% 80% | LL
Standard Penetration (N): 20 40 60 80
Vane Shear (kPa): 25 50 75 100
Unconfined Compression / Penetrometer (kPa): 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0		1 DG/DS	OL	(0.00-0.50) Top soil, black, some gravel, damp.
		2 DS	ML	(0.50-0.75) Silt, black, with cinders. White powder substance in sample.
		3 DG	CL	(0.75-1.25) Clay, black, some top soil, trace gravel, above PL.
1		DS		
		DS		
		5 DG	SP	(1.25-1.50) Sand, brown, with silt, trace gravel, trace clay, saturated.
		DS		
		DS/DG		(1.50-2.50) Sand, brown, coarse grained, saturated.
2		DG		
		DG	GP	(2.50-3.10) Gravel, some sand.
3		7 DS/DG	SP	(3.10-3.70) Sand, brown, fine to coarse grained, some gravel, saturated.
		DG		(3.70-4.30) Sand, coarse grained, with gravel.
4		8 DS/DG		(4.30-4.90) Sand, brown, coarse grained, some gravel, saturated.

(4.90) End of Hole.
No bedrock encountered.

DEPTH (m): 0, 1, 2, 3, 4

WATER TABLE
No water level recorded.

NORTH: -3.000
EAST: 60.000
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 100.87 (m)

EQUIPMENT: Canterra
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling Ltd

COMPLETION: 97/10/27
INSPECTOR: F. Demchenko
DEPTH: 4.90 (m)

STATUS: FINAL: DES 98/11/10 O/B Overlays: SPT UNC1 ATT10 VAN10 LINE USCS TITLE
Printed: 98/05/19 11:46 BG97A GEOTE

Uncontaminated Sand

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GEOTECHNICAL
OVERBURDEN LOG

BRANDON GASSIFICATION PLANT

BG-017
1 OF 1
Prj.# 201001

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL 20% | M.C. 40% 60% | LL 80%

Vane Shear (kPa): 25 50 75 100

Standard Penetration (N): 20 40 60 80

Unconfined Compression / Penetrometer (kPa): 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
			N/R	(0.00-2.10) No record.
1		EG		
2		DG	SP	(2.10-3.60) Sand, brown, fine to coarse grained, with gravel, trace cobbles.
3		DG		
		DG		
		1 DS/DG	ML	(3.60-4.30) Silt, medium brown, some fine grained sand, trace clay, hard insitu, damp.
4		DG		(4.30-6.60) Silt, grey, trace fine grained sand, trace clay, hard insitu, damp.
		2 DS/DG		
5		3 DS/DG		
		4 DS/DG		
6		5 DS/DG		

(6.60) End of Hole.
No bedrock encountered.
Well installed.

WATER TABLE
1) 2.77 (m) 98/09/04
2) 4.30 (m) 97/11/05
3) 4.37 (m) 97/10/29

NORTH: 38.000
EAST: 13.100
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 100.36 (m)

EQUIPMENT: Canterra
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling Ltd

COMPLETION: 97/10/28
INSPECTOR: F. Demchenko
DEPTH: 6.60 (m)

STATUS: FINAL: DES 98/11/10 O/B
Overlays: SPT UNC1 ATT10 VAN10 LINE USCS TITLE
Printed: 99/05/18 11:47 BG97A GEOTE

Contaminated loam!

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OVERBURDEN LOG

BRANDON GASSIFICATION PLANT

BG-021
1 OF 1
Prj.# 201001

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL 20% | M.C. 40% 60% | LL 80%

Standard Penetration (N): 20 40 60 80

Vane Sheer (kPa): 25 50 75 100

Unconfined Compression / Penetrometer (kPa): 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0		1 DS/DG	SP	(0.00-0.50) Sand, brown black, some gravel.
		2 DS/DG		(0.50-1.35) Sand, dark brown, black, some clay, trace gravel, damp.
1		3 DS/DG		
		5 DS/DG	N/R	(1.35-1.50) Sand, with black plastic substance.
				(1.50-2.00) Black substance, with sand.
2		6 DS/DG		(2.00-2.50) Black substance, soft, strong odor.
		7 DS/DG		
		DG	SP	(2.50-3.00) Sand, with black liquid substance.
3		8 DS/DG	ML	(3.00-3.65) Silt, brown, some fine grained sand, stiff insitu, damp, odor.
		9 DS/DG		
		10 DS/DG		(3.65-4.50) Silt, grey, trace fine sand, damp, odor.
4		DG		
			N/R	(4.50-5.00) No recovery.
5		AG	ML	(5.00-6.10) Silt, grey, trace fine grained sand, very hard insitu, damp.
		AG		
6		11 DS/DG		(6.10-6.75) Silt as above, odor.
		DG		(6.75-7.60) Silt, brown, with sand, trace fine gravel, very hard insitu, damp.
7		AG		
				(7.60-7.96) Silt, greyish brown, with sand, some gravel, very hard insitu, damp; "till-like".
				(7.96) End of Hole.
				No bedrock encountered.

WATER TABLE					
No water level recorded.	NORTH: 15.500 EAST: 33.200 TREND: ------ PLUNGE: -90.0 (deg) ELEV G/S: 100.38 (m)	EQUIPMENT: Canterra METHOD: Hollow Stem Auger DRILLER: Paddock Drilling Ltd	COMPLETION: 97/10/29 INSPECTOR: F. Demchenko DEPTH: 7.96 (m)
		STATUS: FINAL: DES 98/10/22 GAB Overlays: SPT UNCT ATT10 VAN10 LINE USCS TITLE	Printed: 98/05/19 11:48 BG97A GEOTE

Contaminated loam 2

MANITOBA HYDRO
Engineering

GEOTECHNICAL

OVERBURDEN LOG

BRANDON GASSIFICATION PLANT

BG-117
1 OF 1
Prj.# 201001

Sampler Type: Split Spoon, Shelby, Auger, Sonic, Auger Barrel, Core Barrel, Special Samplers, Tricone, Bulk Sample, Grab Sample, Air Hammer

Moisture: PL — M.C. — LL; 20% 40% 60% 80%

Standard Penetration (N): 20 40 60 80

Vane Shear (kPa): 25 50 75 100

Unconfined Compression / Penetrometer (kPa): 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0		1 DS/DC	CONC	(0.00-0.12) Concrete.
		1 DS/DC	N/R	(0.12-1.00) Red brick, tar.
		2 DS/DC		
		3 DS/DC		
1		4 DS/DC		(1.00-1.50) Black cinders.
		5 DS/DC	OL	(1.50-2.25) Top soil, black, wood fibers, moist, very strong odor.
2		6 DS/DC		
		7 DS/DC	ML	(2.25-2.50) Silt, dark brown, trace sand, trace clay, moist, strong odor.
		8 DS/DC	CL	(2.50-2.65) Clay, light brown, strong odor, above PL.
3		9 DS/DC	ML-CL	(2.65-2.80) Clay, dark blackish brown, some sand, strong odor.
		10 DS/DC	ML	(2.80-3.00) Clay, brown, with silt, some sand, strong odor, above PL.
		11 DS/DC		(3.00-3.40) Silt, brown, some sand, wet, odor.
		12 DS/DC		(3.40-3.85) Silt, grey, trace fine sand, damp.
		13 DS/DC		(3.85-4.00) Silt, light brown, with fine sand.
		14 DS/DC		(4.00-4.80) Silt, grey, some sand, stiff insitu, damp.
4		15 DS/DC		
		16 DS/DC		
		17 DS/DC		
		18 DS/DC		
5		19 DS/DC	SP	(4.80-5.00) Sand, brown, fine grained, trace silt, damp.
		20 DS/DC		
		21 DS/DC	GP	(5.00-5.25) Sand, grey, fine grained, saturated.
		22 DS/DC		(5.25-6.00) Gravel, coarse grained, some sand, saturated.
6		23 DS/DC	ML	(6.00-7.00) Silt, grey, some sand, trace gravel, stiff insitu, damp; "till-like".
		24 DS/DC		
7		25 DS/DC		

(7.00) End of Hole.
No bedrock encountered.
Well installed.

WATER TABLE
1) 2.31 (m) 98/09/04
2) Saturated at 5.00 (m) 98/01/28

NORTH: 9.700
EAST: 31.100
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 101.46 (m)

EQUIPMENT: Canterra
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling Ltd

COMPLETION: 98/01/28
INSPECTOR: F. Demchenko
DEPTH: 7.00 (m)

STATUS:
FINAL: DES 98/10/22 O/B
Overlays: SPT UNC1 ATT10 VAN10 LINE USCS TITLE
Printed: 98/06/18 11:53 BG07A GEO

Uncontaminated loam

MANITOBA HYDRO
Engineering
GEOTECHNICAL
OVERBURDEN LOG

BRANDON GASSIFICATION PLANT

BG-098
1 OF 1
Prj.# 201001

Sampler Types: Split Spoon; Shelby; Auger; Sonic; Auger Barrel; Core Barrel; Special Samplers; Tricone; Bulk Sample; Grab Sample; Air Hammer

Moisture: PL 20% | M.C. 40% 60% 80% | LL 80%

Standard Penetration (N): 20 40 60 80 *

Vane Shear (kPa): 25 50 75 100

Unconfined Compression △ Penetrometer ▲ (kPa): 100 200 300 400

DEPTH (m)	SAMPLER	SAMPLE #	USCS	SOIL DESCRIPTION
0	DS/DG	1	OL	(0.00-0.50) Top soil, black.
	DS/DG	2	CL	(0.50-1.00) Clay, medium blackish brown, some silt, trace rootlets, below PL.
1	DS/DG	3	CI	(1.00-1.25) Clay, greyish brown, trace silt, trace sand, very stiff insitu. Field pen at 1.00m = 450 kPa.
	DS/DG	4		(1.25-2.25) Clay, medium brown, some silt, trace sand, above PL.
	DS/DG	5		
2	DS/DG	6		Field pen at 1.25m = 250 kPa.
	DS/DG	7		
	DS/DG	8	SP	(2.25-3.50) Sand, dark blackish brown, coarse grained, saturated.
	DS/DG	9		
3	DS/DG	10		
	DS/DG	11		
	DS/DG	12	CL	(3.50-4.00) Clay, medium grey, some silt, medium insitu, above PL. Field pen at 3.50m = 100 kPa.
	DS/DG	13		(4.00-4.50) Clay, medium brown, some silt, some sand, trace gravel, above PL.
4	DS/DG	14		
	D/DG	15	ML	(4.50-4.75) Silt, brown, with sand, fine grained, saturated.
	DS/DG	16		(4.75-5.00) Silt, brown, trace sand, damp.
5	DS/DG	17		(5.00-5.50) Silt, brown, and fine grained sand, saturated.
	DS/DG	18		
	DS/DG	19		(5.50-5.75) Silt, greyish brown, some sand, wet.
	DS/DG	20		(5.75-6.50) Silt, grey, trace sand, damp.
6	DS/DG	21		
	DS/DG	22		
	DS/DG	23		(6.50-7.00) Silt, brown, with fine grained sand, saturated.
7	DS/DG	24		
				(7.00) End of Hole. No bedrock encountered.

WATER TABLE
No water level recorded. Saturated at 2.25m.

NORTH: 10.000
EAST: 265.000
TREND: ------
PLUNGE: -90.0 (deg)
ELEV G/S: 98.44 (m)

EQUIPMENT: Canterra
METHOD: Hollow Stem Auger
DRILLER: Paddock Drilling Ltd

COMPLETION: 98/01/21
INSPECTOR: F. Demchenko
DEPTH: 7.00 (m)

STATUS:
FINAL: OES 98/10/22 O/B
Overlays: SPT UNC1 ATT10 VAN10 LINE USCS TITLE
Printed: 98/05/19 11:52 BG97A GEOTE